TCF3/ACSS2 Axis Promotes Proliferation and Colony-Forming Capacity of Esophageal Squamous Cell Carcinoma Cells

Hongqi Li[1] and Meidong Xu[1,2]*

[1]Endoscopy Center, Zhongshan Hospital, Fudan University, Shanghai 200031, China
[2]Endoscopy Center, East Hospital, School of Medicine, Tongji University, Shanghai 200120, China

ABSTRACT

Esophageal carcinoma (ESCA), particularly esophageal squamous cell carcinoma (ESCC), is a prevalent and challenging health problem. Recent investigations have identified various transcription factors implicated in ESCC tumorigenesis and cancer progression, with potentials to serve as promising therapeutic targets. This study aimed at identifying novel therapeutic targets, particularly transcription factors and downstream effectors associated with ESCC pathogenesis. Data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases were analyzed, and the functional significance of transcription factor 3 (TCF3) in regulating ESCC aggressiveness was further studied. TCF3 was knocked down or overexpressed to investigate the ESCC cell proliferation and colony-forming capacity. RNA sequencing was performed to explore downstream effectors of TCF3. Moreover, acyl-CoA short-chain synthetase family member 2 (ACSS2) knockdown and overexpression was conducted to further examine ESCC cell proliferation and colony-forming capacity. Mouse xenograft models were utilized to access the influence of ACSS2 on ESCC tumor growth *in vivo*. The results revealed that, TCF3 was significantly elevated in ESCA tumors and associated with the of ESCA patient prognosis. TCF3 silencing suppressed ESCC colony-forming capacity and cell proliferation, in contrast, overexpression of TCF3 prominently accelerated ESCC cell proliferation and colony formation. Mechanistically, ACSS2 is a critical nucleocytosolic enzyme that converses acetate to acetyl-CoA, and acts as a TCF3 downstream molecule. TCF3 modulates the aggressiveness of ESCC by regulating the levels of ACSS2. In consistence, ACSS2 knockdown also attenuated ESCC colony-forming capacity and proliferation, while ACSS2 overexpression promoted these two phenotypes. In addition, ACSS2 knockdown cells formed significantly smaller tumors in mouse xenograft models, indicating ACSS2 as an attractive anti-cancer treatment target. In conclusion, this research identified the functional importance of TCF3/ACSS2 axis in regulating ESCC cancer progression and supported it as an attractive treatment option for ESCC treatment.

INTRODUCTION

Esophageal carcinoma (ESCA) ranks as the sixth leading cause of cancer-related death globally, with a 5-year survival rate of lower than 30%. Of note, esophageal squamous cell carcinoma (ESCC) represents one of the most common subtypes of ESCA in China, accounting for approximately 90% of diagnosed patients (Domper Arnal *et al*., 2015; Liang *et al*., 2017; Uhlenhopp *et al*., 2020). ESCC is predominant in China, parts of South America and the "Asian Esophageal Cancer Belt" that extends from Eastern Turkey, through North Iran, Uzbekistan, Kyrgyzstan, Mongolia, to China (Reed and Johnston, 1993; Zhang *et al*., 2012). In comparison, in developed countries, such as United States, United Kingdom, and France, esophageal adenocarcinoma (EAC) represents the predominant subtype (Domper Arnal *et al*., 2015). The high mortality rate of ESCA is attributed to nonspecific symptoms during early stages, delayed diagnosis at advanced stages, aggressive malignant behaviors, and resistance to current therapeutics. Therefore, developing early screening strategies and identifying novel oncogenic drivers for druggable and actionable targets may improve the prognosis of ESCA patients.

Transcriptional dysregulation is known as a critical cancer hallmark. Aberrations of transcription factor

expressions and activities can significantly promote ESCA progression, supporting the possibility of targeting oncogenic transcription factors for ESCA treatment. This study was commenced with a differential expression analysis of transcription factor 3 (TCF3, also known as E2A) in ESCA tissues and non-tumorous tissues from TCGA and GTEx databases. TCF3 belongs to the basic helix-loop-helix (bHLH) transcription factors families, the members of which were known to have critical roles in in eukaryote cell differentiation, tissue development, and disease development (Torres-Machorro, 2021). Previous studies have identified several bHLH transcription factors such as DEC1 (bHLH E40/Stra13/Sharp2), DEC2 (bHLH E41/Sharp1), TCF21 (transcription factor 21), and TWIST1 (Twist family bHLH transcription factor 1), with functions in ESCA progression and response to chemotherapy, either as tumor suppressors (DEC1 and TCF21) or oncoproteins (TWIST1 and DEC2) (Wushou *et al*., 2014; Seino *et al*., 2015; Sato *et al*., 2017; Chen *et al*., 2018; Ardalan Khales *et al*., 2020).

Our analysis of TCF3 expression levels in ESCA tissues and non-tumorous tissues identified the upregulation of TCF3 in ESCA tumors. TCF3 gene encodes two alternatively spliced variants, E12 and E47, which share over 99% identity in amino acid sequences (Murre *et al*., 1989; Henthorn *et al*., 1990). E47 can form homodimer or heterodimer with other bHLH transcription factors, whereas E12 can only form heterodimer (Patel *et al*., 2015). The dimerization of TCF3 with tissue-specific bHLH proteins contributes to various functions of TCF3 in tissue development and differentiation, for example, spermatogenesis and lymphoid specification from hematopoietic stem cells (HSCs) (Dias *et al*., 2008; Patel *et al*., 2015; Zhou *et al*., 2021). Of importance, the oncogenic fusion transcription factors formed by TCF3 and other protein partners due to chromosomal translocation play crucial roles during the tumorigenesis of acute lymphoblastic leukemia (ALL), including t(1;19)/TCF3-PBX1, t(17;19)/TCF3-HLF, t(12;19)/TCF3-ZNF384, t(19;22)/TCF3-TEF, and t(11;19)/TCF3-FL1 (Huang and Bourquin, 2020; Wang *et al*., 2021). In addition, mounting evidence also identified the overexpression of TCF3 in other cancer types and its functional importance in driving tumorigenesis and cancer progression, such as Burkett Lymphoma, breast cancer, prostate cancer, and colorectal cancer, which have qualified TCF3 as an oncoprotein (Patel *et al*., 2015; Jia *et al*., 2020; Yamazaki *et al*., 2020; López-Menéndez *et al*., 2021). By regulating its downstream molecules, for example, signaling proteins, long noncoding RNA (lncRNA), and microRNA (miRNA), TCF3 serves as an essential orchestrator of cancer cell proliferation, invasion, and chemotherapy resistance, thus nominating

TCF3 signaling as a promising therapeutic target (Liu *et al*., 2019; Zheng *et al*., 2019; López-Menéndez *et al*., 2021). However, little is known about its functional importance in ESCA pathogenesis, and in this research, our functional studies revealed that TCF3 can accelerate the proliferation and colony formation of ESCC cells.

