Short communication

ATTENUATION OF ENOYL COENZYME A HYDRATASE SHORT CHAIN 1 EXPRESSION IN GASTRIC CANCER CELLS INHIBITS CELL PROLIFERATION AND MIGRATION IN VITRO

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Abstract: Enoyl coenzyme A hydratase short chain 1 (ECHS1) is an important part of the mitochondrial fatty acid β-oxidation pathway. Altered ECHS1 expression has been implicated in cancer cell proliferation. This study assessed ECHS1 expression in human gastric cancer cell lines and investigated the effects of ECHS1 knockdown on gastric cancer cell proliferation and migration. The human gastric cancer cell lines SGC-7901, BGC-823 and MKN-28, and the immortalized human gastric epithelial mucosa GES-1 cell line were analyzed for ECHS1 protein levels using western blot. The effectiveness of ECHS1-RNA interference was also determined using western blot. Proliferation and migration of the siECHS1 cells were respectively measured with the CCK-8 and transwell assays. Phosphorylation of PKB and GSK3β was assessed using western blot. ECHS1 protein levels were significantly higher in poorly differentiated cells than in well-differentiated cells and immortalized gastric epithelial mucosa cells. Stable expression of ECHS1 shRNA was associated with an over 41% reduction in the ECHS1 protein levels of siECHS1 cells. Constitutive knockdown of the ECHS1 gene in siECHS1 cells was associated with significantly inhibited cell proliferation and migration. We also observed decreased levels of PKB and GSK3β phosphorylation in siECHS1 cells. ECHS1 expression is increased in

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human gastric cancer cells. Increased ECHS1 expression activates PKB and GSK3β by inducing the phosphorylation of the two kinases. ECHS1 may play important roles in gastric cancer cell proliferation and migration through PKB- and GSK3β-related signaling pathways.

Keywords: ECHS1, PKB, GSK3β, Gastric cancer, Cell proliferation, Cell migration, Interference, β-Oxidation pathway, Signaling pathways, Cells

INTRODUCTION

Gastric cancer is one of the most commonly diagnosed cancers, and it is a leading cause for cancer-related mortality, especially in Eastern Asia, Eastern Europe and South America [1, 2]. Rates of early diagnosis and long-term survival in gastric cancer remain very low, so it is crucial to gain mechanistic insights into the oncogenesis of gastric cancer in order to develop effective therapies for this highly lethal disease [3].

Abnormalities in several signaling pathways are known to be involved in gastric cancers [4, 5]. The PI3K-PKB pathway is one example. It has been implicated in many cancers due to its essential role in cell proliferation regulation, cell cycle progression and cell survival [6–8]. The phosphorylation of glycogen synthase kinase 3 (GSK3) by PKB regulates cell differentiation, proliferation and migration, and is also involved in tumorigenesis [9–11]. Reducing PKB activation is a target for gastric cancer treatment, and many therapeutic interventions targeting PKB have been developed for clinical use [12].

ECHS1 has an essential role in the second step of mitochondrial fatty acid oxidation [13]. In recent years, ECHS1 has been implicated in many cancers, including breast, prostate, colon and liver cancer [14–19]. ECHS1 levels were found to be elevated in needle biopsy samples from prostate cancer patients [16] and in liver cancer cells [17, 18]. Reducing ECHS1 expression in hepatocellular carcinoma cells with shRNA inhibits PKB activation and tumor growth in xenograft mice [19]. ECHS1 can inhibit the activity of signal transducer and activator of transcription 3 (STAT3) by repressing its phosphorylation [20]. Proteomic analysis and in vitro study reveal that the PI3K-PKB and STAT3 signaling pathways are functionally linked in cancer [21, 22]. These findings suggest that ECHS1 has a functional role in gastric cancer, which warrants more focused and detailed research on the functions of ECHS1 in human cancer.

In this study, we investigated the roles of ECHS1 in gastric cancer development and progression. We examined the expression of the ECHS1 gene in a variety of gastric cancer cell lines and in an immortalized human gastric epithelial mucosa cell line. We explored the effect of ECHS1 attenuation on cell proliferation, cell migration and PKB and GSK3β protein activation.

