Cellular Signalling 55 (2019) 8–16

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

TCF7L2 and EGR1 synergistic activation of transcription of LCN2 via an ERK1/2-dependent pathway in esophageal squamous cell carcinoma cells

Yan Zhao[a,b,c,1], Qiaoxi Xia[b,d], Yan Liu[e], Wenjing Bai[b,d], Yubin Yao[f], Jiyu Ding[b,d], Ling Lin[b,d], Zhennan Xu[e], Zhixiong Cai[g], Shaohong Wang[a], Enmin Li[b,d], Haixiong Xu[e], Bingli Wu[b,d,*], Liyan Xu[b,c,*], Zepeng Du[a,h,**,1]

[a] Department of Pathology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China
[b] Key Laboratory of Molecular Biology in High Cancer Incidence Coastal Chaoshan Area of Guangdong Higher Education Institutes, Shantou University Medical College, Shantou 515041, China
[c] Institute of Oncologic Pathology, Shantou University Medical College, Shantou 515041, China
[d] Department of Biochemistry and Molecular Biology, Shantou University Medical College, Shantou 515041, China
[e] Department of Neurosurgery, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China
[f] Department of Radiology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China
[g] Department of Cardiology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China
[h] Guangdong Province Key Laboratory of Malignant Tumor Epigenetics and Genes Regulation, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China

ARTICLE INFO

Keywords:
TCF7L2
EGR1
Key transcription activation protein
LCN2
Esophageal squamous cell carcinoma

ABSTRACT

High level expression of lipocalin 2 (LCN2) usually indicates poor prognosis in esophageal squamous cell carcinoma (ESCC) and many other cancers. Our previous study showed LCN2 promotes migration and invasion of ESCC cells through a novel positive feedback loop. However, the key transcription activation protein (KTAP) in the loop had not yet been identified. In this study, we first predicted the most probable KTAPs by bioinformatic analysis. We then assessed the transcription regulatory regions in the human LCN2 gene by fusing deletions of its 5′-flanking region to a dual-luciferase reporter. We found that the region −720/−200 containing transcription factor 7-like 2 (TCF7L2) (−273/−209) and early growth response 1 (EGR1) (−710/−616) binding sites is crucial for LCN2 promoter activity. Chromatin immunoprecipitation (ChIP) experiments demonstrated that TCF7L2 and EGR1 bound directly to their binding sites within the LCN2 promoter as KTAPs. Mechanistically, overexpression of TCF7L2 and EGR1 increased endogenous LCN2 expression via the ERK signaling pathway. Treatment with recombinant human LCN2 protein enhanced activation of the ERK pathway to facilitate endogenous LCN2 expression, as well as increase the expression level of TCF7L2 and EGR1. Treatment with the MEK inhibitor U0126 inhibited the activation by TCF7L2 or EGR1 overexpression. Moreover, overexpression of TCF7L2 or EGR1 accelerated the migration and invasion of ESCC cells. A synergistic effect was observed between TCF7L2 and EGR1 in amplifying the induction of LCN2 and enhancing migration and invasion. Taken together, our study indicates that TCF7L2 and EGR1 are the KTAPs of LCN2, within a positive "LCN2 → MEK/ERK → LCN2" path, to promote the migration and invasion of ESCC cells. Based on their clinicopathological significance, LCN2 and its two expression regulators TCF7L2 and ERG1 might be therapeutic targets for ESCC.

1. Introduction

Globally, esophageal cancer is the eighth most common malignancy, as well as the sixth most common fatal cancer worldwide. Esophageal cancer has two histological types, adenocarcinoma and squamous cell carcinoma, the latter is one of the four common causes of death in China, with an estimated 477,900 new cases and 375,000 deaths for 2015 [1]. Thus, it is important to find out the key molecules in the

* Corresponding authors at: Key Laboratory of Molecular Biology in High Cancer Incidence Coastal Chaoshan Area of Guangdong Higher Education Institutes, Shantou University Medical College, Shantou 515041, China.
** Corresponding author at: Department of Pathology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China.
E-mail addresses: blwu@stu.edu.cn (B. Wu), lyxu@stu.edu.cn (L. Xu), zepdu@126.com (Z. Du).
[1] Author names in bold designate share co-first authorship.

https://doi.org/10.1016/j.cellsig.2018.12.007
Received 11 August 2018; Received in revised form 11 December 2018; Accepted 14 December 2018
Available online 14 December 2018
0898-6568/

process of ESCC.

