



Original Article

Fibroblast Growth Factor 19 Induced Changes in Non-malignant Cholangiocytes

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Abstract

Background and Aims: Fibroblast growth factor (FGF)19 has been implicated in the pathogenesis of murine hepatocellular carcinoma. Whether it plays a role in the development or course of human cholangiocarcinoma remains to be determined. The aim of this study was to determine whether prolonged exposure to FGF19 results in the transformation of non-malignant human cholangiocytes into cells with malignant features. **Methods:** Human SV-40 transfected non-malignant H69 cholangiocytes were cultured with FGF19 (0–50 ng/mL) for 6 weeks, followed by 6 weeks with medium alone. Cell proliferation, invasion, stem cell surface markers, oncofetoprotein expression, state of differentiation, epithelial-mesenchymal transition (EMT) and interleukin (IL)-6 expression were documented at various time intervals throughout the 12-week period. **Results:** FGF19 exposure was associated with significant increases in cell proliferation, de-differentiation, EMT and IL-6 expression. However, each of these effects returned to baseline or control values during the 6-week FGF19 free follow-up period. The remaining cell properties remained unaltered. **Conclusions:** Six weeks of FGF19 exposure did not result in the acquisition of permanent malignant features in non-malignant, human cholangiocytes.

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Keywords: Fibroblast growth factor 19; Cholangiocytes; Cholangiocarcinoma; Farnesoid x receptor; Obeticholic acid.

Abbreviations: CA125, cancer antigen 125; CCA, cholangiocarcinoma; CD, cluster of differentiation; CDH2, Cadherin 2; b-actin, beta-actin; CEA, carcinoembryonic antigen; CYP7A1, cholesterol 7 alpha-hydroxylase; EGF, epidermal growth factor; EMT, epithelial mesenchymal transition; EpCAM, epithelial cell adhesion molecule; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FXR, farnesoid x receptor; HCC, hepatocellular carcinoma; IL, interleukin; IL-6, interleukin-6; OCA, obeticholic acid; SCSM, stem cell surface markers; VIM, vimentin; MUC1, mucin 1.

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Introduction

Fibroblast growth factor (FGF)-fibroblast growth factor receptor (FGFR) signaling plays an important role in cell proliferation, differentiation, migration, morphogenesis and angiogenesis.¹ To date, 22 structurally-related FGFs and 5 cognate receptors (FGFR 1–5) have been identified.^{2–4}

FGF19 is one of only three FGFs that function as an endocrine factor or hormone. Bile acid activation of farnesoid x receptors (FXRs) in the enterocytes of the ileum enhances its expression. FGF19 is then transported to the liver via the portal vein, where it inhibits CYP7A1 expression and thereby bile acid synthesis creating a negative feedback loop.⁵ As a result of these properties, bile acids, such as obeticholic acid (OCA), are presently being employed or tested as treatments for cholestatic liver diseases and non-alcoholic steatohepatitis in humans where toxic bile acids are thought to be involved in the pathogenesis of these disorders.

In addition to its role in inhibiting bile acid synthesis, FGF19 has also been linked to regulating the development and progression of cancers, including hepatocellular carcinoma (HCC), by activating the Wingless/Wnt signaling pathway and resulting in nuclear accumulation of β -catenin and epithelial-mesenchymal transition (EMT).^{6–8} Indeed, ectopic FGF19 expression in transgenic mice results in increased hepatocyte proliferation, oncofetoprotein expression and hepatic tumor formation.⁶ In addition, FGF19 and its cognate receptor FGFR4 are overexpressed in premalignant cirrhotic livers and human HCC.⁷ Finally, FGF19 interference prevents tumor formation in FGF19 transgenic mice treated with the carcinogenic agent diethylnitrosamine.⁷

Relative to hepatocytes, there are a paucity of studies documenting the effects of FGF19 or its inducers on another important cell population of the liver: cholangiocytes. In one of the few papers published to date, OCA exposure exacerbated bile duct injury in a rat model of cholestasis.⁹ In another, FGF19 was found to selectively activate BiP and CHOP in a dose-dependent manner and regulate p-eIF2 α and MAPK protein expression with a bimodal response.¹⁰ Perhaps of more concern are results of a study in which FGFR4 tumor expression was increased in approximately 50–60% of 200 patients with intrahepatic (ICC), perihilar (PCC) or distal (DCC) cholangiocarcinoma (CAA) and served as an independent prognostic factor in ICC and PCC patients.¹¹ In

the same report, the authors described increased proliferation, invasion, and EMT in various malignant CCA cell lines exposed to FGF19.