MATERIAL AND METHODS

Cell lines and culture conditions

The immortalized human gastric epithelial mucosa cell line GES-1, the human gastric cancer cell lines SGC-7901 and BGC-823, and the human embryonic kidney cell line 293T were taken from stocks from our own laboratory. The human gastric cancer cell line MKN-28 was purchased from the Forth Military Medical University Cell Bank. The SGC-7901, BGC-823 and MNK-28 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The GES-1 and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum. The ECHS1 shRNA vector or empty vector stably transfected cell lines were grown in DMEM supplemented with 10% FBS and 1 μg/ml puromycin. All of the cells were cultured in an incubator at 37ºC in a 5% CO_2 atmosphere. Cell passage was done by trypsinization with a 1:1 mixture of 0.02% ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin.

Construction and screening of ECHS1-targeting shRNA vectors

We used the PU6 vector to express shRNA that targets the ECHS1 gene [23]. Two pairs of ECHS1-targeting sense and antisense oligonucleotides were designed, synthesized and annealed. The resulting double-stranded oligonucleotides with overhangs were ligated with PU6 vector digested with BspMI (BveI). The design of the PU6 vector allowed easy insertion of the target double-stranded oligonucleotide fragments in the correct orientation while preventing vector self-ligation. The ECHS1 targeting sequences were designed based on the reference ECHS1 sequence from GeneBank.
The sequences of the oligonucleotide pairs (purchased from Invitrogen) were:
Pair A: sense oligo: 5'-GCU AUG AAA CGA UAU GGG CUU-3', antisense oligo: 5'-GCC CAU AUC GUU UCA UAG CUU-3';
Pair B: sense oligo: 5'-GUU CGU CAC AUC UCA UCU ACU U-3', antisense oligo: 5'-GUA GAU GAG AUG UGA CGA AUU-3'.
Positive colonies harboring the shRNA sequences were selected and cultured in a 37ºC incubator overnight. The bacterial culture (500 μl) was mixed with an equal volume of 30% sterile glycerol to prepare glycerol stock. The glycerol stocks of bacteria containing the constructed plasmids were sequenced by Life Technologies to confirm the inserted ECHS1-targeting shRNA sequences.

ECHS1 shRNA-stably transfected cell lines

To establish ECHS1 shRNA-expressing stably transfected cell lines, BGC-823 cells were seeded in 60-mm culture dishes and cultured with complete medium without antibiotics at 37ºC in a 5% CO_2 atmosphere. When the cells reached 90% confluence, plasmid transfections were performed using Lipofectamine 2000 transfection reagent (Life Technologies) in accordance with the manufacturer's instructions. Cells were transfected with the ECHS1 shRNA plasmid or the empty PU6 vector and cultured for 24 h. Then, 0.5 μg/ml

puromycin was added to the complete medium without antibiotics to select for stably transfected cell clones. The selection medium was changed every 2–4 days when necessary. Puromycin-resistant colonies started to form about 10 days post-transfection. Single colonies were isolated about 14 days post-transfection. The selected stably transfected cell lines were expanded and examined using western blot to confirm ECHS1 knockdown.

Western blot analysis
Cultured cells (about 80% confluent) were rinsed three times with pre-chilled phosphate buffered saline (PBS), detached by trypsinization with 0.25% trypsin, and collected by centrifugation. Cell pellets were lysed with protein lysis buffer with a protease inhibitor cocktail (1:100), and sonicated on ice (5 s sonication with a waiting time of 10 s, at 100–120 W) until the lysate was clear. The cell lysates were centrifuged at 15,000 rpm at 4ºC for 15 min and the supernatants were stored at –80ºC. Protein concentrations were determined using the bicinchoninic acid assay (BCA assay). Equal amounts of protein for each sample were loaded and run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).
The antibodies used for the western blot analysis were: rabbit polyclonal anti-ECHS1 (1:500 dilution, Abcam), anti-actin (1:5000, Cell Signaling), anti-PKB (1:200, Santa Cruz Biotechnology), anti-p-PKB (Ser473; 1:500, Santa Cruz Biotechnology), anti-GSK3β (1:1000, Cell Signaling), anti-p-GSK3β (1:1000, Cell Signaling), and anti-rabbit or anti-mouse IgG conjugated with HRP (Pierce). All of the experiments were repeated three times.