Lipocalin 2 (LCN2), also called neutrophil gelatinase-associated lipocalin (NGAL), is a 25 kDa secreted protein belonging to the lipocalin family. LCN2 tightly binds to bacterial siderophores, possibly serving as a potent bacteriostatic agent by sequestering iron, to play important roles in the acute phase response [2], infection and ischemia/reperfusion injury in kidneys [3], the cardiovascular system [4] and brain [5]. LCN2 forms a heterodimer with MMP-9 through disulfide bonds to induce the invasion of several tumor types, including esophageal, breast, lung, gastric and brain cancers [6–10].

In general, elevated levels of LCN2 are indicative of poor prognosis in various cancers [11]. These features characterize LCN2 as a potential biomarker in malignancy. Our previous research indicates LCN2 is highly expressed in ESCC and is significantly associated with poor prognosis for patients with ESCC [12]. Altered LCN2 expression in disease has led investigators to examine the mechanisms of its transcriptional regulation. We previously found that LCN2 is overexpressed in gastric carcinoma, and its induction by 12-o-tetradecanoylphorbol-13-acetate (TPA) is controlled by CCAAT/enhancer-binding protein beta (C/EBPβ) [13]. IFNγ and TNFα also induce LCN2 expression and secretion through STAT1 and NF-κB signaling [14], and E74-like factor 3 (ELF3) regulates LCN2 expression in chondrocytes in cooperation with NF-κB [15]. LCN2 is induced by Th17 cytokines, such as IL-17A, IL-22, and TNF-α, in colonic epithelial cells [16]. These studies indicate that distinctive regulatory elements, transcription factors and signaling pathways might contribute to the induced expression of LCN2 in different cell types and in response to various factors.

In our previous study, an “LCN2 → MEK/ERK → LCN2” positive feedback loop was shown [17]. LCN2 not only facilitates cancer cell migration and invasion, but also increases the level of pERK1/2, which in turn leads to an increase of LCN2 at both the protein and mRNA levels [17]. However, the key transcriptional activation protein (KTAP) in this loop has not yet been identified. In the present study, we explored the roles of key DNA sequence elements, transcription factors, and the MEK/ERK signal transduction pathway in regulating the “LCN2 → MEK/ERK → LCN2” loop in human ESCC cells.

2. Materials and methods

2.1. Reagents, antibodies and plasmids

Recombinant human LCN2 protein (CF, 1757-LC) was purchased from R&D SYSTEMS (USA). LCN2 (D4M8L) rabbit monoclonal antibody (mAb, #44058), TCF7L2 (C48H11) rabbit mAb (#2569, ChIP Grade), EGR1 (15F7) rabbit mAb (#4153, ChIP Grade) and HRP-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-ERK antibody (E-4, sc-7383) and ERK 1 antibody (G-8, sc-271,269) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GAPDH (MA515738) and secondary goat anti-mouse IgG (H + L) antibody (HRP, 31430) were purchased from Thermo Invitrogen.

Expression plasmids pCMV-TCF7L2, pCMV-TCF7L2-HA, pCMV-EGR1 and pCMV-EGR1-HA were purchased from Sino Biological Inc. of China. The firefly luciferase-expressing plasmid pGL3-Basic (pGLB) and Renilla luciferase-expressing plasmid pRL-TK were purchased from Promega of USA. Eight overlapping DNA fragments of the 5′-flanking region of the human LCN2 promoter (−1, 431/+84, −945/+84, −657/+84, −416/+84, −236/+84, −191/+84, −110/+84, and − 78/+84) were amplified from genomic DNA derived from the ESCC SHEEC cell line by polymerase chain reaction (PCR). The PCR products were cloned into the pGEM-Teasy vector, then directionally subcloned into the pGL3-Basic vector to create the eight constructs, namely pGLB-1431, pGLB-945, pGLB-657, pGLB-416, pGLB-236, pGLB-191, pGLB-110 and pGLB-78. The recombinant clones were identified by restriction endonuclease mapping [18]. All relevant regions of the final constructs were confirmed by DNA sequencing.