Given previous data describing FGF19-FGF4R promotion of HCC, the association between FGF19/FGF4R and established CCA, the documented effects of FGF19 on malignant, CCA cell lines and anticipated long-term use of FXR agonists in patients with chronic liver disorders, we endeavored to determine whether prolonged exposure of nonmalignant human cholangiocytes to FGF19 results in their permanent acquisition of malignant features.

Methods

Cell lines

The human non-malignant, transformed bile duct epithelial cell line H69 was obtained from Dr. Gianfranco Alpini at Texas A&M University (College Station, TX, USA). H69 cells were cultured in Dulbecco's modified Eagle's medium (low glucose; Gibco, Invitrogen, Waltham, MA, USA), 25% Ham's F12 (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 4 mmol/L L-glutamine (Invitrogen), 180 µmol/L adenine (Sigma-Aldrich, St. Louis, MO, USA), 5 mg/L insulin (Invitrogen), 5 mg/L transferrin (Sigma-Aldrich), 2 nmol/L triiodothyronine (Sigma-Aldrich), 1.1 µmol/L hydrocortisone (Sigma-Aldrich), 5.5 µmol/L epinephrine (Sigma-Aldrich), 1.64 µmol/L epidermal growth factor (EGF) (Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Cell cultures

H69 cells were seeded in 6-well plates, cultured in complete H69 medium and exposed to different concentrations of recombinant FGF19 (0, 0.5 ng/mL, 5 ng/mL, and 50 ng/mL) (Invitrogen) for 6 weeks, followed by FGF19-free H69 medium for an additional 6 weeks. Cell features were analyzed after 1, 2, 3, 4, 5, 6, 8 and 12 weeks of culture, unless otherwise stated.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA of cells was extracted using a TRIzol™ Plus RNA Purification Kit (Invitrogen) and quantified by a NanoDrop™ 2000 spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reverse transcribed into complementary DNA (cDNA) following the instructions of iScript™ cDNA synthesis kit Reverse Transcription Kit (BioRad, Hercules, CA, USA). Real-time PCR was performed with SYBR® Green PCR Master Mix (Invitrogen). The specific primers employed are listed in Table 1 and were designed with the respective sequences from GenBank by Oligo 7 software. PCR amplification was initially held at 95°C for 10 m, then carried out by applying 35 cycles comprised of denaturation at 95°C for 15 s, annealing temperature at 55°C for 1 m, elongation at 50°C for 1 m, and followed by a final melting curve stage using a ViiATM 7 Real-time PCR System (Applied Biosystems Inc., Foster City, CA, USA). Data were analyzed by QuantStudio™ Real-time PCR Software (Applied Biosystems Inc.). The relative expression of genes was calculated by means of relative quantification ($2^{-\Delta\Delta Ct}$ method), with β -actin as an internal control.

Table 1. Primers of real-time polymerase chain reaction

Gene		Primers, 5'-3'
KI67	Forward	GCCTGCTCGACCCTACAGA
	Reverse	GCTTGCAACTGCGGTTGC
IL6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGTTTCAGGTTG
CEA	Forward	TCTTGCTGATTGATGGGAAC
	Reverse	CACTGGCTGAGTTATTGGCTT
CA125	Forward	AGCCACCTCATCTATTACCACA
	Reverse	TGTTGCTGCATTGCTTAGGGT
CYP7A1	Forward	GAGAAGGCCAAACGGGTGAAC
	Reverse	GGATTGGCACCAAATTGCAGA
VIM	Forward	AGTCCACTGAGTACCGGAGAC
	Reverse	CATTTACGCATCTGGCGTTC
MUC1	Forward	TGCCGCCGAAAGAACTACG
	Reverse	TGGGGTACTCGCTCATAGGAT
CDH2	Forward	TCAGGCGTCTGTAGAGGCTT
	Reverse	ATGCACATCCTTCGATAAGACTG
β -actin	Forward	TCCTCTCCCAAGTCCACACAGG
	Reverse	GGGCACGAAGGCTCATCATTC

IL6, interleukin 6; CEA, carcinoembryonic antigen; CA125, cancer antigen 125; CYP7A1, cholesterol 7 alpha-hydroxylase; VIM, vimentin; MUC1, mucin 1; CDH2, cadherin 2; β -actin, beta-actin.

Cell proliferation assessment

H69 cultured cells exposed to the different concentrations of FGF19 for the different time periods were seeded at a density of 2,000 cells per well in 96-well plates. After 24 h of plating, cells were incubated with a premixed WST-1 reagent (Takara Bio USA, New York City, NY, USA) at 37°C for 3 h. Absorbance of each well, compared against a blank control, was measured using a microplate reader (Synergy™ 4; BioTek Instruments, Winooski, VT, USA) at 540 nm.