Cell proliferation assay
The CCK-8 assay was used to determine the rate of cell proliferation in BGC-823 cells after ECHS1 shRNA expression. Stably transfected cells with ECHS1 shRNA or the empty PU6 vector and the parental BGC-823 cells growing in logarithmic phase were seeded in 96-well plates at 5000 cells per well in quadruplicate. Blank and control wells were set up accordingly. The CCK-8 reagent (10 μl per well) was added after the cells had attached to the wells. The 96-well plates were then incubated at 37ºC for 90 min. Absorbance values at 450nm were measured using a microplate reader. CCK-8 assays were performed at 12, 24, 36 and 48 h.

Cell migration assay
We used the transwell method to determine cell migration. The cell suspension was prepared by trypsinization of the cultured cells, washing twice with serum-free medium, triturating the cells into a single cell suspension, and adjusting the cell concentration to 1×10^5 cells/ml. The upper chambers of the transwell was placed into a 24-well plate containing 500 μl medium with 20% FBS or chemokine. The cell suspension (200 μl) was added to the upper chamber of the transwell assembly and incubated at 37ºC in a 5% CO_2 atmosphere for 16 h.

Then, the transwell assembly was washed twice with PBS and fixed with pre-chilled methanol at –20ºC for 10 min. The upper chamber of the transwell assembly was washed twice with PBS. The cells that remained on the top surface of the upper chamber were removed with a wet cotton swab. The upper chamber was then washed with PBS three times and air-dried in an inverted position. The chamber membrane was stained with 0.1% crystal violet staining solution (500 μl/well) at 37ºC for 30 min, washed three times, and air-dried. Images of migrated cells on the transwell membrane were taken at 20× magnification. The crystal violet retained on the transwell was eluted with 33% acetic acid and the OD_{570nm} value of the eluent was measured with a microplate reader to indirectly estimate the number of cells that had migrated through the transwell membrane.

Statistical analysis
All of the data were analyzed using the GraphPad Prism 5 statistical software for data processing. Experimental results are presented as the means ± standard deviation. Differences between the two sets of measurements were compared using Student's t-test. Analyses for fitness to normal distribution and homogeneity of variance of the various experimental groups were performed. A p-value of $p < 0.05$ was considered statistically significant.

RESULTS

ECHS1 expression levels in gastric cancer cells correlate with cell differentiation
We measured the expression levels of the ECHS1 gene in the immortalized human gastric epithelial mucosa cell line GES-1 and the human gastric cancer cell lines SGC-7901 and BGC-823. pCMV5-ECHS1-Myc plasmid-transfected 293T cells were used as a positive control for ECHS1 expression. Actin was used as the loading control. ECHS1 was expressed in all of the cells studied.
The expression level of ECHS1 correlated with the degree of differentiation of the gastric cells. Western blot analysis showed that the ECHS1 protein levels were higher in the poorly differentiated BGC-823 cells and moderately differentiated SGC-7901 cells than in the well-differentiated MKN-28 cells and the immortalized gastric epithelia mucosa GES-1 cells (Fig. 1A and B). Since the expression levels of ECHS1 in the gastric cancer cell line increased as the degree of differentiation decreased, and the BGC-823 cells expressed the highest level of ECHS1 protein (Fig. 1A and B), we decided to use BGC-823 cells for further experiments in this study.

Attenuation of ECHS1 expression by shRNA inhibited BGC-823 proliferation
To study the functions of ECHS1 in gastric cancer cells, we established stably transfected BGC-823 cell lines expressing shRNA targeting the ECHS1 gene using the PU6-siECHS1 vector. We named these cell lines siECHS1 cells. We also established BGC-823 cell lines stably transfected with the empty PU6 vector