2.2. Cell culture, transfection and inhibitor treatments

Two human ESCC cell lines were utilized in this study. The KYSE150 cell line was a generous gift from Professor Dong Xie (Shanghai Institutes for Biological Sciences, China). The ECa109 cell line was purchased from the Chinese Academy of Science. KYSE150 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (HYCLONE, USA) with 10% fetal bovine serum. ECa109 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose (Gibco, USA), with 5% fetal bovine serum. All cells were grown in a humidified 5% CO_2 atmosphere at 37 °C, and maintained in media supplemented with antibiotics (100 units/mL penicillin-G and 100 μg/mL streptomycin, HYCLONE, USA). All human ESCC cell lines in this study were authenticated by short tandem repeat (STR) DNA profiling (Shanghai Genechem Co., Ltd).

KYSE150 and ECa109 cells were inoculated into plates and cultured for 12–24 h until 70–90% confluence. Plasmids were transfected into KYSE150 or ECa109 cells using Lipofectamine 3000 (Invitrogen, USA) according to the user's guide. After transfection, cells were incubated for another 48 h before being harvested for further analysis. To investigate the effects of rhLCN2 on signaling pathways, cells were treated with 0–4 μg/mL rhLCN2 for 24 h before being harvested for western blot analysis. All experiments involving treatment with rhLCN2 were performed in serum-free conditions.

MEK Inhibitor U0126 is a chemically synthesized organic compound that inhibits the kinase activity of MAP kinase kinase (MAPKK or MEK). To confirm the MEK/ERK pathway, KYSE150 cells were grown in RPMI medium for 16 h, and then the MEK inhibitor U0126 (Promega, USA) was added to the medium to a final concentration of 20 μM. TCF7L2 or EGR1 was then transfected into cells, and the cells were incubated for 48 h before harvesting and lysing. Protein samples were analyzed by SDS-PAGE and western blot.

2.3. Online database analyses

Transcription factors (TFs) that potentially target the LCN2 promoter were predicted by the combination of the UCSC genome browser (http://genome.ucsc.edu/) and the JASPAR (http://jaspar.genereg.net/) CORE database [19,20]. The 1Kb promoter region before the transcription start site of LCN2 was observed in USCS, and the track “JASPAR” containing transcription factor names and positions were selected to be visualized. The 1Kb promoter nucleic acid sequence of LCN2 was downloaded from USSC, then scanned in the JASPAR database, using a threshold of 90%, to find potential transcription factor binding sites.

Differential expression of TCF7L2, EGR1 and LCN2 genes in multiple cancers, including esophageal cancer, were obtained from the Oncomine database (https://www.oncomine.org/resource/main.html), which is a collection of cancer microarrays with a web-based data-mining platform. Oncomine needs a registration and log in before use. The analysis type selected was the Cancer vs. Normal Analysis [21]. The threshold was designed with the following parameters: *p*-value of 1E-4, fold-change of 2, and gene ranking of 10% of all genes measured. TCF7L2 and EGR1 expression levels from the dataset of Aoyagi et al. were applied for the survival analyses [22]. The X-tile 3.6.1 program was applied to define the optimal cutoff point for the expression level of TCF7L2 and EGR1, in order to classify ESCC patients into high and low expression groups, following the Kaplan–Meier and log-rank test survival analyses by GraphPad Prism7.

2.4. Western blotting

Protein expression levels were analyzed by western blot as described previously [17]. Cells were washed with ice-cold PBS, lysed on ice in RIPA buffer containing 1× Halt™ Protease and Phosphatase Inhibitor Cocktail (78,445, EDTA-free, 100×, Thermo, USA). Protein

concentration in the lysates was detected using a BCA protein assay kit (Pierce Biotechnology, USA). Then equal amounts of whole cell lysates (20 μg or 40 μg) were boiled at 100 °C for 10 min in loading buffer, and were resolved by 12% SDS polyacrylamide gel electrophoresis, using standard methodology, followed by transfer onto 0.22 μm PVDF membranes (Roche, Switzerland). Membranes were blocked in TBST containing 5% nonfat dry milk for 1 h at room temperature, and then incubated with primary antibody at 4 °C overnight. All primary antibodies were diluted at 1:1000, except anti-GAPDH (1:5000). After washing with TBST three times (5 min each), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:1000 or goat anti-mouse, 1:5000) for 1–2 h at room temperature. Antigen-antibody complexes were detected using Super-Signal West Pico PLUS Chemiluminescent Substrate (34,578, Thermo Fisher Scientific, USA). Photography and quantitative analyses of related immunoreactive bands were performed using a ChemiDoc XRS + Imaging System (Bio-Rad, USA). Experiments were repeated three times.