Invasion assay

Twenty-four well Transwell permeable chambers with 8-µm pores (Corning, Corning, NY, USA) were used to measure cell invasion. To the upper chambers, 1×10^5 H69 cells exposed to the different concentrations of FGF19 for the different time periods were added to 100 µL H69 serum-free medium. Lower chambers were filled with 650 µL medium and 10% FBS. After 24 h incubation, cells from the upper surface were removed with cotton swabs. Penetrated cells were dissociated and collected by a cell dissociation buffer (Invitrogen). Collected cells were quantified by a cell counter (Cellometer® Auto 2000; Nexcelom Biosciences, Boston, MA, USA).

Stem cell surface markers (SCSMs) and differentiation

H69 cultured cells exposed to the different concentrations of FGF19 for different time periods (1, 6, 8 and 12 weeks) were

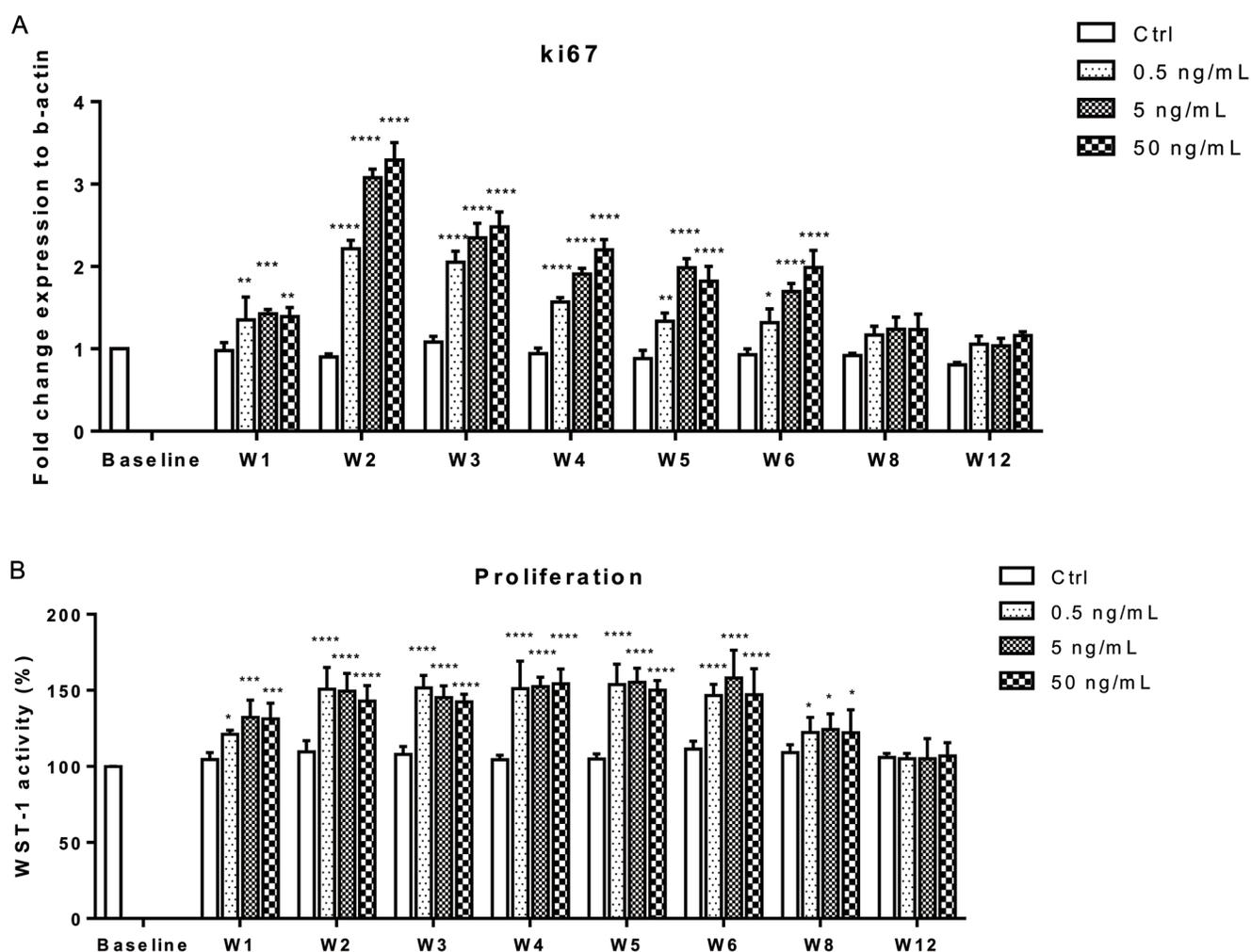


Fig. 1. Effects of FGF19 exposure on H69 cholangiocyte proliferative activity as determined by Ki67 expression (A) and WST-1 activity (B). Data represent mean±standard deviation of at least three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. FGF, fibroblast growth factor.