as controls, which we named PU6 cells. Compared to the parental BGC-823 cells, there was a more than 41% decrease in ECHS1 protein levels in the siECHS1 cells (Fig. 2A and B). No obvious decrease in ECHS1 protein levels in the control PU6 cells was observed. We concluded that we had successfully established stably transfected siECHS1 cells that expressed less ECHS1 protein than the parental cells.
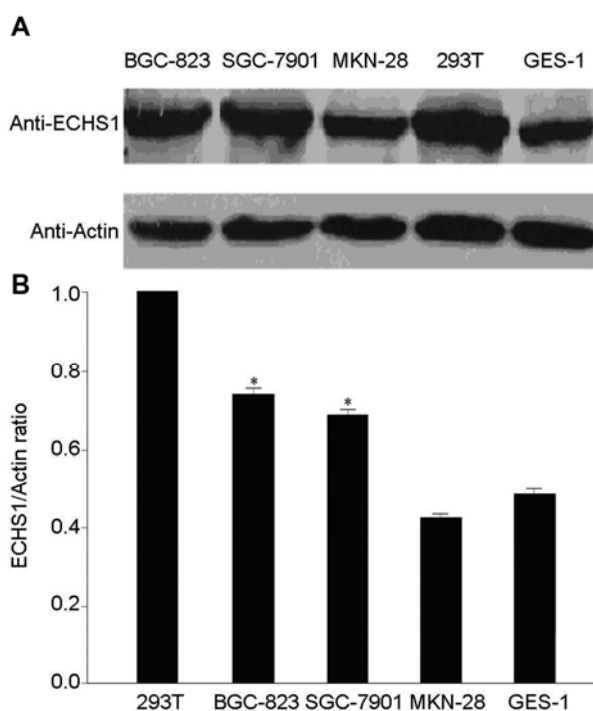

Fig. 1. ECHS1 expression in gastric cancer cells. A − Western blot analysis of ECHS1 protein levels in human gastric cancer cell lines BGC-823, SGC-7901 and MKN-28, the immortalized human gastric epithelial mucosa cell line GES-1, and pCMV-ECHS1-MYC plasmid-transfected 293T cells. Actin was used as the loading control. The quantity of the ECHS1 protein in the different cell lines is represented as the ratio of ECHS1 to actin, where the ratio in the 293T cells was arbitrarily set as 1.00. B − Comparison of ECHS1 expression in BGC-823, SGC-7901, MKN-28, GES-1 and 293T cells (means ± SD; n = 3). *p < 0.05, using Student's t-test.

We compared the proliferation of siECHS1 cells to that of the PU6 and parental BGC-823 cells using the CCK-8 assay. Cells were cultured for 12, 24, 36 or 48 h before the assay. The number of living cells was considered to be proportional to the OD_{450nm} value. The OD_{450nm} of siECHS1 cells was significantly lower than that of the PU6 or parental cells starting from 24 h after seeding (Fig. 3, p < 0.05). It remained significantly lower 36 h and 48 h after seeding (p < 0.05). These results suggested that attenuation of ECHS1 in BGC-823 cells inhibited cell proliferation via the PKB and GSK3β pathways.
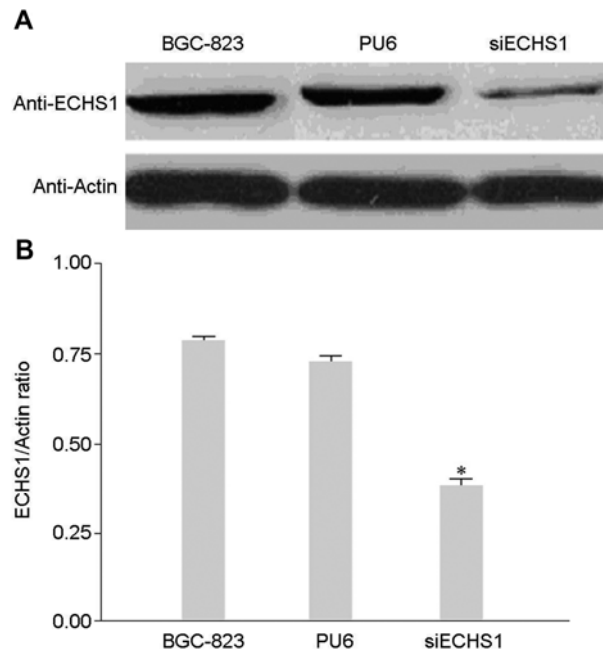
Fig. 2. Reduced ECHS1 protein levels in siECHS1 cells. A – Western blot analysis of the parental BGC-823 cells and stably transfected BGC-823 cell lines with the ECHS1 shRNA vector or the empty PU6 vector. Results for two independent cell lines are shown. siECHS1: BGC-823 cells expressing ECHS1 shRNA. PU6: BGC-823 cells stably transfected with the empty PU6 vector. B – Comparison of ECHS1 expression in BGC-823, PU6 and siECHS1 cells (means ± SD; n = 3). *p < 0.05, Student's t-test.
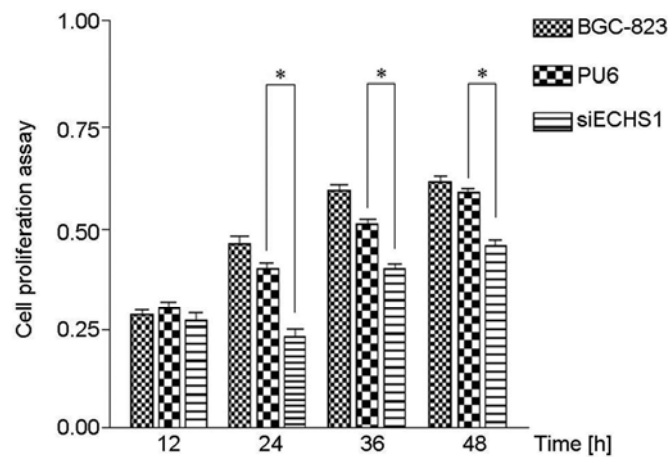

