Suppression of FHL2 Expression Induces Cell Differentiation and Inhibits Gastric and Colon Carcinogenesis

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Background & Aims: **FHL2 (4-1/2 LIM protein 2) is an adapter and modifier in protein interactions that is expressed mainly in the heart and ovary. It functions in a cell type- or promoter-specific manner. The aims of this study were to examine its expression in gastrointestinal cancers and to determine its role in cell differentiation and tumorigenesis.** ***Methods:*** **FHL2 expression in cancerous and normal gastrointestinal cells was detected by reverse-transcription polymerase chain reaction, immunoblotting, and immunohistochemistry. The effect of FHL2 suppression by both antisense and siRNA methods on cell differentiation and growth were evaluated in vitro and in vivo.** ***Results:*** **FHL2 expression was up-regulated in gastrointestinal cancer, compared with matched normal tissues. Stable transfection of gastric cancer cell line, AGS, and colon cancer cell line, Lovo, with antisense *FHL2* induced lengthened or shuttle-shape morphologic changes with long or dendritic-like cytoplasmic processes and decreased the nuclear:cytoplasmic ratio. FHL2 antisense induced expressions of carinoembryonic antigen and E-cadherin and the maturation of F-actin. Furthermore, *FHL2* antisense inhibited the transcriptions of some oncogenes including *cox-2*, *survivin*, *c-jun*, and *hTERT*, and suppressed the promoter activity of activator protein-1 and hTERT. Suppression of FHL2 inhibited serum-dependent, anchorage-dependent and -independent cell growth, and suppressed de novo tumor formation in nude mice xenograft.** ***Conclusions:*** **Suppression of FHL2 induces cell differentiation and inhibits tumorigenesis. Antisense or siRNA methods targeting FHL2 is a promising strategy for treatment of gastrointestinal cancers.**

FHL2 (4-1/2 LIM protein 2), also known as DRAL (down-regulated in rhabdomyosarcoma LIM protein),[1] was initially cloned by its abundant expression in the human heart. It is the second member of a small family of 5 proteins with 4-1/2 LIM domains. The acronym LIM is derived from the names of 3 transcription factors, Lin-11, Isl-1, and Mec-3, in which such a domain was first identified. LIM domains are characterized by the cysteine-rich consensus CX2CX16-23HX2CX2CX2CX16-21CX2-3(C/H/D), and function as adapters and modifiers in protein interactions and those alterations of transcription networks lead to leukemia.[2,3] LIM domains are present in many proteins that have diverse cellular roles as regulators of gene expression, cyto-architecture, cell adhesion, cell motility, and signal transduction.[3,4]

FHL2 is particularly intriguing because it can function as either a repressor or activator of target proteins in a cell type-dependent fashion, and interacts with other proteins. FHL2 serves as a transcriptional co-activator of androgen receptor,[5] activator protein-1 (AP-1), CREB, CREM, ERK2, BRCA1, WT-1, NF-κB, IGFBP-5, hNP220, presenilin 2, and hCDC47 in transformed cell types.[5–10] It also acts as a transcriptional co-repressor of the promyelocytic leukemia zinc-finger protein in muscle cells.[11] Thus, FHL2 represents a network-forming protein common to several signaling pathways.

In adults, FHL2 is expressed mainly by healthy or diseased heart tissue and, to a lesser extent, by ovarian tissue.[12] It exerts different functions in different types of cells. FHL2 protein can shuttle between cytoplasm and nucleus, and may be involved in heart muscle differentiation and the maintenance of the heart phenotype. C_2C_{12} mouse myoblasts stably expressing FHL2 show increased myogenic differentiation reflected by accelerated myotubule formation and expression of muscle-specific proteins.[7,9] However, the role of FHL2 in the differentiation of other tissues and transformed cells remain elusive.

Abbreviations used in this paper: AP-1, activator protein-1; ATRA, *all-trans* retinoic acid; FBS, fetal bovine serum; CEA, carcinoembryonic antigen; COX, cyclooxygenase; FHL2, 4-1/2 LIM protein 2; GI, gastrointestinal; hTERT, telomerase reverse transcriptase subunit; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide; RLU, relative luciferase unit; RT-PCR, reverse-transcription polymerase chain reaction.

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It has been reported that FHL2 is expressed in myoblasts but down-regulated in malignant rhabdomyosarcoma cells.[1] However, other studies reported that FHL2 was highly expressed by various types of cancer cells, and acts as an oncogene. For example, expression of FHL2 protein in epithelial ovarian cancer and hepatoblastoma tissues was markedly up-regulated,[8,13] compared with the matched normal tissues. Colon cancer cell line SW480, cervical cancer cell line HeLa, and some breast cancer cell lines also expressed high levels of FHL2.[12,14] In addition, FHL2 interacted with and activated oncogenic proteins, AP-1, and β-catenin[6,8] transient transfection of FHL2 into melanocyte stimulated cell proliferation.[15] These findings suggest that FHL2 may play an important role in carcinogenesis. However, direct evidence is still lacking.

In this study, we first showed that FHL2 expression is elevated in gastrointestinal cancer cell lines and cancer tissues. Because FHL2 is highly expressed by cancer cells, we suppressed its expression by antisense and RNA interfering strategies, and determined the effect on cell differentiation and tumorigenicity. Results from this study demonstrated, for the first time, that suppression of FHL2 promoted cell differentiation and inhibited carcinogenesis of gastrointestinal (GI) cancer cells.

Materials and Methods

Chemicals, Tissue Specimens, and Cell Lines

Sodium butyrate and *all-trans* retinoic acid (ATRA) were purchased from Sigma (St. Louis, MO). Rhodamine-phallotoxin was purchased from Molecular Probes (Eugene, OR). Three pair of gastric and 11 pair of colon cancer and their adjacent normal tissues were obtained from patients by surgical resection in the Nanfang Hospital (Guangzhou, China). Tissue specimens were snap-frozen in liquid N_2 and stored at −70°C until use. Tissue slices were subjected to histopathologic review, and tumor specimens composed of at least 80% carcinoma cells were chosen for molecular analysis.

To exclude the possibility that the adjacent "normal" tissues contain cancer cells or cells with dysplasia, we also collected colon tissues from 15 cancerous and 15 noncancerous patients under colonoscopy. All tissue specimens and slides were examined by an experienced pathologist. Serial sections were prepared for hematoxylin-eosin staining and immunohistochemical analysis of FHL2 expression.