suspended in phosphate-buffered saline with 1.5% FBS, 25 mM HEPES pH7.0, and 1 mM EDTA (Sigma-Aldrich) at a concentration of 1×10^6 cells/mL, stained with fluorescence-conjugated antibodies (anti-CD13-APC, BD Biosciences, San Jose, CA, USA; anti-CD24-APC-eFlour 780, Life Technologies, Carlsbad, CA, USA; anti-CD44-eFlour 450, Life Technologies; anti-CD133-PE, Miltenyi Biotec, Auburn, CA, USA; anti-EpCAM-FITC, Miltenyi Biotec; and 7-AAD, Beckman-Coulter, Brea, CA, USA). Cells stained with isotype-matched antibodies (BD Biosciences) served as negative controls. Flow cytometer analysis was performed on FACSCanto-II Digital Flow Cytometry Analyzer (BD Biosciences) using FlowJo (Tree Star, Ashland, OR, USA) software.

Statistical analysis

All experiments were performed in triplicate. Data were analyzed by Prism6 statistical software (GraphPad Software, Inc., San Diego, USA). Measurement data were presented as mean ± standard deviation. Significant differences were determined by repeated measures of ANOVA and/or Tukey's multiple comparison post-hoc test. A Student's *t*-test was used for comparisons between two groups. Differences with

p values of < 0.05 were considered to be statistically significant.

Results

Cell proliferation

The effects of FGF19 exposure on H69 cholangiocyte proliferative activity as reflected by Ki67 and WST-1 expression are shown in Figures 1A and B, respectively. After 1 week of exposure, the proliferative activity detected by both parameters was significantly increased in FGF19-exposed cells relative to buffer alone and remained elevated throughout the 6 weeks of exposure to FGF19, before returning towards buffer control levels at 8–12 weeks. FGF4R expression remained relatively stable throughout this period (data not shown).

Cell invasion

Cell invasion, as determined by the Transwell migration as-

Transwell Activity

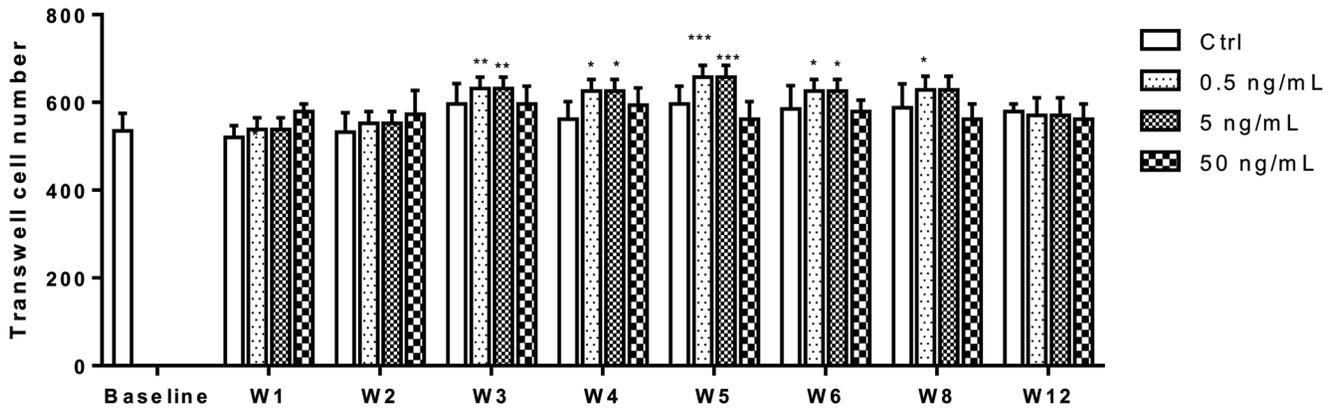


Fig. 2. Effects of FGF19 exposure on H69 cholangiocyte invasion as determined by the Transwell cell invasion assay. Data represent mean±standard deviation of at least three experiments. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. FGF, fibroblast growth factor.

say, was not consistently altered by exposure to FGF19 (Fig. 2).

SCSMs

As shown in Table 2, CD13 and CD90 were highly expressed in H69 cells but the levels of expression did not change with exposure to FGF19. CD24, CD133 and EpCAM expression levels were expressed in less than 10% of cells and expression remained low following FGF19 exposure. Finally, CD44 expression was evident in approximately 25–35% of cells and remained within that range on exposure to all concentrations of FGF19. Thus, overall SCSMs did not appreciably

change as a result of FGF19 exposure.

De-differentiation

Despite the absence of changes in SCSMs, the state of cell dedifferentiation as reflected by CEA (Fig. 3A) and CA125 (Fig. 3B) expression significantly increased on exposure to FGF19. In both cases, expression of these markers increased at essentially all FGF19 concentrations following 1–2 weeks of exposure, remained elevated throughout the 6-week FGF19 exposure period, and to a lesser extent following withdrawal of FGF19.