Fig. 3. Cell proliferation analysis of siECHS1 cells. BGC-823, PU6 and siECHS1 cells were seeded at the same density and cultured for 12, 24, 36 or 48 h. The CCK-8 assay was performed at these time points to estimate the number of living cells (means ± SD; n = 3). *p < 0.05, Student's t-test.

Knockdown of ECHS1 expression suppressed tumor cell migration
We investigated the effect of ECHS1 knockdown on BGC-823 cell migration using the transwell assay. Fewer siECHS1 cells migrated through the transwell membrane than PU6 cells (Fig. 4A). We estimated the number of migrated cells by measuring the OD_{570nm} value of the eluted crystal violet stain retained on the transwell membrane. About 30% fewer cells in the siECHS1 group migrated than in the PU6 group (Fig. 4B). The decrease in cell migration is statistically significant (Fig. 4B; $p < 0.05$). These results suggested that the decrease in ECHS1 levels suppressed the cell migration of the BGC-823 cells via the PKB and GSK3β pathways.
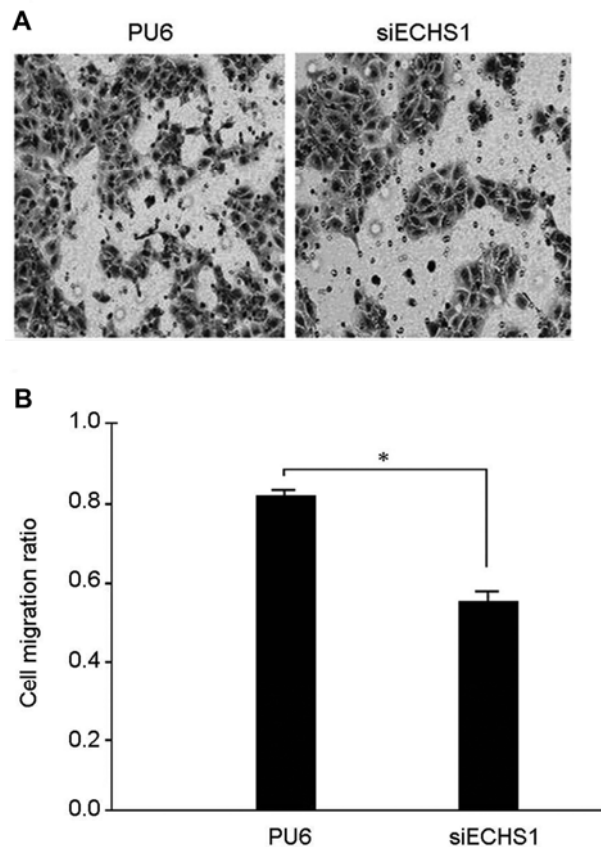

Fig. 4. Cell migration analysis of siECHS1 cells. A – Light microscopy images of crystal violet-stained PU6 or siECHS1 cells that migrated through the transwell membrane. Magnification: 20×. B – Quantification and comparison of the migration of PU6 and siECHS1 cells (means ± SD; n = 3). *p < 0.05, Student's t-test.