An immortalized esophageal squamous epithelial cell NE6–E6E7 was obtained from Professor S. W. Tsao, Department of Anatomy, University of Hong Kong, and grown in a defined keratinocyte serum-free medium (Gibco, Rockville, MD, and Invitrogen, Carlsbad, CA).[16] An immortalized normal gastric epithelial cell line GES-1 was obtained from Cancer Research Institute of Beijing, China.[17] Esophageal squamous cancer cell line, ECA 109, gastric cancer cell lines AGS, Kato-III, and colon cancer cell lines DLD1, SW480, HCT15, SW1116, HT-29, Lovo, and Colo205 were obtained from American Type Culture Collection (ATCC, Rockville, MD). Gastric cancer cell line MKN45 and BCG 823 were maintained by our laboratory as previously described.[18] Cells were maintained in RPMI1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 μ/mL penicillin in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

Immunohistochemistry

For visualization of FHL2 expression, antigen retrieval and an indirect immunoperoxidase technique were applied as described. The anti-FHL2 monoclonal antibody (11–134, MBL International Incorporation, Woburn, Japan; dilution 1:50) and the biotin-linked antimouse IgG (Dako, Copenhagen, Denmark) in combination with the ABC complex (Vectastain, Vector) were used. Normal mouse IgG (Sigma) was used as an isotype control for anti-FHL2 antibody to verify specificity of the staining.

Constructs, Transient Transfection, and Establishment of Stable Transfectants Expressing FHL2-AS and FHL2

FHL2 full-length cDNA was isolated from an ovary cDNA library (Clontech, Palo Alto, CA). The cDNA fragment encoding the full-length human FHL2 was cloned in frame in the pCMV-tag epitope tagging mammalian expression vector pCMV-tag 3b (Stratagene, Tokyo, Japan). The primer sequences were as follows: forward, 5′-ATGACTGAGCGCTTTGACTG-3′, reverse, 5′-TCAGATGTCTTTCCCACAGT-3′. An *Eco*RI site was added into the 5′ terminus of the forward primers, and a *Sal*I site was added into the reverse primer. Full-length *FHL2* cDNA was isolated by *Eco*RI and *Sal*I digestion from pCMV-tag3b-*FHL2*, and subcloned into the corresponding sites of pcDNA3.1(+) and pcDNA3.1(−), respectively, to generate pcDNA3.1(+)–*FHL2*-sense (pcDNA3.1–*FHL2*) and pcDNA3.1(−)–*FHL2*-antisense (pcDNA3.1–*FHL2*-AS). Transient transfection was carried out with LipofectAMINE2000 as reported previously.[18] Whole-cell lysates were prepared 48 hours later for evaluation of the protein expression.

To establish stable transfectants, Lovo cells transfected with empty pcDNA3.1 vector and pcDNA3.1–*FHL2*-AS were passaged at 1:15 (vol/vol) and cultured in RPMI 1640 medium supplemented with Geneticin (G418) at 1000 μg/mL for 4 weeks. Stably transfected clones were selected by immunoblotting for FHL2 expression, and maintained in the medium containing 600 μg/mL G418 for additional studies. AGS cells transfected with pcDNA3.1 vector and pcDNA3.1-*FHL2*-AS, cultured in the medium with Geneticin but without clone selection, were also generated and defined as pooled AGS/vector and AGS/*FHL2*-AS. Similarly, stable transfectants of pooled DLD/vector and DLD/*FHL2* were also generated

by transfecting DLD1 cells with pcDNA3.1 vector and pcDNA3.1–*FHL2* followed by G418 selection.

Immunoblotting

The whole-cell lysates were prepared with lysis buffer (20 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L ethyle glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetracetic acid, 1 mmol/L sodium vanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.5% NP-40, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μg/mL pepstatin A). The protein concentration was determined by the bicinchoninic acid assay (BCA protein assay kit, Pierce, Rockford, IL). Equal aliquots of total cell lysates (20 μg) were solubilized in sample buffer and electrophoresed on denaturing sodium dodecyl sulfate-PAGE gel (5% stacking gel and 12% separating gel). The proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were probed with primary mouse antihuman FHL2 antibody (11–134, MBL International Incorporation) followed by the HRP-conjugated antigoat secondary antibody. Goat antihuman actin antibody (I-19, Santa-Cruz, CA) was used as internal control. Antigen–antibody complexes were visualized by the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont Buckinghamshire, England).

Reverse-Transcription Polymerase Chain Reaction

Cells were harvested, and total RNA was extracted using TRIzol Reagent (Gibco BRL and Life Technologies). RNA was reversely transcribed to cDNA by Thermoscript RT system reagent (Gibco BRL) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using 2 μL of resulting cDNA, 0.3 unit Hotstart DNA polymerase. The sequence of the primers was as follows: CEA forward: 5′-AACCCTTCATCACCAGCAAC-3′; carcinoembryonic antigen (CEA) reverse: 5′-CAGGAGAGGCTGAGGTTCAC-3′; E-cadherin forward: 5′-CGACCCAACCCAAGAATCTA-3′, E-cadherin reverse: 5′- GCTGGCTCAAGTCAAAGTCC-3′, survivin forward: 5′-GGACCACCGCATCTCTACAT-3′, survivin reverse: 5′- GACAGAAAGGAAAGCGCAAC-3′, cyclooxygenase 2 (COX-2) forward: 5′- TTCAAATGAGATTGTGGGAAAATTGCT-3′, COX-2 reverse: 5′-AGATCATCTCTCCTGAGTATCTT-3′, c-jun forward: 5′- GGAGTGTCCAGAGAGCCTTG-3′, c-jun reverse: 5′- GAAAGGCTTGCAAAAGTTCG-3′, telomerase reverse transcriptase subunit (hTERT) forward, 5′-CGGAAGAGTGTCTGGAGCAA-3′, hTERT reverse: 5′-GGATGAAGCGGAGTCTGGA-3′ GAPDH forward: 5′-GACCACAGTCCATGCCATCAC-3′, GAPDH reverse: 5′-CCACCACCCTGTTGCTGTA-3′. Hotstart PCR was performed for 32 cycles with 95°C denaturation for 30 minutes (first cycle), 94°C denaturation for 45 seconds, 55°C annealing for 45 seconds, and 72°C elongation for 45 seconds and 10 minutes (final cycle). The expected size of PCR products were 340, 386, 223, 305, 204, 145, and 449 bp, respectively for *CEA*, *E-cadherin*, *survivin*, *COX-2*, *c-jun*, *hTERT,* and *GAPDH*.

Generation of Promoter–Luciferase Constructs

AP-1 promoter–luciferase reporter pGL3 construct was purchased from Promega (Madison, WI),[19] which contains −73 to +67 collagenase promoter region with 1 AP-1 binding site; 330 bp and 1365 bp 5′-flanking regions containing the core promoter of human telomerase reverse transcriptase (*hTERT)* were inserted into the pGL3 luciferase reporter vector. The shared proximal (reverse) primer (217 to 236 nt, TCCTTCAGGCAGGACACCTG) was used. The distal primer sequences were CCCGGGTCCGCCCGGAGCAGCTGC (−306 nt to −330 nt) and TACAAGACGAGGCTAACCTC (−1346 nt to −1365 nt). The upstream nucleotide adjacent to the translation starting ATG codon was here defined as −1. Promoter segments were obtained by PCR amplification. After digestion of both the pGL3basic vector (Promega) and the PCR products with *Kpn*I and *Bgl*II, the purified products were inserted in the forward orientation upstream of a luciferase reporter gene of pGL3 basic vector to generate pLuc-330 and pLuc-1365 constructs.