Our subsequent investigation of potential TCF3 downstream signaling molecules indicated that ACSS2 (acyl-CoA short-chain synthetase family member 2) may be modulated by TCF3 and function as a TCF3 downstream effector. ACSS2 is known to converse acetate to acetyl-CoA, the critical metabolite for tricarboxylic acid cycle, fatty acid synthesis, and histone and transcription factor acetylation (Comerford *et al*., 2014; Moffett *et al*., 2020). By acting as the only known enzyme that utilizes free acetate to produce acetyl-CoA, ACSS2 can promote the acetylation reactions of transcription factors and histones in the nucleus (Moffett *et al*., 2020). Owing to its role in accelerating histone and transcription factor acetylation, ACSS2 is significantly implicated in driving tumorigenesis and cancer aggressiveness (Moffett *et al*., 2020). Furthermore, malignant tumor cells that have evolved with metabolic reprogramming for survival, are able to utilize acetate as a nutritional source in an ACSS2-dependent manner under stress conditions, such as limited nutrition and hypoxia (Comerford *et al*., 2014). Intriguingly, numerous evidence has identified the oncogenic roles of ACSS2 in diverse malignant tumors, which include breast cancer, renal cell carcinoma (RCC), and cervical squamous cell carcinoma (CESC) (Zhang *et al*., 2018; Yao *et al*., 2020).

This study aimed to explore the functional roles of TCF3 in ESCA tumorigenesis and cancer progression, thus elucidating the transcriptional dysregulation mechanisms in ESCA development and identifying possible therapeutic targets for ESCA treatment. The TCF3 expression levels in ESCA tissues and non-tumorous tissues from TCGA and GTEx databases were analyzed and compared. The functional roles of TCF3 during the progression of ESCC were identified by genetically manipulating its expression in ESCC cells. To explore the downstream effectors of TCF3, RNA sequencing was performed in TCF3-overexpressing ESCC cells and multiple proteins, for example, ACSS2, were investigated to be prominently upregulated in TCF3-overexpressing cells. Subsequently, the oncogenic functions of ACSS2 in driving ESCC cell proliferation and colony-forming capacity, and *in vivo* tumor growth as the downstream molecule of TCF3 were further identified. These investigations revealed the oncogenic roles of TCF3 and ACSS2 in ESCC pathogenesis and indicated TCF3/ACSS2 axis as a novel and promising therapeutic target for ESCC treatment. This research provides practical

and informative insights into the molecular mechanisms of ESCA pathogenesis by focusing on a novel signaling pathway, TCF3/ACSS2 axis, and identifies novel potential therapeutic strategies for ESCA treatment.

MATERIALS AND METHODS

Cell culture

Human ESCC cell lines, ECA109 and KYSE150, were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and routinely verified for mycoplasma contamination using Universal Mycoplasma Detection Kit (Manassas, VA, USA) according to manufacturer's instruction. ECA109 and KYSE150 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Corning, CA, USA) and 1% penicillin/streptomycin. Both cell lines were maintained in a 37°C humidified incubator with 5% CO_2.

MTT cell proliferation assay and colony formation assay

ECA109 and KYSE150 cells were seeded at 2000 cells/well in four 96-well plates, and incubated with humidity for 72 h. Subsequently, MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Biosharp, Hefei, China) was added into each plate and incubated at 37°C for 4 h. The supernatant was then carefully discarded, and DMSO (dimethyl sulfoxide) was added. The plate was shaken on an orbital shaker in darkness for 10 min, followed by measuring the absorbance (OD) at 570 nm. The same procedure was repeated for the second and third plates on day 5 and 7, respectively. Cell proliferation curves were plotted based on the OD values collected from at least three independent experiments.

Cells (8000 cells/well) were suspended in 1 mL of culture medium containing 0.4% agar and applied on top of solidified culture medium containing 0.6% agar (1 mL) in the wells of 6-well plates (Li *et al*., 2020). Cells were cultured in triplicate for 15 days, followed by staining with 0.4% crystal violet for visualization. After staining, colonies from each well were counted using ImageJ software.

Western blot

Cells were lysed in RIPA buffer (Solarbio, Beijing, China) and subjected to protein denaturation at 95°C. After being resolved in SDS-polyacrylamide gels, protein samples were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA). Membranes were incubated with primary antibodies TCF3 (1:1000, ab243152, Abcam, Cambridge, UK), ACSS2 (1:500, ab264391, Abcam) and GAPDH (1:2000, ab8245,

Abcam) overnight at 4°C. The next day, membranes were washed and then incubated with horseradish peroxidase (HRP)-conjugated IgG (1:5000 diluted in 5% non-fact milk; Bioss, Beijing, China). Protein bands were visualized and acquired using the GelDoc Go Gel Imaging System (Bio-Rad, Hercules, CA, USA), and the gray values of each band were measured using ImageJ. All protein gray values were normalized to the loading control to plot bar graphs.

Establishment of knockdown and overexpressing cell lines

The shRNA plasmids targeting human TCF3 (shTCF3: ATTGTGCCTAAGCGAAATATT) and ACSS2 (shACSS2: GCTTCTGTTCTGGGTCTGAAT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The shRNA plasmid, pMD2.G and psPAX2 plasmids were introduced into HEK293T cells (Procell Life Science & Technology, Wuhan, China) by transfection with polyethylenimine (PEI; Beyotime, Shanghai, China) to generate lentivirus for 48 h (Yang *et al*., 2016). Lentiviral supernatant was then collected, filtered and applied to infect ESCC cells for 24 h with the addition of polybrene (10 μg/mL; Millipore). Next day, lentiviral supernatant was discarded and fresh medium was added, followed with the knockdown cells selected under puromycin treatment (1 μg/mL, Beyotime) for at least 7 days. TCF3 and ACSS2 knockdown was subsequently validated through western blot.

The TCF3 or ACSS2 open reading frames (ORFs) were cloned into pSIN4-IRES-puro vectors (Addgene, Watertown, MA, USA), respectively. Lentiviral packaging and transduction of the expression plasmids into ESCC cells were performed as described above. Overexpression of TCF3 and ACSS2 was validated by western blot.

RNA extraction, real-time PCR, and RNA sequencing

Total RNA was extracted from TCF3 parental and ACSS2 knockdown or overexpressed cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described previously (Li *et al*., 2020). The extracted RNA was then reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). TaqMan real-time PCR (RT-PCR) assays were then conducted using the cDNA produced from reverse transcription, primers, SYBR Green (Bio-Rad) and TaqMan enzyme depending on the manufacturer's instruction. Each sample was prepared in triplicate with GAPDH serving as a housekeeping control. Primers added for TaqMan RT-PCR assays were listed in Table I.