Additional evidence of dedifferentiation was obtained

Table 2. Effects of FGF19 exposure on SCSM expression

		CD13	CD24	CD44	CD90	CD133	EpCAM
Baseline	Ctrl	99.6±0.5	0.6±0.3	33.8±3.3	100	0.1±0.03	8.3±0.6
	0.5 ng/mL	97.6±0.8	1.0±0.3	25.0±1.0	99.5±0.5	0.3±0.3	7.5±1.4
	5 ng/mL	98.6±0.7	1.1±0.3	25.1±1.5	99.9±0.2	0.4±0.1	6.6±0.6
Week 1	50 ng/mL	97.1±1.1	1.1±0.3	25.7±1.5	100	0.5±0.1	7.1±1.0
Week 6	Ctrl	99.3±1.2	1.5±0.7	31±1	99.7±0.6	0.1±0.03	6.7±1.6
	0.5 ng/mL	98.4±1.4	0.9±0.6	25.3±0.6	99.9±0.2	0.3±0.2	5.7±1.1
	5 ng/mL	98.3±0.6	1.2±0.5	25.6±2.0	99.6±0.6	0.1±0.1	6.8±0.3
	50 ng/mL	97.9±1.0	1.0±0.3	26.9±1.7	100±0.1	0.2±0.2	7.7±1.1
Week 8	Ctrl	98.2±1.1	1.3±0.6	31.9±2.5	99.6±0.5	0.1±0.1	7.6±1.2
	0.5 ng/mL	96.7±2.5	1.1±0.4	27.8±1.1	100	0.5±0.3	6.4±0.5
	5 ng/mL	97.2±0.8	1.0±0.3	27.6±0.5	100	0.5±0.3	6.7±2.0
	50 ng/mL	97.6±1.5	0.8±0.2	28.2±2.0	99.6±0.5	0.1±0.1	7.3±0.6
Week 12	Ctrl	97.2±1.3	1.1±0.7	31.4±2.2	99.9±0.1	0.2±0.02	5.6±0.5
	0.5 ng/mL	95.3±1.2	1.1±0.5	29.6±2.9	99.8±0.3	0.6±0.1	7.4±1.4
	5 ng/mL	96.1±1.0	1.3±0.6	28.1±1.8	100	0.3±0.02	6.6±0.7
	50 ng/mL	98.3±1.2	1.1±0.5	30.2±0.7	99.7±0.6	0.4±0.1	7.2±1.1

CD, cluster of differentiation; EpCAM, epithelial cell adhesion molecule.