ECHS1 knockdown decreased PKB and GSK3β phosphorylation in BGC-823 cells

Activation of the PKB- and GSK3β-related signaling pathways is known to promote cell proliferation and migration [19]. We therefore analyzed PKB and GSK3β phosphorylation in gastric cancer cells after knockdown of ECHS1 expression. Compared to BGC-823 and PU6 cells, the level of PKB phosphorylation at serine 473 was significantly decreased in siECHS1 cells (Fig. 5A and B). The phosphorylation level of the PKB downstream protein, GSK3β, was also decreased in siECHS1 cells (Fig. 5A and B). These results indicate that the pro-proliferation and pro-migration functions of ECHS1 might act through the PKB- and GSK3β-related signaling pathways.
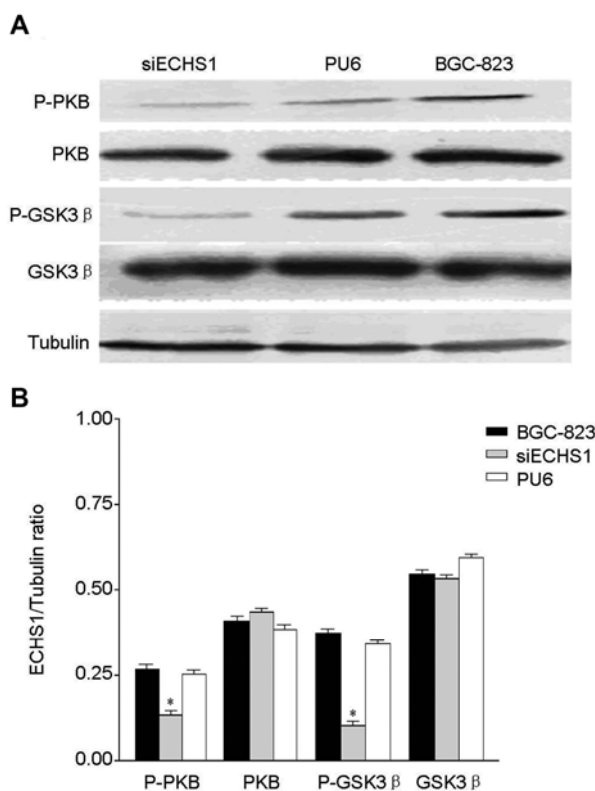
Fig. 5. Effects of ECHS1 knockdown on PKB and GSK3β phosphorylation. A – Western blot analysis of PKB phosphorylation at serine 473 (P-PKB[473]) in siECHS1, PU6 and the parental BGC-823 cells. Western blot analysis of GSK3β phosphorylation (P-GSK3β) in siECHS1, PU6 and the parental BGC-823 cells. Tubulin was used as the loading control. B – Quantification and comparison of PKB and GSK3β in BGC-823, PU6 and siECHS1 cells (means ± SD; n = 3). *p < 0.05, Student's t-test.

DISCUSSION

Although ECHS1 has been identified by proteomic or gene expression profiling in many cancer cell types and patient biopsies [14], only a few functional studies have been reported. In this study, we examined ECHS1 expression levels in gastric cancer cells and found that ECHS1 is highly expressed in these cell lines. The expression level of ECHS1 in gastric cancer cells correlated with the degree of cell differentiation. Poorly differentiated gastric cancer cells had higher levels of ECHS1 protein than well-differentiated cells or immortalized human gastric epithelial mucosa cells. Cell proliferation and migration were inhibited in BGC-823 cells, which had ECHS1 protein levels that were reduced by constitutive expression of ECHS1-specific shRNA. ECHS1 knockdown also suppressed PKB and GSKβ phosphorylation. Our results indicate that ECHS1 is involved in gastric cancer cell proliferation and migration and may function through PI3K-PKB- and GSKβ-related signaling pathways.