Promoter–Luciferase Reporter Expression

For the luciferase assay, the cells were transiently transfected with various luciferase reporter constructs by Lipofectamine 2000 as previously described.[19] PRL-CMV (Promega) was used to normalize the reporter gene activity. Forty-eight hours after transfection, cells were solubilized in 1× passive lysis buffer (Promega) and scraped with a rubber policeman. Soluble protein lysates were assayed for firefly and renilla luciferase activities using the Dual-Luciferase reporter assay system (Promega) with a model TD-20/20 Luminometer (EG&G Berthold, Australia). Firefly luciferase activity value was normalized to renilla activity value. Promoter activity was presented as the fold induction of relative luciferase unit (RLU) compared with the basic vector control (RLU is the value of firefly luciferase unit/value of renilla luciferase unit).

Cell Growth Assays

Cell growth was measured by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[18] The ratio of the absorbance of cells relative to that of the control cells was calculated and expressed as the index (%) of cell proliferation. Assay of anchorage-dependent cell growth was performed as previously described.[20] The number of cells per well was expressed as mean ± SD at the indicated number of days after plating. Assay for anchorage-independent cell growth was performed as previously described.[18] Each

treatment was triplicated, and the result was expressed as mean ± SD.

siRNA Transfection

The siRNA duplexes consisted of 21 base pairs with a 2-base deoxynucleotide overhang (Proligo, Singapore). The sequences of the *FHL2* siRNA were as follows (sense strand): siRNA 1, CGAAUCUCUCUUUGGCAAGdTdT, siRNA 2, UCUCUCUUUGGCAAGAAGUdTdT, siRNA 3, GGACUUGUCUUACAAGGACdTdT. The control siRNA, GL2 (CGUACGCGGAAUACUUCGA) was directed against the luciferase gene. The cells were transfected with siRNA duplexes using Oligofectamine (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence

For staining of F-actin, cells were fixed with 3.7% formaldehyde, incubated with rhodamine-conjugated phallotoxin (5 U/mL, Molecular Probes) in PBS at room temperature. Coverslips were washed, mounted, and visualized using Zeiss Axioscop fluorescence microscope. For staining of α-tubulin, cells were fixed and permeabilized in ice-cold methanol for 20 min. Antibodies to α-tubulin (clone DM1A, 1:100 dilution) and fluorescein isothiocyanate-conjugated goat antimouse antibodies were purchased from Sigma. Nuclei were stained with 1 μg/mL Hoechst 22358, and cells were analyzed using fluorescence microscope.

Tumorigenicity in Nude Mice

Single cell suspensions of stable Lovo/vector transfectant and pooled stable Lovo/*FHL2*-AS transfectant were trypsinized and collected. Cell viability was >95% as determined by trypan blue exclusive staining. Cells (5×10^6) in a 0.1-mL volume of RPMI were inoculated subcutaneously into the right flank of 5–6-week-old female BALB/c-*nu/nu* mice (Laboratory Animal Unit, The University of Hong Kong). Institution guidelines were followed in handing the animals. The mice were maintained under sterile conditions. Tumor formation was observed 6 weeks later. The volumes of tumor were calculated as follows: $V = (4/3)\ R1^2\ R2$, where $R1$ is radius 1 and $R2$ is radius 2 and $R1 < R2$. All tumors formed were removed and dissociated. The Committee on the Use of Live Animals in Teaching and Research, University of Hong Kong, Hong Kong, approved the protocol.

Statistical Analysis

Results obtained from triplicate luciferase and cell growth experiments were expressed as mean ± SD. The results with different treatments were compared using a 2-tailed Student's t test and considered significant if P value was <.05.

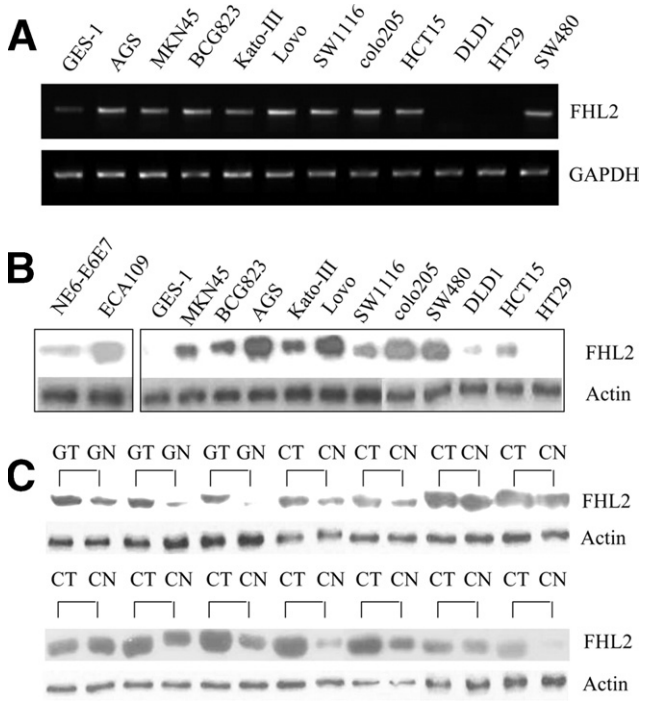

Figure 1. GI cancer cells expressed higher levels of FHL2 than normal cells. (*A*) Expression of *FHL2* in immortalized gastric epithelial cell line GES-1 and GI cancer cell lines as detected by RT-PCR. (*B*) Protein expression of FHL2 in GES-1, GI cancer cell lines, immortalized esophageal epithelial cell line NE6–E6E7, and esophageal squamous cancer cell line ECA-109, as detected by immunoblotting. (*C*) FHL2 expression in gastric (GT) and colon (CT) cancer tissues and matched normal gastric (GN) and colon (CN) tissues as detected by immunoblotting. All of these experiments were repeated 3 times with identical findings. *GAPDH* and actin were used as the internal control for RT-PCR and immunoblotting respectively.

Results

Cancer Cells Expressed Higher Levels of FHL2

We showed that most gastric and colon cancer cell lines expressed high levels of FHL2 (Figure 1*A* and *B*). *FHL2* expression was nearly undetectable at both mRNA and protein levels in the immortalized normal gastric epithelial cell line GES-1. We also measured FHL2 expression in an immortalized but normal esophageal squamous cell line, NE6–E6E7, and showed that this cell line expressed a lower level of FHL2 than esophageal squamous cancer cell line, ECA109. We then measured FHL2 expression in 14 pairs of matched gastric (G) and colon (C) normal (N) and cancerous (T) tissues by immunoblotting. Of the 14 cancerous tissues, 13 expressed higher levels of FHL2 than normal tissues (Figure 1*C*).