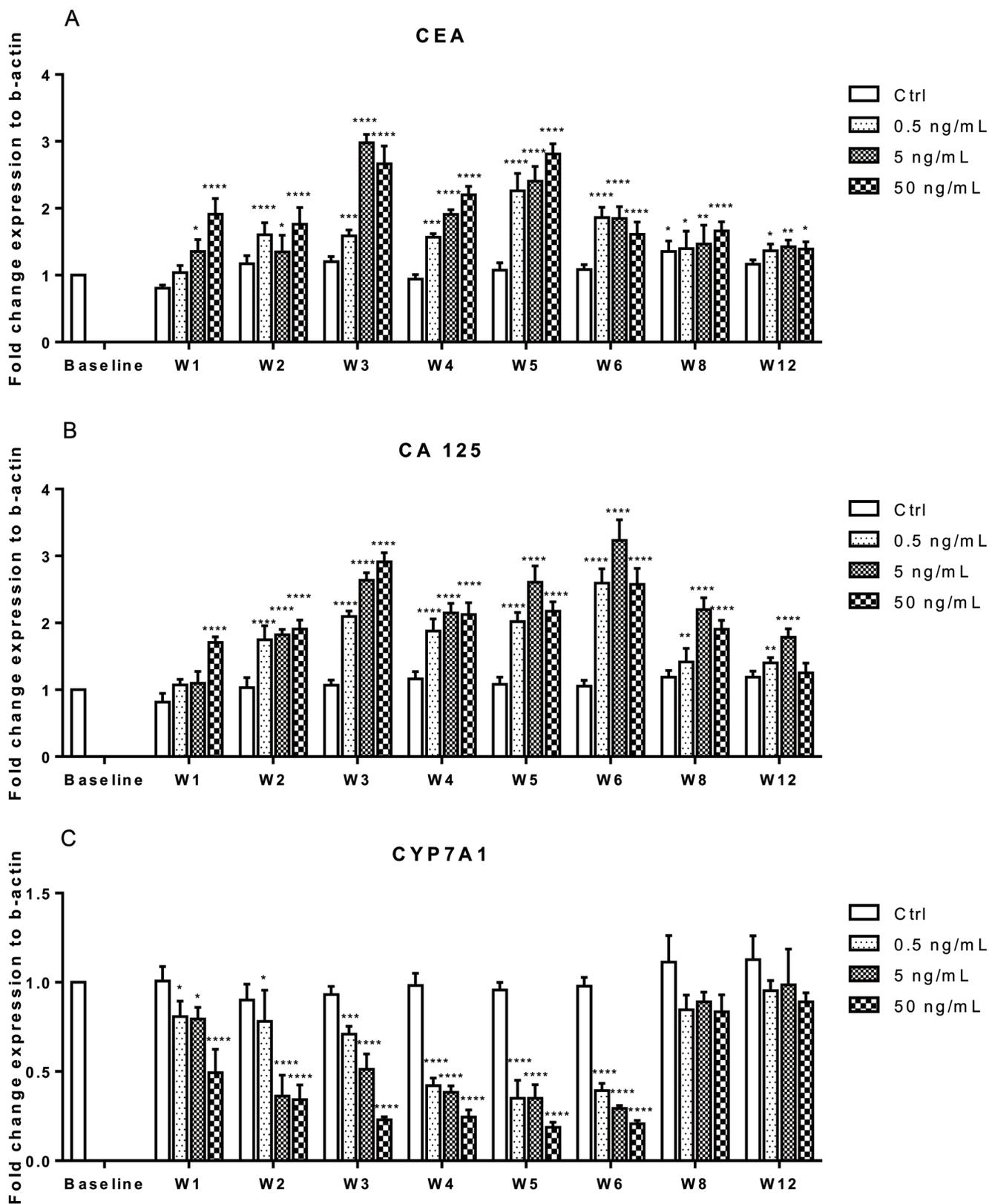


Fig. 3. Effects of FGF19 exposure on H69 cholangiocyte differentiation as determined by CEA (A), CA125 (B) and CYP7A1 (C) expression. Data represent mean±standard deviation of at least three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CYP7A1, cholesterol 7 alpha-hydroxylase; FGF, fibroblast growth factor.