2.5. Dual-luciferase reporter assay

Cells were co-transfected with the firefly luciferase-expressing reporter plasmid (100 ng), and Renilla luciferase-expressing plasmid pRL-TK (2 ng) as the internal control, using Lipofectamine 3000 reagent according to the user guide after culturing in 96-well plates for 12–24 h. In the TCF7L2 or EGR1 overexpression experiments, ESCC cells were also transfected with pCMV-TCF7L2 or pCMV-EGR1 plasmids, respectively. After 48 h, luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega, USA) as described [23]. All transfection experiments were repeated four times.

2.6. Chromatin immunoprecipitation (ChIP)

KYSE150 cells were grown to 70–90% confluence in 10 cm plates (approximately 4×10^6 cells for each immunoprecipitation), and transfected with pCMV-TCF7L2 or pCMV-EGR1 plasmids, respectively, for 48 h. Then ChIP was performed using a Simple ChIP® Enzymatic Chromatin IP Kit (#9003, Magnetic Beads, Cell Signaling Technology). Briefly, cells were fixed with formaldehyde to cross-link histone and non-histone proteins to DNA. Chromatin was digested with micrococcal nuclease into 150–900 bp DNA/protein fragments. The enzymatically-sheared chromatin was pre-cleared with protein-G-coated magnetic beads that facilitated the capture of the immune complexes, and an aliquot was saved for use as a positive control (input DNA). Aliquots of the pre-cleared sheared chromatin were then immunoprecipitated using 2 μg antibodies against IgG, H3, TCF7L2, and EGR1, respectively, or without using an antibody. Cross-links were reversed, and DNA was purified and ready for analysis. Purified DNA was analyzed by PCR with 2 × TransStart FastPfu PCR SuperMix (Trans Gen Biotech). PCR of immunoprecipitated DNA was carried out using human LCN2 promoter-specific primers as follows: 5′-CCACCCTTCCCTGACCCTT-3′ and 5′-GTATGTGCCCTGTGGTCCC-3′ (for amplification of the LCN2–416/−206 sequence, covering the TCF7L2 binding sites), and 5′-AAGCAA CAGGTGTCCAGAGC-3′ and 5′-GGACCCTTCCTCTCCTGTGA-3′ (for amplification of the LCN2 -750/-551 sequence, covering the EGR1 binding sites).

2.7. Cell migration and invasion assays in vitro

Migration and invasion assays for transiently-transfected cells were performed using transwell chambers as described previously [17]. For invasion assays, 1×10^5 cells were inoculated onto the top chamber of a 24-well Matrigel-coated membrane with 8-μm pores (BD Falcon, USA), and the bottom chamber was filled with medium containing 10% fetal bovine serum (for the migration assay, cells were directly plated on an uncoated chamber). After 48 h, membranes were fixed and stained with hematoxylin. Cell numbers were quantified by counting 10 random fields under a light microscope (200 ×). The mean value was calculated from data obtained from 10 random fields. All migration and invasion assays were repeated three times.

A scratch assay was also performed. ESCC cells were grown for 12–18 h to 70–90% confluence, then transfected with either the pCMV-TCF7L2 or pCMV-EGR1 plasmid. Cells were incubated until 100% confluence was reached. A 200 μL pipet tip was used to scratch a wound through the cell monolayer in the center of the well. The plate was washed once and replaced with the desired medium containing 2% fetal bovine serum. Cells were observed and photographed under a microscope immediately after the scratch (0 h), and at 24 intervals until the scratch closed.

2.8. Cell proliferation assay

For the cell proliferation assay, ESCC cells were inoculated at 8000 cells per well in a 96-well plate. A CellTiter 96 aqueous one solution cell proliferation assay (MTS) was performed at 0, 24, 48, 72, and 96 h, according to the manufacturer's instructions (Promega, USA). A 20 μL volume of MTS was added to each well, and plates were incubated for 2 h at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was detected using a plate microplate reader (Multiskan MK3, Thermo). Raw data were normalized against those of the medium blank control.