Although Chan et al[12] reported that *FHL2* mRNA is undetectable in both normal gastric and intestinal tissues, we found that some "adjacent normal tissues" expressed detectable level of FHL2 protein. Some of them even expressed higher level of FHL2 than other cancer tissues. Because our matched "normal" tissues were ob-

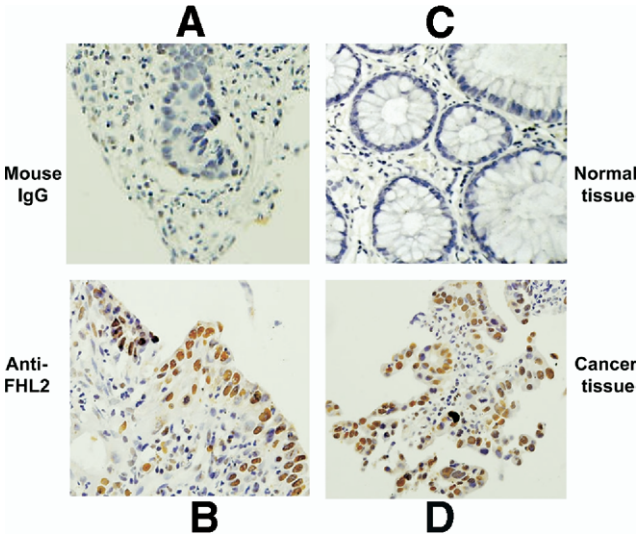

Figure 2. Cancerous but not normal colon epithelial cells expressed FHL2. FHL2 expression in normal (*C*) or cancerous colon tissue specimens (*B, D*) was detected by immunohistochemistry assay. Normal mouse IgG was used as the isotype control for the first antibody (*A*). These figures were the representatives of colon tissues from 15 cancerous and 15 noncancerous patients. Original magnification, 200×.

tained from the surgical resection tissues, they were only macroscopically tumor free. To exclude the possibility that these tissues contain some micrometastatic cancer cells or cells with dysplasia, we detected FHL2 expression in situ by immunohistochestry in tissue specimens collected from colon with noncancerous or cancerous diseases under colonoscopy. We showed that specific nuclear FHL2 protein was only expressed in the carcinoma cells of all colon cancer samples as exemplified in Figure 2*B* and *D*. On the contrary, normal colon tissues did not express FHL2 protein. Figure 2*C* shows a representative picture of normal colon tissues.

These findings demonstrated that FHL2 was overexpressed in gastrointestinal cancer cells.

Antisense FHL2 Induced Morphologic Changes in Cancer Cells

We established cell transfectant that stably expressed antisense *FHL2*. Colon cancer cell Lovo was transfected with pcDNA3.1 and pcDNA3.1–*FHL2*-AS. After G418 selection, 3 clones transfected with empty vector (Lovo/vector) and 10 clones transfected with pcDNA3.1–*FHL2* AS (Lovo/*FHL2*-AS) were picked up, spread, and collected. FHL2 expression was examined by immunoblotting. Because we found no difference with regard to FHL2 expression between untransfected Lovo cells and Lovo/vector transfectants (data not shown), we used 1 Lovo/vector transfectant as the control for additional experiments. Pooled AGS/*FHL2*-AS was also generated.

Six clones of Lovo/*FHL2*-AS expressed lower levels of FHL2 protein compared with Lovo/vector. However, only 2 of them survived after identification. As shown in Figure 3*A*, stable transfection of *FHL2*-AS suppressed FHL2 expression in both Lovo and AGS cells.

To characterize *FHL2*-AS transfectants, we first examined the morphologic features of these cells. The parent cells or stable transfectants of vector displayed a round or flat morphology with a short cytoplasmic process (Figure 3*B1* and *B3*). However, *FHL2*-AS transfectant exhibited lengthened or shuttle-shape morphology. Long or dendritic-like cytoplasmic processes were visible under phase-contrast microscope (Figure 3*B2* and *B4*). These morphologic changes were also found in transient transfected Lovo and AGS cells (data not shown), and maintained in stable transfectants for more than 20 passages.

To distinguish cytoplasm and nucleus, we stained the stable transfectants with anti-α-tubulin antibody by immunofluorescence to display the cytoplasm and Hochest 22358 to display the nuclei. Similar morphologic changes were found (Figure 3*C1* and *C2*). Furthermore, Lovo/*FHL2*-AS stable transfectants exhibited a decreased nuclear:cytoplasmic ratio by mainly increasing

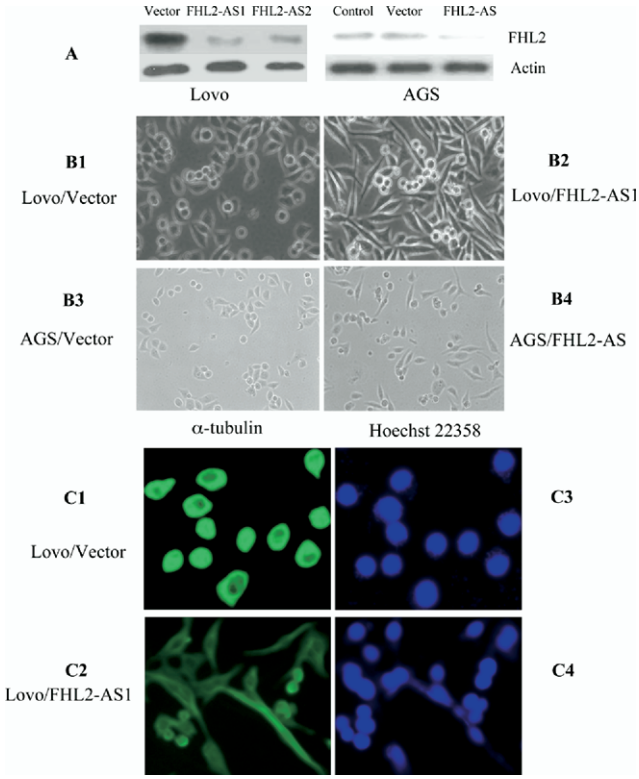

Figure 3. Antisense *FHL2* induced morphologic changes in cancer cells. (*A*) FHL2 expression in stable transfectants of Lovo and AGS cells expressing vector or antisense FHL2 as detected by immunoblotting. Actin was used as the internal control. (*B*) Morphology of stable transfectants of Lovo/vector (*B1*), Lovo/*FHL2*-AS1 (*B2*), AGS/vector (*B3*), and AGS/*FHL2*-AS (*B4*) as visualized under phase-contrast microscope. (*C*) Stable transfectants of Lovo cells stained with anti-α-tubulin with immunofluorescence showing the cytoplasm (*C1* and *C2*) and Hochest 22358 showing the nuclei (*C3* and *C4*) under fluorescent microscopy. These pictures were representatives of 3 independent experiments with identical results.