ECHS1 was discovered to be an important mitochondrial enzyme for fatty acid metabolism [13]. In recent years, roles in cancer cell proliferation and apoptosis have been reported for it. ECHS1 directly interacts with hepatitis B surface antigen (HBsAg) [18, 24]. Binding of HBsAg reduces ECHS1 levels and induces apoptosis of the hepatocellular carcinoma HepG2 cells [18, 25]. Proteomic analysis showed that ECHS1 expression is lower in hepatocellular carcinoma patients with hepatitis C virus infections [26]. In breast cancer MCF-7 cells, drug-induced downregulation of ECHS1 leads to MCF-7 apoptosis [14]. These studies indicate that ECHS1 has an anti-apoptotic function. Therefore, increased ECHS1 expression may lead to overproliferation of cancer cells.

Consistent with previous reports regarding colorectal, prostate, and liver cancers [15, 16, 19, 27, 28], our results showed that ECHS1 is highly expressed in gastric cancer cells, correlated with the degree of cell differentiation. Attenuation of ECHS1 expression by shRNA inhibited BGC-823 cell proliferation. Furthermore, shRNA-mediated knockdown of ECHS1 protein expression inhibited PKB activation in hepatocellular carcinoma HepG2 cells [19]. We also observed PKB inhibition in gastric cancer BGC-823 cells due to ECHS1 knockdown. Therefore, the activation of the PKB signaling may be a common mechanism for ECHS1-induced cell proliferation in cancer cells. Future efforts should focus on investigating the detailed mechanism of ECHS1-mediated PKB activation.

Most cancer cells rely on abnormal aerobic glycolysis to generate adequate energy for the incorporation of nutrients to support fast proliferation [29]. As ECHS1 is an essential mitochondrial enzyme for fatty acid metabolism, its proper expression is important for mitochondrial integrity and function. Since ECHS1 can regulate carbohydrate synthesis and glycolysis, it may have a key role in cancer cell survival during tumor growth. In human cancer cells, activation of the epidermal growth factor receptor (EGFR) can induce nuclear localization of ECHS1 and subsequent ECHS1-β-catenin interaction. ECHS1

binding to β-catenin results in histone acetylation and cyclin-D1 expression. This interaction has been associated with cell proliferation in brain tumors such as malignant glioma. These studies suggest that EGFR activation and ECHS1 expression promotes tumor growth, especially through the non-canonical Wnt signaling-mediated activation of β-catenin [11].

In this study, we found that ECHS1 knockdown suppressed GSK3β phosphorylation. PKB-dependent phosphorylation of GSK3β has been reported to regulate the Wnt signaling pathway [10]. Whether or not ECHS1 acts similarly in gastric cancers needs further exploration.

Cell migration is an important property of malignant cancers. Previous studies have established the key roles of the PI3K-PKB signaling and GSK3β in the regulation of cell migration [11, 30, 31]. Transducer of ErbB-2.1-mediated decrease of PKB and GSK3β phosphorylation in MKN28 and AGS cells inhibits the proliferation and migration of both gastric cancer cell lines [30]. In addition, GSK3β also regulated expressions of miR-96, miR-182 and miR-183, all of which are known to affect proliferation and migration of gastric cancer cells [31]. By contrast, Wnt5a induced the phosphorylation of GSK3β and enhanced tumor cell migration [11]. In accordance with the above studies, our data showed that GSK3β phosphorylation and cell migration were reduced in siECHS1 cells compared with the PU6 cells. These results strongly suggest that ECHS1 regulates gastric cancer cell migration through the GSK3β-related pathways.

Many genes related to metabolism also have important regulatory roles in cell proliferation and migration, because energy supply is a limiting factor for tumor growth and metastasis. Modulation of these metabolic genes may be a plausible strategy for cancer treatment. According to our results, shRNA-mediated decrease of ECHS1 expression was able to suppress gastric cancer cell proliferation and migration. It is still unclear whether the decreased proliferation and migration of siECHS1 cells were due to the direct functional defects of ECHS1 via PKB- or GSK3β-related pathways or the indirect effects of other molecules. Our future efforts will focus on deciphering factors that interact with ECHS1 in gastric cancer cells, to gain mechanistic insight into ECHS1 overexpression and oncogenesis.

ECHS1 could be a potential therapeutic target for gastric cancer treatment. Since ECHS1 is highly expressed in gastric cancer cells, it may also be a candidate as a biomarker for gastric cancer diagnosis. Future study will further investigate whether ECHS1 is a novel target in the control of gastric cancer.

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