2.9. Immunofluorescence

TCF7L2-HA-overexpressing ESCC cells or EGR1-HA-overexpressing ESCC cells were grown on slides, in 12-well plates, that were pretreated with 10 μg/mL fibronectin at 37 °C for 2 h. When cells were 80%–90% confluent, medium was aspirated, and cells were rinsed gently three times with 500 μL PBS for 5 min each time. Then cells were fixed in 4% formaldehyde for 10 min and washed as described above. After washing with PBS, the cells were permeabilized by 0.1% Triton X-100 in PBS for 3 min at room temperature. After washing with PBS, the cells were blocked for 1 h in PBS containing 5% donkey serum. After washing with PBS, the cells were incubated with anti-HA-Tag (rabbit mAb, #3724, CST, 1:1600 in blocking buffer) overnight at 4 °C. After washing with PBS, the cells were incubated for 1 h at room temperature with donkey anti-rabbit IgG (H + L) secondary antibody (two drops per mL buffer to stain, Thermo) and Acti-stain™ 488 Fluorescent Phalloidin (# PHDG1, Cytoskeleton, 100 nM) in the dark, followed by washing with PBS. Nuclei were then counterstained with DAPI, and the slides were mounted, and viewed using an upright microscope (DP73, Olympus).

2.10. Statistical analysis

Data analysis was performed using SPSS 19.0 and GraphPad Prism 7.0 software. Differences were considered statistically significant at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Data are plotted as mean ± S.D.

3. Results

3.1. Identification of probable KTAPs via bioinformatic analysis

The "LCN2 → MEK/ERK → LCN2" positive feedback loop in ESCC may be partly controlled by transcription factors (TFs) that bind to the LCN2 promoter. To better understand the transcriptional regulation of LCN2 in ESCC, the region −1431 ~ +84 from the transcriptional start site of the human *LCN2* gene was cloned from ESCC cells and sequenced [18]. We sought to delimit the promoter sequences and predict potential TFs that target LCN2. The promoter sequence of LCN2 was obtained from the UCSC genome browser, and subjected to prediction of transcription factor binding sites in the JASPAR database. We found that