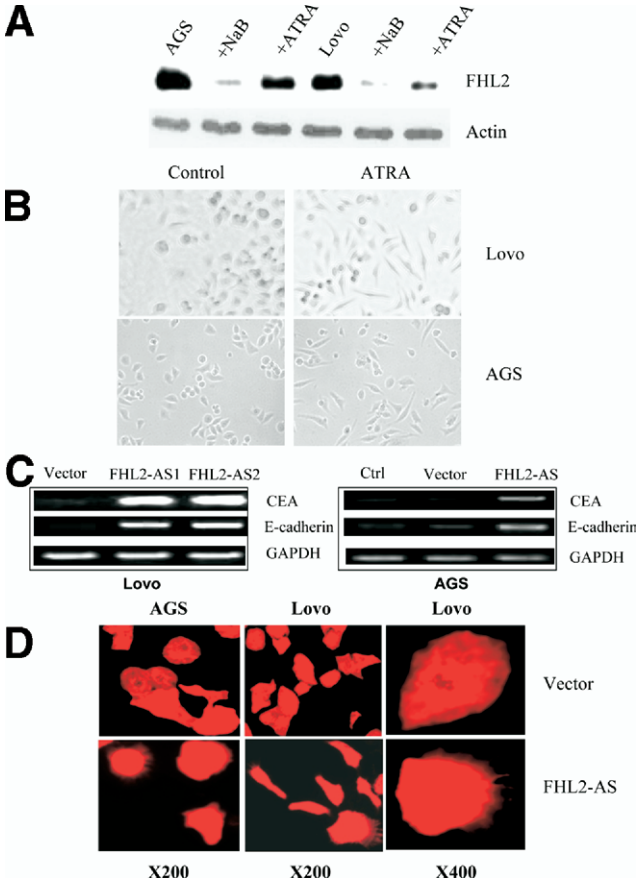

Figure 4. Suppression of FHL2 induced cell differentiation. (*A*) FHL2 expression in AGS and Lovo cells treated without or with sodium butyrate (NaB) and *all-trans* retinoic acid (ATRA) for 48 hours, as detected by immunoblotting. (*B*) Morphology of Lovo and AGS cells in control or after ATRA (50 μmol/L) treatment for 48 hours, as visualized under phase-contrast microscopy. (*C*) Expression of CEA and E-cadherin in stable transfectants, as detected by RT-PCR. (*D*) Stable transfectants stained with rhodamine-phallotoxin, with F-actin filaments being visualized under fluorescent microscopy. All of these experiments were repeated 2 to 3 times with similar findings.

the cytoplasm plot and slightly decreasing the size of nuclei (Figure 3*C*). These morphologic changes and the decreased nuclear:cytoplasmic ratio were all features of cytoplasm differentiation of normal cells,[20,21] and thus suggesting that suppression of FHL2 might induce differentiation of gastrointestinal cancer cells.

Suppression of FHL2-Induced Cell Differentiation

To examine the effect of FHL2 in cell differentiation, we first investigated the effect of pro-differentiation agents on FHL2 expression. AGS and Lovo cells were treated with sodium butyrate (sodium butyrate, 5 mmol/L and ATRA, 50 μmol/L) for 48 hours. Both agents suppressed FHL2 expression significantly (Figure 4*A*). Notably, pretreatment with ATRA induced similar morphologic changes to that of *FHL2*-AS stable transfectant (Figure 4*B*). Next, we showed by reverse-transcription polymerase chain reaction (RT-PCR) in stable transfectants that suppression of FHL2 increased expression of differentiation markers, CEA and E-cadherin.[22–25] in both Lovo and AGS cells (Figure 4*C*). We then stained F-actin in stable transfectants with rhodamine-phallotoxin. Weak and aggregated F-actin filaments exhibited in vector transfectants of both AGS and Lovo cells (Figure 4*D*). Suppression of FHL2 displayed uniform and highly structured array of thick F-actin filaments at cell-adherent regions and throughout the cytoplasm (Figure 4*D*). Importantly, typical microvilli-like actin filaments that protruded out of the cytoplasm plot were observed only in *FHL2*-AS transfectants (Figure 4*D*).

Suppression of FHL2 Inhibited the Expression of Oncogenes

We evaluated the effect of antisense *FHL2* on transcription of several oncogenes. The mRNA expression of *survivin*,[18,26] *cox-2*,[27,28] *hTERT*,[27] and *c-jun*[29] were down-regulated in stable transfectants of *FHL2* antisense (Figure 5*A*). The same results were obtained in pooled AGS/*FHL2*-AS stable transfectants except that AGS cell did not express *cox-2* (Figure 5*A*). Then we examined the

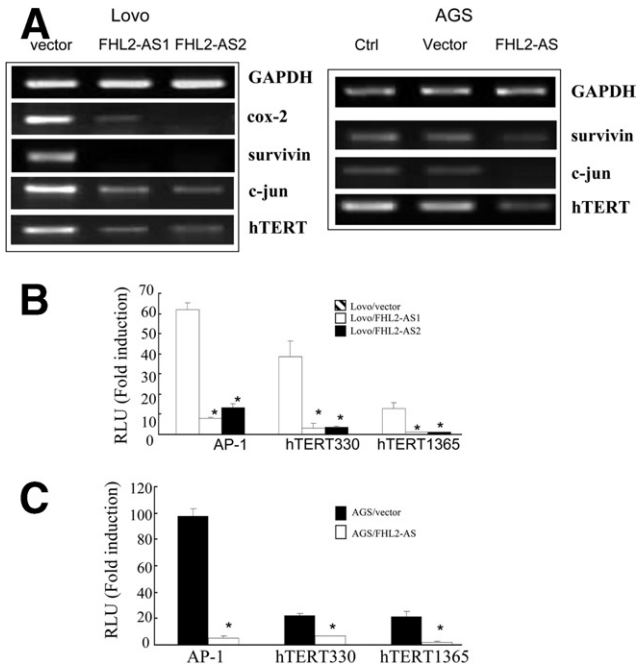

Figure 5. Antisense FHL2 suppressed oncogene transcription. (*A*) The expression of *cox-2*, *survivin*, *c-jun,* and *hTERT* in stable transfectants of Lovo and AGS cells as detected by RT-PCR. These figures were representatives of 4 independent experiments with similar results. (*B, C*) Stable transfectants of Lovo and AGS cells were transfected transiently with 1 AP-1 and 2 (pLuc330 and pLuc1365) hTERT promoter-driven luciferase reporter constructs. Luciferase activities were assayed 48 hours later. Promoter activity was presented as the fold induction of relative luciferase unit (RLU) compared with the basic pGL3 vector control. RLU = values of firefly luciferase unit/values of renilla luciferase unit. All experiments were performed in triplicate, and the results were expressed as means ± SD. *$P < .05$ compared with the Lovo/vector.