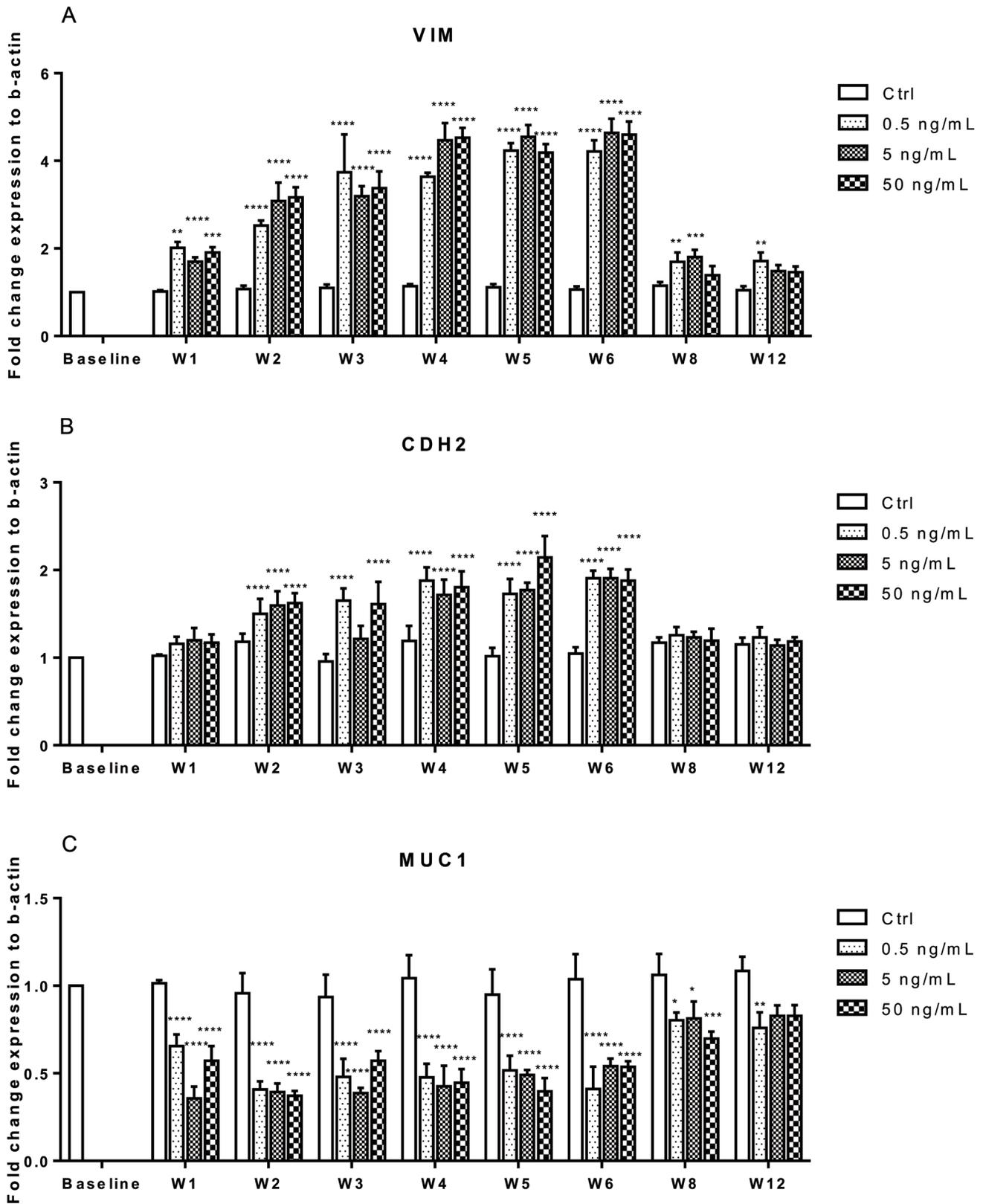


Fig. 4. Effects of FGF19 exposure on H69 cholangiocyte EMT as reflected by vimentin (A), CDH2 (B) and MUC1 (C) expression. Data represent mean±standard deviation of at least three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. EMT, epithelial mesenchymal transition; FGF, fibroblast growth factor; VIM, vimentin; CDH2, Cadherin 2; MUC1, mucin 1.

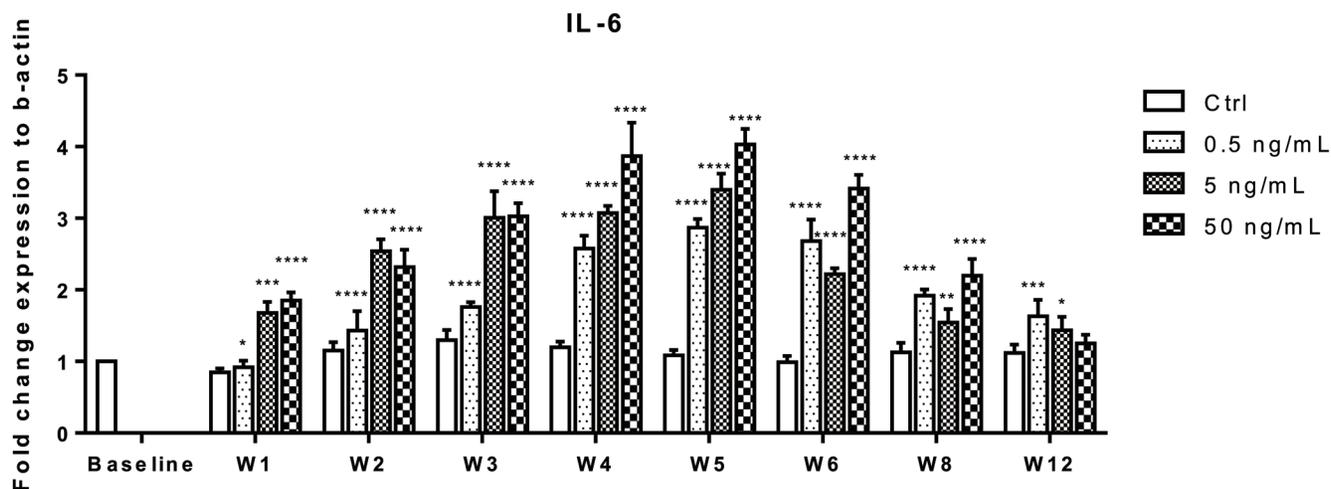


Fig. 5. Effects of FGF19 exposure on H69 cholangiocyte IL-6 expression. Data represent mean±standard deviation of at least three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. FGF, fibroblast growth factor; IL-6, interleukin-6.

from the loss of differentiated function. CYP7A1 is a CYP450 enzyme involved in the metabolism of cholesterol to bile acids. Within 1 week of FGF19 exposure, CYP7A1 expression was significantly downregulated at all three FGF19 concentrations and remained downregulated throughout the FGF19 exposure period (Fig. 3C). However, recovery of this activity was prompt and not significantly different from buffer controls by week 8.

EMT

As occurs with the onset of EMT, expression of the mesenchymal cell marker vimentin significantly increased following 1 week of FGF19 exposure, remained elevated throughout the 6-week exposure period, then rapidly fell towards buffer control levels of expression at weeks 8 and 12 (Fig. 4A). Similarly, CDH2, another marker of the EMT process, significantly increased with FGF19 exposure before returning to buffer control levels at weeks 8 and 12 (Fig. 4B). Conversely, MUC1 expression, an epithelial cell marker, was significantly lower at week 1 and remained low until its expression increased, but for the most part not to buffer control levels at weeks 8 and 12 (Fig. 4C).