For RNA sequencing, library preparation was carried out. RNA isolated from cells was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to determine the quantity. Subsequently, high-

quality RNA was applied for sequencing using HiSeq2500 (Illumina Inc., San Diego, CA, USA) at Genechem Biotechnology Co., Ltd. (Shanghai, China) (Li *et al.*, 2020).

Table I. Primer sequences used for TaqMan real-time PCR assays.

Gene	Primer (5'–3')
Human *ACSS2*	F GGATCACTGGTCATTCCTAC
	R GTGCTGTGTAGAACTTGGTC
Human *TCF3*	F GCAGCCTAGACACGCAGCCC
	R GTAGGCGGTGGCATCCCTGC
Human *GAPDH*	F TGACTTCAACAGCGACACCCA
	R CACCCTGTTGCTGTAGCCAAA

Animal studies

To study the influence of ACSS2 on ESCC tumor growth *in vivo*, a mouse xenograft model was established. Specifically, ten 4-week-old male BALB/c nude mice obtained from Vital River Laboratory (Beijing, China) were maintained under sterile conditions at 25°C with 50% humidity. Mice were randomly divided into two groups (n=5 per group): ECA109 parental cell injection and ACSS2 knockdown cell injection. ECA109 cells suspended in PBS/Matrigel mixture (1:1) were injected as 5×10^5 tumor cells per flank into the left and right flanks of the mice. Length and width of the subcutaneous tumors were then measured weekly for eight weeks, and the volumes were calculated as: $V = 0.5 \times length \times width^2$.

Data analysis

The expression levels of TCF3 in ESCA and non-tumor tissues from both TCGA and GTEx databases were analyzed and compared using the gene expression profiling interactive analysis 2 (GEPIA2) web server (Tang *et al.*, 2019). Overall survival (OS) analysis was conducted using Kaplan-Meier and log-rank test with high (25%) and low (25%) cutoffs in GEPIA2. Hazard ratios (HR) were calculated using the Cox proportional hazards (PH) model.

All data analyses were conducted by GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined using two-way ANOVA (analysis of variance) and unpaired Student's *t*-test, with significance considered at *P*-value < 0.05.

RESULTS

TCF3 overexpression is associated with poor prognosis in ESCA patients

We first compared the expression levels of TCF3 in ESCA tissues and non-tumorous tissues by analyzing

TCGA and GTEx databases. As shown in Figure 1A, the analysis of TCGA and GTEx datasets containing 182 cases of ESCA and 286 cases of non-tumor tissues indicated a significant upregulation of TCF3 in ESCA tumors when compared to non-tumorous esophagus tissues. In addition, ESCA patients with prominently higher TCF3 expression showed significantly lower overall survival rate when compared to those with lower TCF3 levels (Fig. 1B). These findings hinted the potential functional importance of TCF3 in ESCA pathogenesis, which was further investigated in this study.
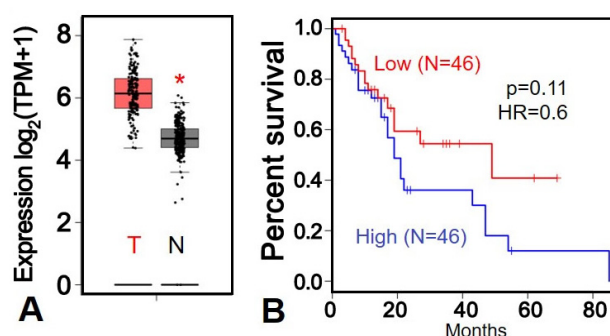

Fig. 1. TCF3 expression levels in ESCA and non-tumor tissues from TCGA and GTEx databases.
(A) Boxplot comparing TCF3 levels in ESCA tumors (red, N = 182) and normal tissues (gray, N = 286) from TCGA and GTEx databases. * *P* < 0.01. TPM, transcripts per million; T, ESCA tumors; N, normal tissues (B) Kaplan-Meier survival analysis shows the survival prognosis of ESCA patients with cutoff-high (25%) and cutoff-low (25%) TCF3 levels from the TCGA database. HR, hazard ratio, calculated by the Cox PH model in GEPIA2.

TCF3 silencing suppresses and overexpression promotes ESCC cell growth and colony formation

ESCC represents the most common ESCA histological subtype in China; therefore, two ESCC cell lines, KYSE150 and ECA109, were used for the subsequent functional studies. In order to explore the potential functions of TCF3 in ESCC, TCF3 was first silenced in both cell lines using shRNA-mediated gene knockdown, as evidenced by downregulated TCF3 mRNA (Fig. 2A) and proteins levels (Fig. 2B). Next, functional studies demonstrated that TCF3 knockdown ECA109 and KYSE150 cells formed fewer colonies than control cells (Fig. 2C-F). The colony-forming capacity of tumor cells in semisolid medium is connected to tumor cell aggressiveness *in vivo* (Mori *et al.*, 2009). Therefore, TCF3 knockdown-induced colony-forming suppression indicated the potential role of TCF3 in regulating ESCC tumor aggressiveness. Furthermore, TCF3 silencing also attenuated the proliferation rates of

ECA109 and KYSE150 cells in contrast to control cells, which further supported the oncogenic roles of TCF3 in ESCC pathogenesis (Fig. 2G, H).
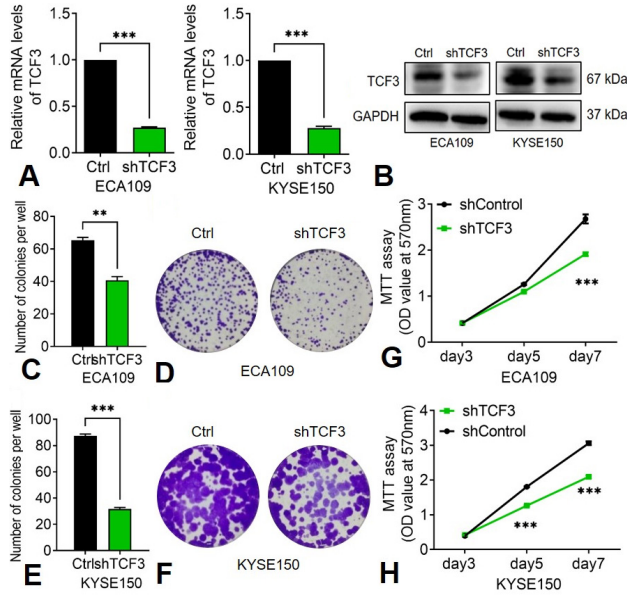