effect of *FHL2* suppression on promoter activities of AP-1 and hTERT. We transiently transfected stable transfectants with 1 AP-1 and 2 hTERT promoter–luciferase reporter constructs. We showed that RLU fold inductions of AP-1 construct were 62.4 ± 3.3, 8.3 ± 0.3, and 13.8 ± 1.6, respectively in Lovo/vector, Lovo/*FHL2*–AS1, and Lovo/*FHL2*–AS2 (Figure 5*B*). RLU fold inductions of hTERT pLuc330 were 39.1 ± 7.8, 3.6 ± 2.2, and 3.8 ± 0.4, while those of pLuc1365 were 13.3 ± 2.6, 1.6 ± 0.1, and 1.4 ± 0.3, respectively, in Lovo/vector, Lovo/*FHL2*–AS1 and Lovo/*FHL2*–AS2 (Figure 5*B*). Similar results were obtained in AGS cells (Figure 5*C*). Suppression of FHL2 decreased the transcription activity of AP-1 and hTERT significantly ($P < .05$).

Antisense FHL2 Inhibited Serum-Dependent Cancer Cell Growth

As shown in Figure 6*A*, cell proliferation of Lovo/vector were 113.8% ± 5%, 126.4% ± 6%, and 162.5% ± 5% when cultured in the presence of 0.4%, 2%, and 10% FBS, respectively, while those of Lovo/*FHL2*-AS1 and Lovo/*FHL2*-AS2 were 103.3% ± 3%, 110.3% ± 5%, and 115.2% ± 9%, and 107.8% ± 6%, 113.5% ± 3%, and 135.5% ± 4%, respectively. Significant difference was found between stable transfectants of Lovo/vector and Lovo/*FHL2*-AS ($P < .05$).

We synthesized 3 pairs of *FHL2* siRNA. All of them suppressed FHL2 expression (Figure 6*B*). Subsequently, cell growth in response to FBS was assessed after transfection of control siRNA (GL2) or *FHL2* siRNA. As shown in Figure 6*C*, indices of cell proliferation of AGS cells transfected with GL2 siRNA were 230.2% ± 7%, 245.7% ± 11%, and 310.3% ± 6% when cultured in the presence of 0.4%, 2%, and 10% FBS, while those for *FHL2* siRNA were 131.3% ± 8%, 160.14% ± 6%, and 206.9% ± 6% for *FHL2* siRNA1, 173.7% ± 4%, 183.1% ± 6%, and 200.2% ± 7% for *FHL2* siRNA2, and 177.3% ± 7%, 196.9% ± 6%, and 232.0% ± 6% for *FHL2* siRNA3, respectively. Significant differences were found between control siRNA and *FHL2*–siRNA ($P < .05$). Similar results were observed in Lovo cells (Figure 6*C*). These data suggest that *FHL2* suppression inhibits cancer cell growth.

To further elucidate the role of FHL2 in cancer cell growth, we transfected vector control or sense *FHL2* construct into DLD1 cell, which expressed a low level of FHL2 (Figure 1). Stable transfectants were established as identified by immunoblotting (Figure 6*D*). As shown in Figure 6*E*, the proliferation indices of DLD1 control were 131.9% ± 8%, 148.8% ± 12%, and 272.9% ± 26%, respectively, in response to 0.4%, 2%, and 10% of FBS, while those for the DLD1/vector were 121.6% ± 10%, 158.2% ± 19%, and 291.6% ± 18%, and those for DLD1/*FHL2* were 223.8% ± 12%, 358.4% ± 19%, and 835.8% ± 12%, respectively. Overexpression of FHL2 promoted DLD1 cell proliferation, compared with vector control ($P < .05$).