Cytokine expression

Interleukin (IL)-6 is a proinflammatory cytokine that has been implicated in the pathogenesis of FGF19-induced HCC in rodents. As shown in Figure 5, FGF19 significantly increased IL-6 expression in H69 cells within 1 week of exposure and essentially remained increased (albeit to a lesser extent) until week 12.

Discussion

The results of this study indicate that FGF19 significantly influences non-malignant, human cholangiocyte features including cell proliferation, state of differentiation, EMT and IL-6 expression without altering cell invasion or SCSM expression. However, in essentially all instances, the significant changes identified reverted to baseline or control values within 2–6 weeks of FGF19 withdrawal. These findings

suggest that FGF19 exposure is unlikely to be carcinogenic for nonmalignant human cholangiocytes.

As stated earlier, the principal purpose of this study was to determine whether prolonged exposure to FGF19 results in the permanent acquisition of malignant features in non-malignant human cholangiocytes. While the findings of reversibility were somewhat reassuring, it should be noted that 6 weeks of exposure may not have been sufficient to achieve malignant transformation. Also to be noted is the fact that FGF19 induced HCC in mice was documented *in vivo*, where the contributions of extracellular influences to tumor formation including side populations and angiogenesis may have been relevant.^{6,7} Finally, whether SV-40 transfected cells, such as the H69 cell line employed in this study, have the same threshold for malignant transformation as primary cholangiocytes has yet to be determined.

A secondary objective of the study was to document the effects of FGF19 on cholangiocyte properties that might alter the severity of those liver disorders where FXR agonists are being employed. Here, some interesting findings were observed. Specifically, FGF19 significantly increased H69 proliferation which may be relevant to the replacement of injured cholangiocytes in conditions such as primary biliary cholangitis or primary sclerosing cholangitis.^{12,13} Of note, similar findings were reported by Hannan *et al.*¹⁰ The increased proliferative activity may also explain the observed dedifferentiation of H69 cells (as manifest by increases in the oncofetoproteins CEA and CA125).¹⁴ That FGF19 exposure-downregulated CYP7A1 expression in H69 cells was a somewhat unexpected finding in that cholesterol metabolism is generally considered a hepatocyte rather than cholangiocyte function.¹⁵ However, Jung *et al.*¹⁶ reported similar findings and identified the mechanism to involve FGF15/19 binding to FGF4R via the p38 kinase pathway.

EMT is a process observed in fetal tissue development and various carcinomas.¹⁷ It is also observed in tissue repair where epithelial cells transition into fibrogenic cells and serve as modulators of metalloproteins.¹⁷ Thus, the EMT that occurred in H69 cells exposed to FGF19 may represent an important step in the repair of damage associated with chronic liver disease. Conversely, the upregulation of the proinflammatory cytokine IL-6 suggests FGF19 exposure might enhance the severity of those chronic liver disorders.

There are a number of limitations to this study that warrant emphasis. First, as mentioned earlier, 6 weeks of FGF19 exposure may not have been sufficient to effect ma-

lignant transformation. Second, although the concentrations of FGF19 were selected to approximate those reported in the blood of humans being treated with FXR agonists, the intrahepatic concentrations of FGF19 in these individuals is unknown. Third, the duration of FGF19 exposure and recovery period following exposure (a total of 12 weeks) necessitated the use of transformed cholangiocyte cells rather than primary cholangiocytes. Fourth, additional assays such as Ki-67 qIHC and MTT, and those measuring cyclins and proinflammatory cytokines beyond IL-6, and further experiments such as inducing overexpression of the FGF19 gene would have been helpful in confirming and/or extending our findings. Finally, because the effects of human FGF19 and the murine analogue FGF15 vary in mice, it was considered important to perform these experiments with human FGF19 and human cholangiocytes. Thus, species dependency experiments were not undertaken.

In conclusion, nonmalignant human cholangiocytes exposed to various concentrations of FGF19 for up to 6 weeks did not undergo malignant transformation. However, the effects observed could theoretically enhance the growth of established CCA. Alternatively, FGF19 exposure may be more relevant to modifying the extent of hepatic injury in patients being treated with FXR agonists.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (GYM), acquisition of data (JY, DS), analysis and interpretation of data (GYM, JY, SK), drafting of the manuscript (GYM), critical revisions of the manuscript for important intellectual content (JY, SK), and administrative, technical, or material support, study supervision (GYM).

Data sharing statement

Data and materials are available upon reasonable request to the corresponding author.

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