Fig. 2. TCF3 knockdown inhibits ESCC cell proliferation and colony-forming capacity. (A, B) Relative mRNA and protein levels of TCF3 in control (Ctrl) and TCF3 knockdown (shTCF3) ECA109 and KYSE150 cells. (C, E) Number of colonies formed by shTCF3 ECA109 and KYSE150 cells. (D, F) Representative images of the colonies. (G, H) Proliferation curves of shTCF3 ECA109 and KYSE150 cells by MTT assay. Western blots are representatives of at least three independent repeats. Data were exhibited as the mean ± standard error of the mean (SEM) from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

Next, we ectopically overexpressed TCF3 in ECA109 and KYSE150 cells (Fig. 3A), which reflected the elevation of TCF3 in ESCA tumors as presented in Figure 1A. TCF3 overexpression significantly enhanced the colony-forming capabilities of ESCC cells compared with control cells in both ECA109 and KYSE150 cell lines (Fig. 3B-E). In addition, ECA109 and KYSE150 proliferation rates were also prominently augmented when TCF3 was overexpressed in both cell lines (Fig. 3F, G). Taken together, these data revealed that TCF3 silencing can suppress ESCC cell colony-forming capacity and proliferation, while TCF3 overexpression can facilitate ESCC colony-forming capacity and cell proliferation, supporting the oncogenic functions of TCF3 in driving ESCC progression. The enhancement of cell colony-forming capacity and proliferation induced by TCF3

overexpression may also explain the association between high TCF3 expression and poor outcome in ESCA patients as shown in Figure 1B.
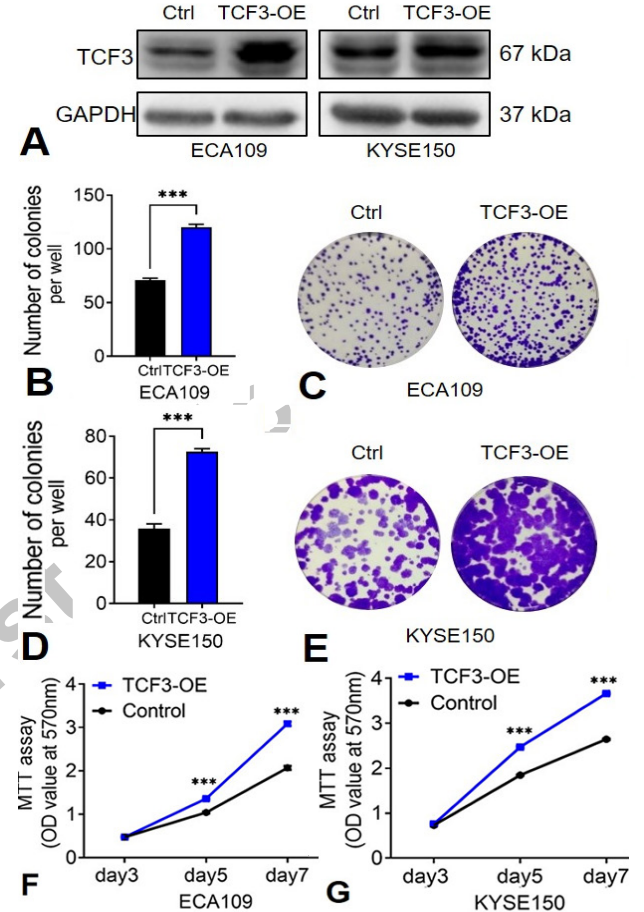

Fig. 3. TCF3 overexpression enhances ESCC cell proliferation and colony-forming capacity.
(A) Protein levels of TCF3 in TCF3-overexpressing (TCF3-OE) ECA109 and KYSE150 cells. (B, D) Number of colonies formed by TCF3-OE ECA109 and KYSE150 cells. (C, E) Representative images of the colonies. (F, G) Proliferation curves of TCF3-OE ECA109 and KYSE150 cells by MTT assay. Western blots are representatives of at least three experiments. Data exhibited as mean ± SEM from three independent experiments. ***, $P < 0.001$.

TCF3 regulates ACSS2 expression in ESCC cells

To further identify a downstream effector of TCF3 in regulating ESCC colony-forming capacity and cell proliferation, we analyzed the possible downstream targets of TCF3 using RNA sequencing technique (Fig. 4A). Strikingly, a variety of proteins were upregulated when TCF3 was overexpressed in ECA109 cells, for example, PARP2 (Poly [ADP-ribose] polymerase 2),

PARP4, PLK4 (polo-like kinases 4), and ACSS2. Among them, the elevation of ACSS2 in TCF3-overexpressing ECA109 and KYSE150 cells were further confirmed by western blot (Fig. 4B). Moreover, TCF3 silencing induced the downregulation of ACSS2 in ECA109 and KYSE150 cells (Fig. 4C). These results demonstrated the regulation of TCF3 on ACSS2 expression in ESCC cells, which were consistent with the RNA sequencing data, and the functional roles of ACSS2 in ESCC were further examined.

ACSS2 knockdown inhibits and overexpression promotes ESCC colony formation and cell proliferation

To investigate the roles of ACSS2 in regulating ESCC colony-forming capacity and cell proliferation, we silenced ACSS2 expression in ECA109 and KYSE150 by shRNA-mediated gene knockdown as confirmed by ACSS2 protein (Fig. 5A) and mRNA levels (Fig. 5B). In consistence with the effects of TCF3 knockdown, ACSS2 silencing also prominently attenuated the colony-forming capacities of both ESCC cells (Fig. 5C, D). Similarly, ACSS2 silencing also reduced the proliferation rates of both ESCC cells (Fig. 5E, F).
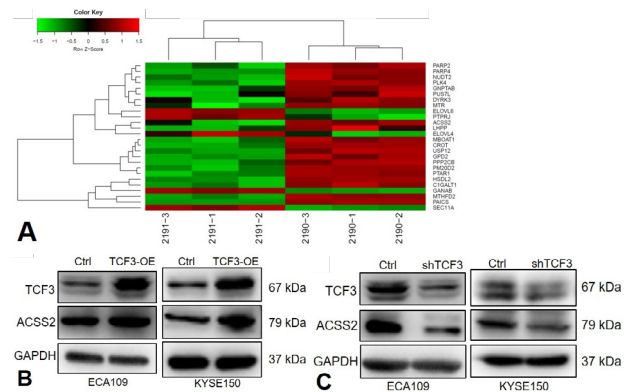