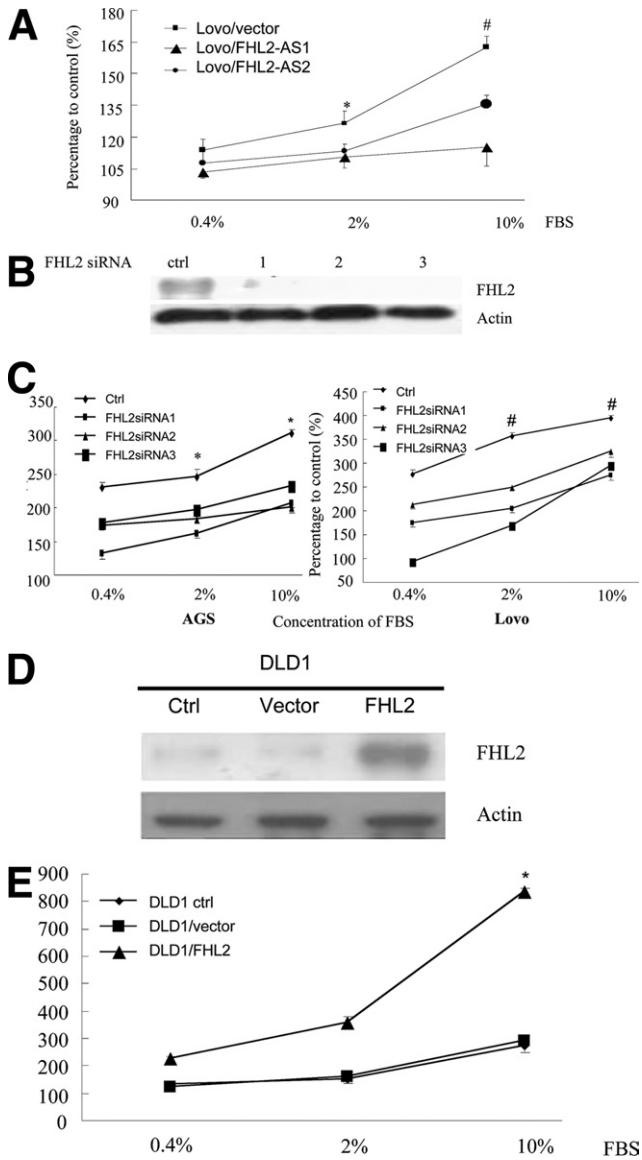

Figure 6. Antisense *FHL2* inhibited serum-dependent cancer cell growth. (*A*) Lovo/vector and Lovo/*FHL2*-AS stable transfectants were seeded into a 96-well plate in triplicate at a density of 10,000 cells/well for 12 hours. The medium was replaced with RPMI 1640 containing 0.1%, 0.4%, 2%, or 10% FBS for an additional 48 hours, and then cell growth was assessed by MTT assay. All experiments were performed in triplicate, and the results were expressed as means ± SD. *#$P < .05$, compared with the Lovo/vector. (*B*) Lovo cells seeded in a 24-well plate were transfected with 3 pairs of *FHL2* siRNA, and FHL2 expression was detected by immunoblotting 48 hours later. Actin was used as the internal control. These figures were representatives of 3 independent experiments with identical results. (*C*) AGS and Lovo cells were seeded into a 96-well plate for 12 hours and transfected with *FHL2* siRNAs subsequently. The medium was replaced with RPMI 1640 containing 0.1%, 0.4%, 2%, or 10% FBS 24 hours later for an additional 48 hours, and cell growth was assessed by MTT assay. *#$P < .05$, compared with the *FHL2* siRNA. (*D*) DLD1 cells with or without stable transfection of vector or *FHL2* were identified by detecting FHL2 expression with immunoblotting. (*E*) Stable transfectants of DLD1 cells were seeded into 96-well plates in the presence of 0.1%, 0.4%, 2%, or 10% FBS for 72 hours; cell growth was assessed by MTT assay. *$P < .05$, compared with the vector control. All MTT experiments were performed in triplicate, and the results were expressed as means ± SD.

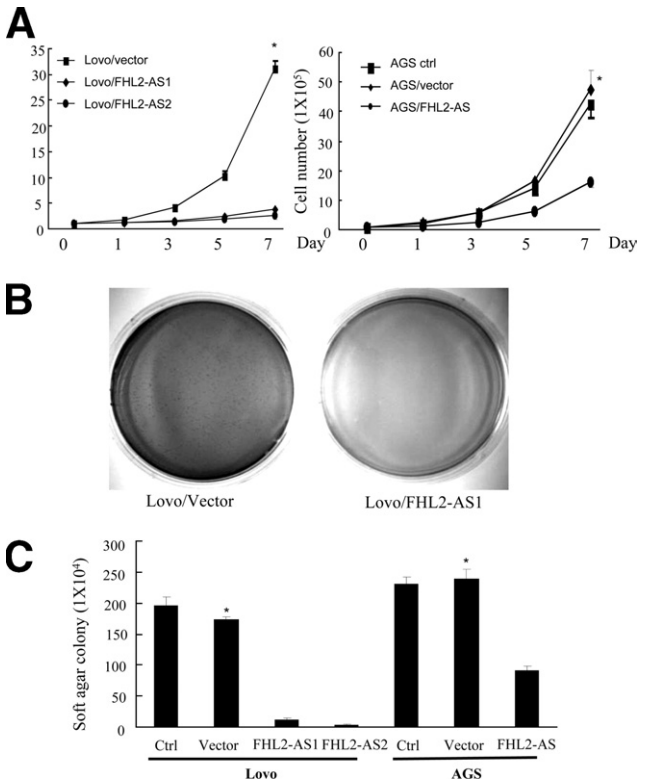

Figure 7. Suppression of FHL2 inhibited anchorage-dependent and -independent cell growth. (*A*) Stable transfectants were seeded into 6-well plates. Cells from triplicate wells were collected every other day. Cell numbers were determined using a Coulter counter. The number of cells per well was expressed as mean ± SD. *$P < .05$, compared with *FHL2*-AS transfectants. (*B*) Stable transfectants of Lovo cells (5×10^3) were plated in a tissue culture dish with complete culture medium containing 0.35% agar on top and 0.5% agar at bottom and cultured for 12 days. Cell colonies were visulized after staining with 0.005% crystal violet. (*C*) Anchorage-independent cell growth of stable transfectants was evaluated by soft-agar assay. Colonies were scored 12 days later. Colonies containing >50 cells were considered viable. All experiments were performed in triplicate, and the results were expressed as means ± SD. *$P < .01$, compared with *FHL2*-AS transfectants.

Suppression of FHL2 Inhibited Anchorage-Dependent and -Independent Cell Growth

Further, we assessed the effect of antisense *FHL2* on the capacity of anchorage-dependent and -independent cell growth of cancer cells because the acquisition of anchorage-independent growth ability positively correlated with tumorigenicity.[18,20] We showed that FHL2 suppression inhibited anchorage-dependent cell growth considerably in both Lovo and AGS cells (Figure 7*A*, $P < .05$). In addition, *FHL2*-AS transfectants displayed significantly loss of colony-forming capacity (Figure 7*B* and *C*, $P < .01$).

Antisense FHL2 Inhibited In Vivo Tumorigenesis of Cancer Cells

To verify the effect of antisense *FHL2* in tumorigenesis in vivo, we evaluated the ability of a colon cancer cell line, Lovo/vector, and pooled Lovo/*FHL2*-AS transfectants in tumor formation in vivo. We showed that all of the 4 mice inoculated with Lovo/vector developed tumors with the mean volume of 1069.5 ± 364.8 mm^3 (Figure 8*A*). However, only 1 of the 4 mice inoculated with pooled Lovo/*FHL2*-AS transfectants developed a slow-growing tumor, with a volume of 549 mm^3 (Figure 8*A*). After detection of *FHL2* expression in tumor extracts by immunoblotting, we found that the tumor formed by Lovo/*FHL2*-AS expressed a comparable level of FHL2 protein with those developed in mice inoculated with Lovo/vector (Figure 8*B*), suggesting that this tumor was formed by cells without efficient expression of *FHL2*-AS gene. These data suggest that suppression of FHL2 inhibits tumorigenesis in vivo.

Discussion

In this study, we characterized the role of FHL2 in cell growth and differentiation of gastrointestinal cancers. We found that FHL2 was overexpressed in most gastrointestinal cancer cell lines and cancerous tissues. Suppression of FHL2 induced cell differentiation, inhibited cell growth, and suppressed de novo tumor formation in nude mice. These findings suggest that FHL2 plays a pivotal role in gastrointestinal carcinogenesis.