Fig. 4. TCF3 regulates the expression of ACSS2. (A) Heatmap showing the downstream genes of TCF3. Left three groups are control groups and right three groups are TCF3 overexpression groups. (B) Expression levels of TCF3 and ACSS2 in TCF3-overexpressing (TCF3-OE) ECA109 and KYSE150 cells. (C) Expression levels of TCF3 and ACSS2 in shTCF3 ECA109 and KYSE150 cells. Western blots are representatives of at least three repeats.
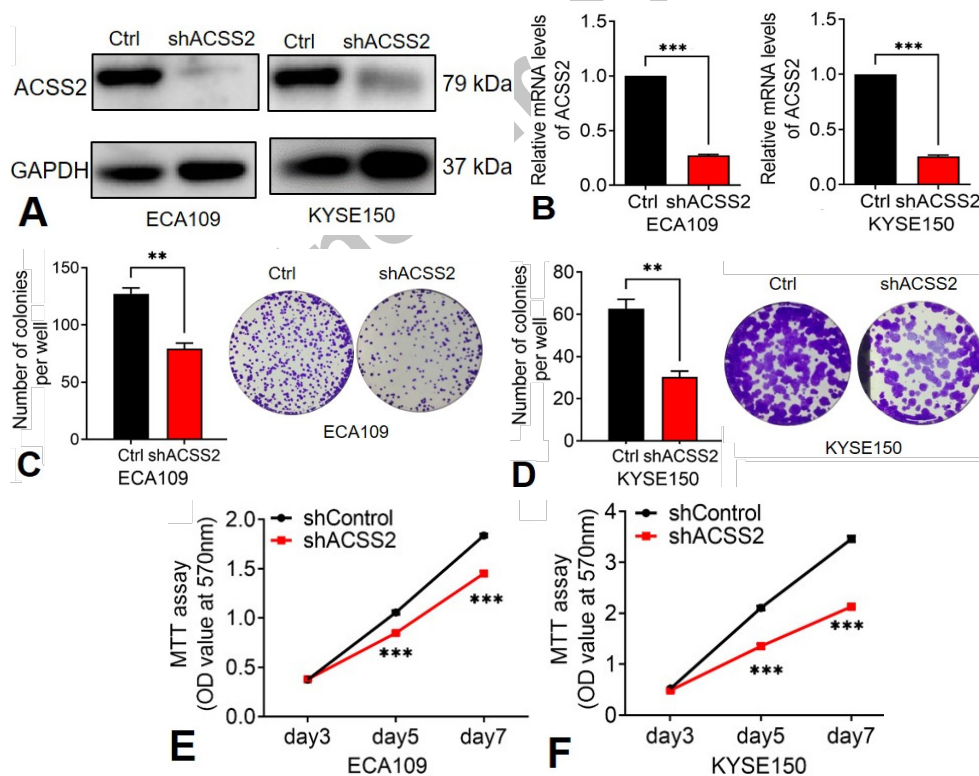

Fig. 5. ACSS2 silencing inhibits ESCC cell proliferation and colony-forming capacity. (A, B) The protein and relative mRNA levels of ACSS2 in control (Ctrl) and ACSS2 knockdown (shACSS2) ECA109 and KYSE150 cells. (C, D) Numbers of colonies formed by shACSS2 ECA109 and KYSE150 cells with representative images of the colonies shown on the right. (E, F) Proliferation curves of shACSS2 ECA109 and KYSE150 cells by MTT assay. Western blots are representative of at least three independent experiments. Data were exhibited as mean ± SEM from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.
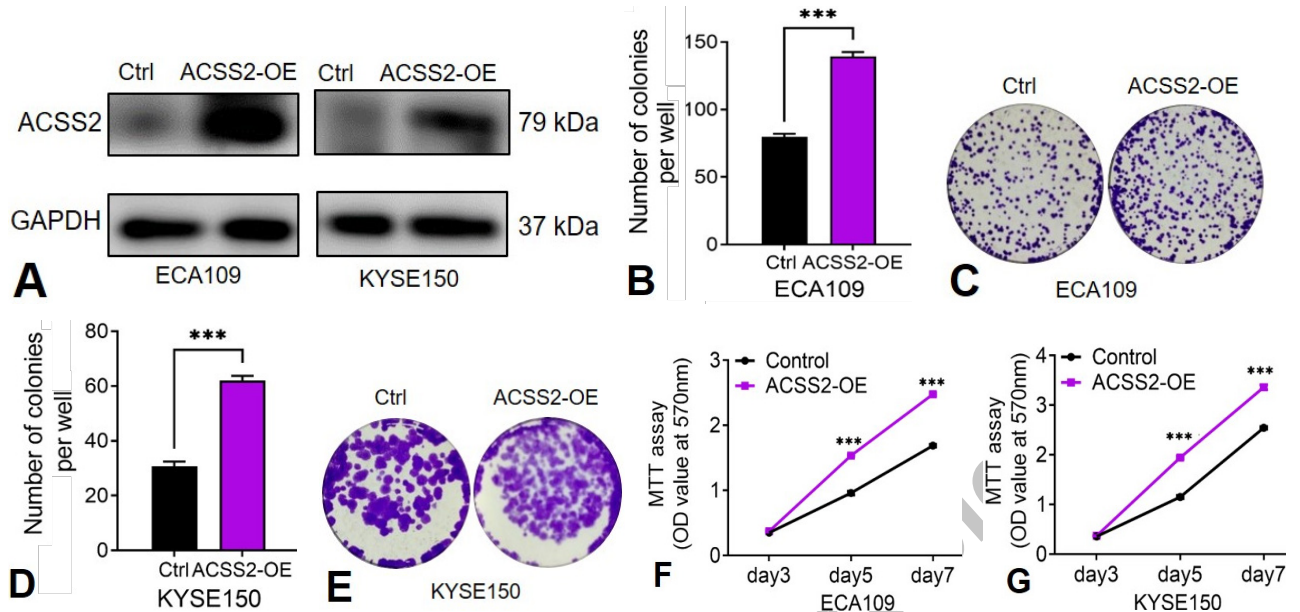
Fig. 6. ACSS2 overexpression promotes ESCC cell proliferation and colony-forming capacity. (A) Protein levels of ACSS2 in control (Ctrl) and ACSS2-overexpressing (ACSS2-OE) ECA109 and KYSE150 cells. (B, D) Numbers of colonies formed by ACSS2-OE ECA109 and KYSE150 cells with representative images of the colonies shown in C and E. (F, G) Proliferation curves of ACSS2-OE ECA109 and KYSE150 cells. Western blots are representative of at least three repeats. Data were exhibited as mean ± SEM of three independent experiments. ***, $P < 0.001$.

Of note, when ACSS2 was overexpressed in ECA109 and KYSE150 cells (Fig. 6A), the colony-forming capacities of both cell lines were significantly enhanced as shown in Figure 6B-E. Additionally, ACSS2-overexpressing ECA109 and KYSE150 cells also exhibited notably increased proliferation rates compared to controls (Fig. 6F, G). Taken together, these findings revealed the important roles of ACSS2 in regulating ESCC cell proliferation and colony-forming capacity and further supported the oncogenic roles of TCF3/ACSS2 axis in ESCC pathogenesis.