Owing to the strong expression in heart tissues and the coactivator function of LIM domain, most studies on FHL2 have focused on its role in differentiation and signal pathways in muscle cells.[7–9,12] FHL2 expressions in tissues other than heart and ovary are weak or undetect-

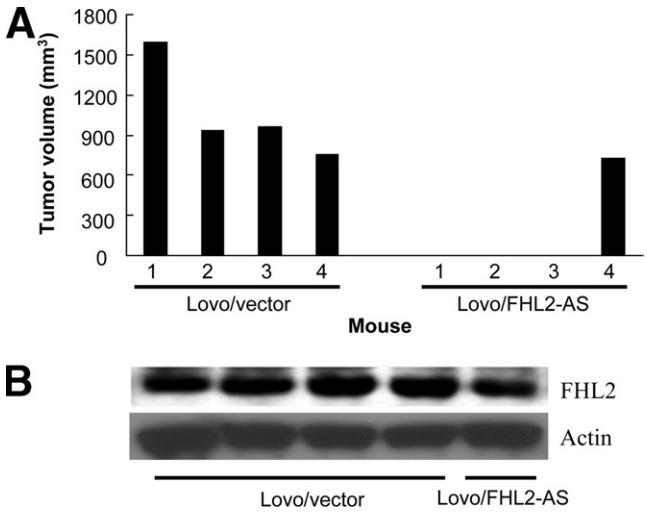

Figure 8. Suppression of FHL2 inhibited in vivo tumorigenicity. Cells (5×10^6) of pooled Lovo/vector and Lovo/*FHL2*-AS stable transfectants in 0.1-mL volume of RPMI were inoculated subcutaneously into the right flank of 5–6-week-old female BALB/c-*nu/nu* mice with 4 mice for each transfectant. (*A*) Tumor formation was observed 6 weeks later. The volume of tumor was calculated as follows: $V = (4/3)\,R1^2\,R2$, where $R1$ is radius 1, $R2$ is radius 2 and $R1 < R2$. (*B*) All tumors formed were removed and dissociated. Protein extracts were prepared, and the expression of FHL2 was detected by immunoblotting with actin being used as the internal control.

able.[12] On the contrary, FHL2 is overexpressed in prostate,[30] breast,[14,31] hepatoblastoma,[8] ovarian,[13] and colon cancer cells.[12] These reports are consistent with our findings that gastrointestinal cancers expressed higher level of FHL2 than normal tissues. In our study, FHL2 expression in HT-29 was undetectable. This finding was consistent with those reported in previous studies that HT-29 was a partially differentiated colon cancer cell line[32,33] characterized by the production of CEA and well-differentiated tumor formation in nude mice (http://www.ATCC.com).

FHL2 localizes mainly in the nucleus, and can shuttle between cytoplasm and nucleus. It has been reported to participate in the differentiation of heart muscle through forming a multicomplex with human DNA-binding nuclear protein (hNP220) in the nucleus.[9] On the other hand, some FHL2-interacting proteins such as SKI,[15] ERK,[34] and nuclear stabilized β-catenin[7,8] have been implicated in the dedifferentiation of cancer cells including gastrointestinal cancers,[35–37] suggesting that FHL2 may also be involved in differentiation control of cancer cells. By establishing stable transfectants expressing antisense FHL2, we observed that suppression of FHL2 not only induced morphologic changes and reduced the nuclear: cytoplasmic ratio similar to those of well-differentiated gastrointestinal epithelial cells,[20] but also increased the expression of 2 differentiation markers for gastrointestinal epithelial cells, CEA, and E-cadherin.[22–25]

A dynamic actin cytoskeleton characterizes normal epithelial cells. It mediated the roles of intercellular (eg, E-cadherin/catenin complex) and cell-matrix (eg, integrins) adhesion molecules in regulating cell polarity, differentiation, proliferation, migration, and invasion. In colorectal cancer, cells lose actin cytoskeletal organization and normal cell adhesion when they become invasive.[38] As the maturation form of actin, F-actin expression has been reported in previous studies to be higher in differentiated cells than that of undifferentiated cells.[38] The colonic adenocarcinoma cells only displayed actin aggregates and did not possess any organized stress fibers.[38,39] Our finding that FHL2 suppression induced maturation of F-actin filaments in cancer cells not only implicated the role of FHL2 in dedifferentiation of cancer cells, but also implied its role in the regulation of the invasion capacity of cancer cells.[40,41] FHL2 might exert its effect through the direct interaction with actin protein as reported by Cannalt,[10] or through modulating the expression and function of E-cadherin/catenin complex.[6,8] In fact, our data here have shown that suppression of FHL2 increased the expression of E-cadherin.

Studies have shown that FHL2 interacted with and activated 2 AP-1 components c-fos and c-Jun.[6,42,43] Our current study showed that suppression of FHL2 indeed down-regulated or completely abrogated the transcription of *c-jun*, *hTERT*, *survivin,* and *cox-2*. Although the mechanisms to inhibit *hTERT* and *cox-2* remain to be elucidated, the effect of *FHL2* antisense on *survivin* suppression is consistent with that reported previously.[44] FHL2 interacted with and activated β-catenin in transformed cells, while β-catenin positively regulated survivin transcription.[5,7,8,44] Similarly, c-jun was also the target gene of β-catenin/TCF complex; thus, FHL2 might modulate c-jun expression through interaction with β-catenin as well. Because survivin, hTERT, and Cox-2 are overexpressed in gastrointestinal cancer and are able to induce cancer cell proliferation,[18,26–28] our observations indicated that FHL2 suppression reversed malignant phenotypes of cancer cells.

Studies reported that overexpression of FHL2 stimulated proliferation of melanocyte[15] and inhibited FOXO1-induced apoptosis in prostate cancer cells.[30] A recent clinical study reported that forty (47%) of 85 breast cancer samples expressed low level of FHL2, whereas 45 tumors (53%) expressed high level of FHL2. Patients with tumors expressing low amounts of FHL2 were characterized by a significantly better survival compared with those with high intratumoral FHL2 expression.[31] This study indicated that the expression of FHL2 in primary breast cancer is a potentially relevant prognostic factor, suggesting the potential role of FHL2 in tumor cells' growth.[31] To examine the direct effect of FHL2 on growth of gastrointestinal cancer cells, we suppressed FHL2 expression by both antisense and RNA interference methods. We showed that FHL2 suppression inhibited serum-dependent, anchorage-dependent, and -independent growth of gastrointestinal cancer cells. To validate our in vitro observations, we collected pooled Lovo stable transfectant expressing antisense *FHL2* and tested its capacity in tumor formation in vivo. Only a small tumor with expression of FHL2 grew in 1 out of 4 nude mice. This tumor might be formed by cells with loss of *FHL2* antisense expression. Because the ability of anchorage-independent growth and in vivo tumor formation are 2 characterized features of transformed cells, these findings support the above notion that FHL2 plays an important role in carcinogenesis. Although this conclusion is required to be validated by using other sources of cancers such as prostate and breast cancer cells in vivo, our finding concerning the effect of FHL2 on cell growth is consistent with the following observation. FHL2 physically interacted with E4F1 in the nuclear compartment and inhibits the capacity of E4F1 in blocking cell proliferation.[45]

In conclusion, this study shows that gastrointestinal cancer cells express higher levels of FHL2 than normal tissues. Suppression of FHL2 induces cell differentiation, inhibits cell growth in vitro and in vivo. Thus, targeting of *FHL2* by antisense or RNA interference methods may have a promising role in the management of gastrointestinal cancer.

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