ACSS2 knockdown suppresses tumor growth in mouse xenograft models

To investigate the influence of ACSS2 on ESCC tumor growth *in vivo* and provide preliminary support for targeting TCF3/ACSS2 in ESCC treatment, we established the mouse xenograft models with ACSS2 knockdown ECA109 cells and control cells inoculated in nude mice. As shown in Figure 7A, ACSS2 knockdown ECA109 cells formed substantially smaller tumors in mice than control cells. A clear difference in tumor growth between the two groups is further displayed in the tumor growth curves (Fig. 7B). These results support the critical roles of ACSS2 in ESCC2 tumor growth and the potential for pharmacological intervention targeting this molecule to inhibit ESCC tumor growth.
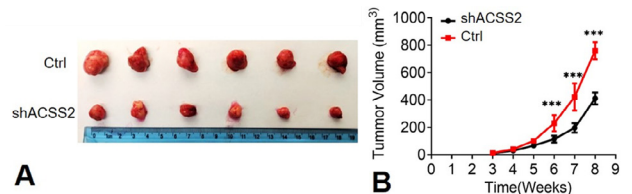

Fig. 7. *In vivo* tumor growth formed by ACSS2 knockdown ECA109 cells. (A) Representative subcutaneous tumors formed by control (Ctrl) and ACSS2 knockdown (shACSS2) ECA109 cells in nude mice (N = 5). (B) Tumor growth curves of subcutaneous tumors formed by control (Ctrl) and shACSS2 ECA109 cells in nude mice. ***, $P < 0.001$.

DISCUSSION

Dysregulated transcriptional programs caused by genetic alterations during the multi-step tumorigenesis can result in the prominent dependence of neoplastic cells on the critical regulators of gene expression, such as transcription factors (Bradner *et al*., 2017). Extensive studies have demonstrated the transcriptional addiction of ESCA cells to a variety of transcription factors, which nominated these regulators as compelling therapeutic targets (Bradner *et al*., 2017). Transcription factors, such

H. Li and M. Xu

as TP63 (Tumor Protein P63), Sox2, and KLF5 (Kruppel like factor 5), have been identified as vital regulatory factors in ESCC cells due to their essential functions in determining epigenetic modifications and gene expression patterns. Pharmacological disruptions of their functions displayed strong suppression of tumor growth in mouse models. Recently identified ECA-specific master regulator transcription factors (E74-like factor 3, ELF3; KLF5; GATA-binding factor 6, GATA6; and ETS homologous factor, EHF), are involved in epigenomic dysregulation and metabolic alterations in ECA (Ma *et al*., 2021). These core transcription factors implicated in ESCA pathogenesis provided novel insight into the mechanistic elucidation of ESCA tumorigenesis and progression, as well as the identification of potential therapeutic targets.

TCF3 is a member of the bHLH transcription factor family that serves as an important regulator in lymphoid development and cancer progression. Our study commenced with the comparisons and analyses of TCF3 expression levels in ESCA tumors and non-tumor subjects from TCGA and GTEx databases. Strikingly, TCF3 was substantially elevated in ESCA tissues in comparison to non-tumor tissues and ESCA patients with lower TCF3 levels had favorable prognosis than those with higher TCF3 (Fig. 1), suggesting its potential functional importance in ESCA pathogenesis. ESCC and EAC are two major ESCA subtypes; however, in USA, where TCGA and GTEx samples were collected, EAC represents the predominant subtype of ESCA, whereas in China, ESCC is the most frequent subtype. To further identify the clinical significance of TCF3 in ESCC subtype, it is particularly necessary to investigate the TCF3 expression profiles and analyze the prognostic impact of TCF3 in Chinese ESCC patients.

This research discovered that silencing TCF3 significantly attenuated ESCC colony-forming capacity and cell proliferation, whereas TCF3 overexpression resulted in enhanced ESCC colony-forming capacity and cell proliferation (Figs. 2 and 3), supporting the oncogenic functions of TCF3 in ESCC pathogenesis. However, targeting transcription factors such as TCF3 for cancer treatment, though feasible, remains challenging due to the lack of therapeutics to interrupt these transcription factors (Bradner *et al*., 2017). To investigate the druggable targets implicated in ESCC aggressiveness, the downstream effectors of TCF3 were identified using RNA sequencing technique, revealing a diversity of proteins regulated by TCF3, including ACSS2 (Fig. 4A). Further investigations validated that ACSS2's significant elevation upon TCF3 overexpression and downregulation following TCF3 knockdown (Fig. 4B, C). Notably, in addition to ACSS2, diverse oncogenes, for example, PARP2, PARP4, and

PLK4, were also prominently upregulated in TCF3-overexpressing ESCC cells (Fig. 4A), revealing their potential roles in driving ESCC aggressiveness, which were largely unestablished. This analysis also hinted TCF3 as a possible master regulator in ESCC. Furthermore, although ACSS2 was evidenced as a TCF3 downstream protein, whether TCF3 directly regulates the expression of ACSS2 remains largely unknown, and their possible interaction will be explored by ChIP assay in the future. Silencing ACSS2 significantly suppressed colony formation and cell growth of cultured ESCC cells, as well as tumor growth in xenograft mouse models, whereas ACSS2 ectopic overexpression facilitated ESCC colony-forming capacity and cell proliferation (Figs. 5 and 6). These aforementioned findings are consistent with a previous study that identified ACSS2 as driving ESCC cell proliferation via ACSS2/AMPK/PCNA pathway (Mi *et al*., 2019). However, the mechanisms concerning how ACSS2 regulates ESCC cell viability remain incompletely unknown. It has been documented that ACSS2 can facilitate RCC cell migration via promoting histone acetylation in regulatory regions of snail homolog 1 (SNAI1), thus augmenting the expression of SNAI1, a key mediator of epithelial-mesenchymal transition (EMT) (Yao *et al*., 2020). Additionally, ACSS2 can promote RCC invasion through elevating the levels of autophagy-related factor lysosomal-associated membrane protein 1 (LAMP1) (Yao *et al*., 2018). The mechanistic foundation of ACSS2-driven ESCC aggressiveness could be associated with its roles in driving epigenetic alterations of other genes or modulating autophagy, and these hypotheses will be further tested.

Furthermore, ACSS2 knockdown suppressed tumor growth in mouse xenograft models (Fig. 7). Of importance, a variety of small molecule inhibitors targeting ACSS2 have been developed, and some of them have been tested in malignant tumor models with prominent anti-cancer effects (Kargbo, 2019; Wen *et al*., 2019; Sabnis, 2021). Future attention will also be paid to investigating the potential of these ACSS2 inhibitors in ESCC models and whether these inhibitors could be used synergistically with current ESCC anti-cancer treatment.

CONCLUSION

Our study demonstrated that TCF3 silencing could suppress ESCC colony-forming capacity and cell growth, whereas TCF3 overexpression prominently accelerated ESCC cell proliferation and colony formation. Mechanistically, TCF3 can regulate ESCC aggressiveness by modulating ACSS2 expression. Of note, ACSS2 knockdown also attenuated ESCC colony-forming capacity and cell growth, while ACSS2 overexpression, promoted

ESCC colony-forming capacity and cell proliferation. Moreover, ACSS2 knockdown cells formed significantly smaller tumors in mouse bearing xenografts. In summary, this research identified the oncogenic functions of TCF3/ACSS2 axis in driving ESCC aggressiveness and indicated the potential of ACSS2 as a therapeutic target for ESCC treatment.

Ethical compliance
Research experiments conducted in this article with animals were approved by the animal care and use committee of Fudan University (No. HSRF2019-0017), following all guidelines, regulations, legal, and ethical standards as required for animals.

IRB approval
Not applicable.

Statement of conflict of interests
The authors have declared no conflict of interest.

REFERENCES

Ardalan Khales, S., Abbaszadegan, M.R., Majd, A. and Forghanifard, M.M., 2020. TWIST1 upregulates matrix metalloproteinase (MMP) genes family in esophageal squamous carcinoma cells. *Gene Expr. Patterns*, **37**: 119127. https://doi.org/10.1016/j.gep.2020.119127

Bradner, J.E., Hnisz, D. and Young, R.A., 2017. Transcriptional addiction in cancer. *Cell*, **168**: 629-643. https://doi.org/10.1016/j.cell.2016.12.013

Chen, Y., Zhang, C., Chen, J., Zhang, B., Zhang, H., Yang, X., Liu, J. and Wu, Q., 2018. Expression of transcription factor 21 (TCF21) and upregulation its level inhibits invasion and metastasis in esophageal squamous cell carcinoma. *Med. Sci. Monit.*, **24**: 4128-4136. https://doi.org/10.12659/MSM.909138

Comerford, S.A., Huang, Z., Du, X., Wang, Y., Cai, L., Witkiewicz, A.K., Walters, H., Tantawy, M.N., Fu, A., Manning, H.C., Horton, J.D., Hammer, R.E., McKnight, S.L. and Tu, B.P., 2014. Acetate dependence of tumors. *Cell*, **159**: 1591-1602. https://doi.org/10.1016/j.cell.2014.11.020

Dias, S., Månsson, R., Gurbuxani, S., Sigvardsson, M. and Kee, B.L., 2008. E2A proteins promote development of lymphoid-primed multipotent progenitors. *Immunity*, **29**: 217-227. https://doi.org/10.1016/j.immuni.2008.05.015

Domper Arnal, M.J., Ferrández Arenas, Á. and Lanas Arbeloa, Á., 2015. Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries. *World J. Gastroenterol.*, **21**: 7933-7943. https://doi.org/10.3748/wjg.v21.i26.7933

Henthorn, P., Kiledjian, M. and Kadesch, T., 1990. Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. *Science*, **247**: 467-470. https://doi.org/10.1126/science.2105528

Huang, Y. and Bourquin, J.P., 2020. Targeting the oncogenic activity of TCF3-HLF in leukemia. *Mol. Cell. Oncol.*, **7**: 1709391. https://doi.org/10.1080/23723556.2019.1709391

Jia, H., Wu, D., Zhang, Z. and Li, S., 2020. TCF3-activated FAM201A enhances cell proliferation and invasion via miR-186-5p/TNKS1BP1 axis in triple-negative breast cancer. *Bioorg. Chem.*, **104**: 104301. https://doi.org/10.1016/j.bioorg.2020.104301

Kargbo, R.B., 2019. Inhibition of ACSS2 for treatment of cancer and neuropsychiatric diseases. *ACS Med. Chem. Lett.*, **10**: 1100-1101. https://doi.org/10.1021/acsmedchemlett.9b00295

Li, H., Li, Q., Lian, J., Chu, Y., Fang, K., Xu, A., Chen, T. and Xu, M., 2020. MLL2 promotes cancer cell lymph node metastasis by interacting with RelA and facilitating STC1 transcription. *Cell Signal.*, **65**: 109457. https://doi.org/10.1016/j.cellsig.2019.109457

Liang, H., Fan, J.H. and Qiao, Y.L., 2017. Epidemiology, etiology, and prevention of esophageal squamous cell carcinoma in China. *Cancer Biol. Med.*, **14**: 33-41. https://doi.org/10.20892/j.issn.2095-3941.2016.0093

Liu, Z., Wu, M., Shi, H., Huang, C., Luo, S. and Song, X., 2019. DDN-AS1-miR-15a/16-TCF3 feedback loop regulates tumor progression in cervical cancer. *J. Cell. Biochem.*, **120**: 10228-10238. https://doi.org/10.1002/jcb.28307

López-Menéndez, C., Vázquez-Naharro, A., Santos, V.,

Dubus, P., Santamaría, P.G., Martínez-Ramírez, Á., Portillo, F., Moreno-Bueno, G., Faraldo, M.M. and Cano, A., 2021. E2A modulates stemness, metastasis, and therapeutic resistance of breast cancer. *Cancer Res.*, **81**: 4529-4544. https://doi.org/10.1158/0008-5472.CAN-20-2685

Ma, S., Zhou, B., Yang, Q., Pan, Y., Yang, W., Freedland, S.J., Ding, L.W., Freeman, M.R., Breunig, J.J., Bhowmick, N.A., Pan, J., Koeffler, H.P. and Rowsey, D.C., 2021. A transcriptional regulatory loop of master regulator transcription factors, PPARG, and fatty acid synthesis promotes esophageal adenocarcinoma. *Cancer Res.*, **81**: 1216-1229. https://doi.org/10.1158/0008-5472.CAN-20-0652

Mi, L., Zhou, Y., Wu, D., Tao, Q., Wang, X., Zhu, H., Gao, X., Wang, J., Ling, R., Deng, J., Mao, C. and Chen, D., 2019. ACSS2/AMPK/PCNA pathway-driven proliferation and chemoresistance of esophageal squamous carcinoma cells under nutrient stress. *Mol. Med. Rep.*, **20**: 5286-5296. https://doi.org/10.3892/mmr.2019.10735

Moffett, J.R., Puthillathu, N., Vengilote, R., Jaworski, D.M. and Namboodiri, A.M., 2020. Acetate revisited: a key biomolecule at the nexus of metabolism, epigenetics, and oncogenesis – Part 2: Acetate and ACSS2 in health and disease. *Front. Physiol.*, **11**: 580171. https://doi.org/10.3389/fphys.2020.580171

Mori, S., Chang, J.T., Andrechek, E.R., Matsumura, N., Baba, T., Yao, G., Kim, J.W., Gatza, M., Murphy, S. and Nevins, J.R., 2009. Anchorage-independent cell growth signature identifies tumors with metastatic potential. *Oncogene*, **28**: 2796-2805. https://doi.org/10.1038/onc.2009.139

Murre, C., McCaw, P.S. and Baltimore, D., 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell*, **56**: 777-783. https://doi.org/10.1016/0092-8674(89)90682-X

Patel, D., Chinaranagari, S. and Chaudhary, J., 2015. Basic helix loop helix (bHLH) transcription factor 3 (TCF3, E2A) is regulated by androgens in prostate cancer cells. *Am. J. Cancer Res.*, **5**: 3407-3421.

Reed, P.I. and Johnston, B.J., 1993. The changing incidence of oesophageal cancer. *Endoscopy*, **25**: 606-608. https://doi.org/10.1055/s-2007-1010414

Sabnis, R.W., 2021. Amide-substituted condensed pyridine derivatives as ACSS2 inhibitors for treating cancer. *ACS Med. Chem. Lett.*, **12**: 1870-1871. https://doi.org/10.1021/acsmedchemlett.1c00571

Sato, H., Wu, Y., Kato, Y., Liu, Q., Hirai, H.,

Yoshizawa, T., Morohashi, S., Watanabe, J. and Kijima, H., 2017. DEC2 expression antagonizes cisplatin-induced apoptosis in human esophageal squamous cell carcinoma. *Mol. Med. Rep.*, **16**: 43-48. https://doi.org/10.3892/mmr.2017.6571

Seino, H., Wu, Y., Morohashi, S., Kawamoto, T., Fujimoto, K., Kato, Y., Takai, Y. and Kijima, H., 2015. Basic helix-loop-helix transcription factor DEC1 regulates the cisplatin-induced apoptotic pathway of human esophageal cancer cells. *Biomed. Res.*, **36**: 89-96. https://doi.org/10.2220/biomedres.36.89

Tang, Z., Kang, B., Li, C., Chen, T. and Zhang, Z., 2019. GEPIA2: An enhanced web server for large-scale expression profiling and interactive analysis. *Nucl. Acids Res.*, **47**: W556-W560. https://doi.org/10.1093/nar/gkz430

Torres-Machorro, A.L., 2021. Homodimeric and heterodimeric interactions among vertebrate basic helix-loop-helix transcription factors. *Int. J. mol. Sci.*, **22**: 12855. https://doi.org/10.3390/ijms222312855

Uhlenhopp, D.J., Then, E.O., Sunkara, T. and Gaduputi, V., 2020. Epidemiology of esophageal cancer: update in global trends, etiology and risk factors. *Clin. J. Gastroenterol.*, **13**: 1010-1021. https://doi.org/10.1007/s12328-020-01237-x

Wang, Y., Xue, Y.J., Lu, A.D., Jia, Y.P., Zuo, Y.X. and Zhang, L.P., 2021. Long-term results of the risk-stratified treatment of TCF3-PBX1-positive pediatric acute lymphoblastic leukemia in China. *Clin. Lymphoma Myeloma Leuk.*, **21**: e137-e144. https://doi.org/10.1016/j.clml.2020.09.009

Wen, H., Lee, S., Zhu, W.G., Lee, O.J., Yun, S.J., Kim, J. and Park, S., 2019. Glucose-derived acetate and ACSS2 as key players in cisplatin resistance in bladder cancer. *Biochim. biophys. Acta Mol. Cell Biol. Lipids*, **1864**: 413-421. https://doi.org/10.1016/j.bbalip.2018.06.005

Wushou, A., Hou, J., Zhao, Y.J. and Shao, Z.M., 2014. *Twist-1* up-regulation in carcinoma correlates to poor survival. *Int. J. mol. Sci.*, **15**: 21621-21630. https://doi.org/10.3390/ijms151221621

Yamazaki, T., Liu, L., Conlon, E.G. and Manley, J.L., 2020. Burkitt lymphoma-related *TCF3* mutations alter TCF3 alternative splicing by disrupting hnRNPH1 binding. *RNA Biol.*, **17**: 1383-1390. https://doi.org/10.1080/15476286.2020.1772559

Yang, S., Shi, H., Chu, X., Zhou, X. and Sun, P., 2016. A rapid and efficient polyethylenimine-based transfection method to prepare lentiviral or retroviral vectors: useful for making iPS cells and

transduction of primary cells. *Biotechnol. Lett.*, **38**: 1631-1641. https://doi.org/10.1007/s10529-016-2123-2

Yao, L., Guo, X. and Gui, Y., 2018. Acetyl-CoA synthetase 2 promotes cell migration and invasion of renal cell carcinoma by upregulating lysosomal-associated membrane protein 1 expression. *Cell. Physiol. Biochem.*, **45**: 984-992. https://doi.org/10.1159/000487293

Yao, L., Jiang, L., Zhang, F., Li, M., Yang, B., Zhang, F. and Guo, X., 2020. Acetate promotes *SNAI1* expression by ACSS2-mediated histone acetylation under glucose limitation in renal cell carcinoma cell. *Biosci. Rep.*, **40**: BSR20200382. https://doi.org/10.1042/BSR20200382

Zhang, H.Z., Jin, G.F. and Shen, H.B., 2012. Epidemiologic differences in esophageal cancer between Asian and Western populations. *Chin. J. Cancer*, **31**: 281-286. https://doi.org/10.5732/cjc.011.10390

Zhang, S., He, J., Jia, Z., Yan, Z. and Yang, J., 2018. Acetyl-CoA synthetase 2 enhances tumorigenesis and is indicative of a poor prognosis for patients with renal cell carcinoma. *Urol. Oncol.*, **36**: 243.e9-243.e20. https://doi.org/10.1016/j.urolonc.2018.01.013

Zheng, L., Hu, N. and Zhou, X., 2019. TCF3-activated LINC00152 exerts oncogenic role in osteosarcoma through regulating miR-1182/CDK14 axis. *Pathol. Res. Pract.*, **215**: 373-380. https://doi.org/10.1016/j.prp.2018.12.031

Zhou, D., Fan, J., Liu, Z., Tang, R., Wang, X., Bo, H., Zhu, F., Zhao, X., Huang, Z., Xing, L., Tao, K., Zhang, H., Nie, H., Zhang, H., Zhu, W., He, Z. and Fan, L., 2021. TCF3 regulates the proliferation and apoptosis of human spermatogonial stem cells by targeting PODXL. *Front. Cell Dev. Biol.*, **9**: 695545. https://doi.org/10.3389/fcell.2021.695545