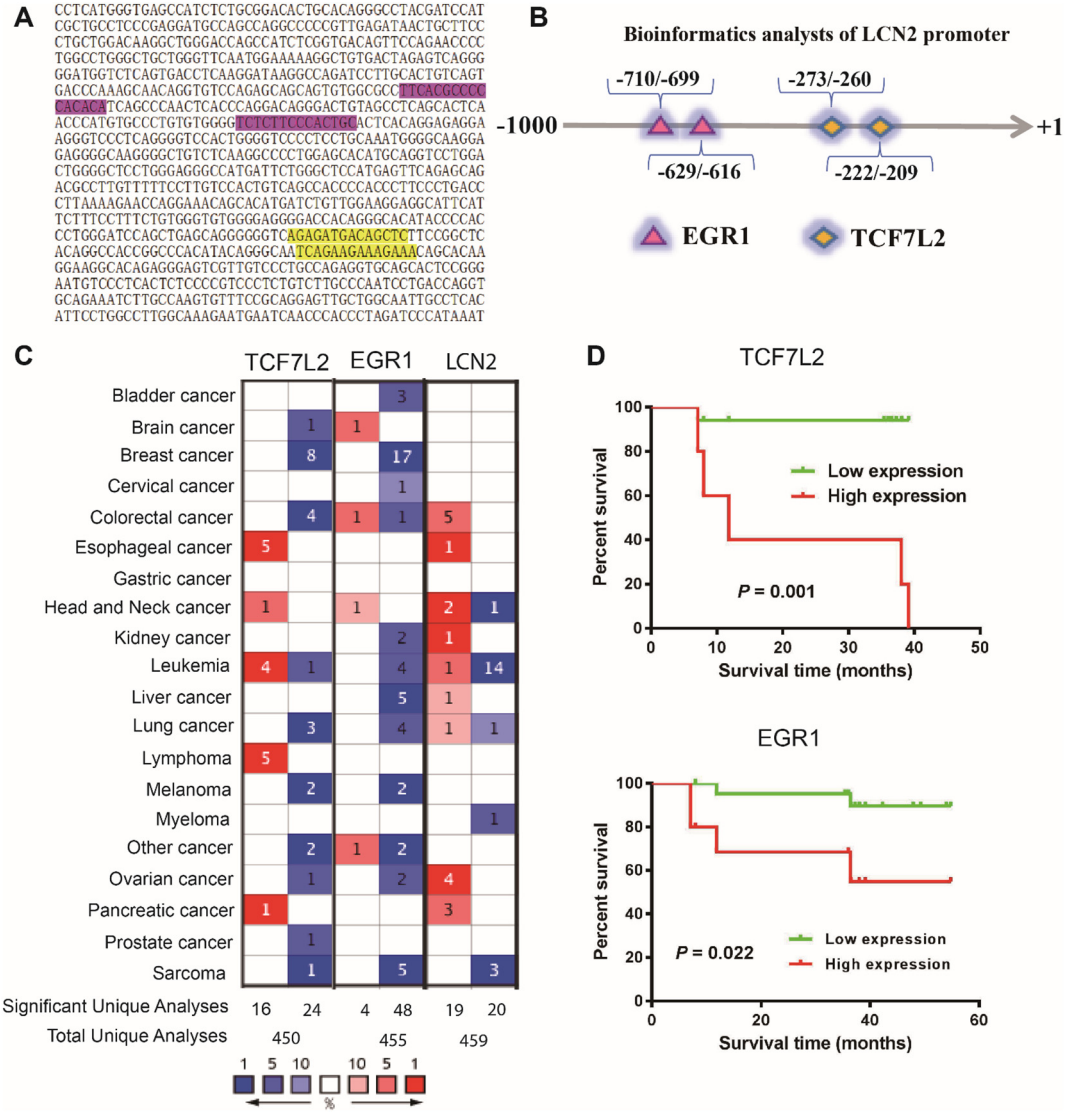

Fig. 1. Nucleic acid sequences (A) and schematic representation of the predicted transcription factor binding sites for TCF7L2 and EGR1 in the LCN2 promoter (B). (A-B) Predicted EGR1 binding sites at −710/−699 and −629/−616, and TCF7L2 binding sites at −273/−260 and −222/−209 are indicated based on analysis using the UCSC and JASPAR databases. (C) TCF7L2, EGR1 and LCN2 mRNA expression level in esophageal cancer from Oncomine datasets. This graphic compares the number of datasets that had significant mRNA overexpression (left column, red) and underexpression (right column, blue) of the three genes when comparing cancer versus normal tissue. The red blocks represent overexpression and the blue blocks represent underexpression. The white blocks indicate that significant differences in expression were not found from the current collected data, or did not meet the threshold set in this study. The number in the block indicated the amount of significant results according to the rank within the top 10%. The levels of expression are based on the best gene rank percentile for the analyses within the cell. The intensity of the color signifies the best ranking of genes in those analyses. (D) Kaplan-Meier survival curve for TCF7L2 (upper panel) and EGR1 (lower panel) in ESCC patients from the Aoyagi esophagus dataset. High expression of bothTCF7L2 and EGR1 correlate with lower survival in ESCC patients. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transcription factor 7-like 2 (TCF7L2) and early growth response 1 (EGR1) had the greatest potential for being KTAPs (Fig. 1A-B).

To address the mRNA expression differences of TCF7L2, EGR1 and LCN2 between tumor and normal tissues in multiple cancers, especially esophageal cancer, we performed analyses using the Oncomine database, which collects various expression dataset of different cancers. We adopted a Cancer vs. Normal Analysis Type to compare the expression levels of TCF7L2 or LCN2 in normal esophagus and patients with esophageal cancer. As shown in Fig. 1C, the database contained a total of 450, 455 and 459 unique analyses for TCF7L2, EGR1 and LCN2, respectively. In 40 studies, TCF7L2 was ranked within the top 10% of all genes showing significant statistical differences, 16 of which revealed overexpression in tumors compared to normal tissues, while 24 analyses displayed the opposite result. 39 significant unique analyses revealed that the mRNA expression level of LCN2 varied with the type of tumor. When considering their expression in esophageal cancer, at least five analyses and one study supported a significant elevation of TCF7L2 and LCN2, but differences in EGR1 expression were not determined from the current collected data.

Based on the evidence we obtained, we speculated a tight association between TCF7L2 or EGR1 expression levels and the life expectancy of ESCC patients. We utilized Kaplan-Meier curves to compare overall survival differences between “high” and “low” expression groups, and calculated *p*-values using the log-rank test method. According to the curve, we obtained a trend showing that high expression of both TCF7L2 and EGR1 was associated with lower survival, even in the first three years, after commencement of follow-up, compared to low expression (Fig. 1D).

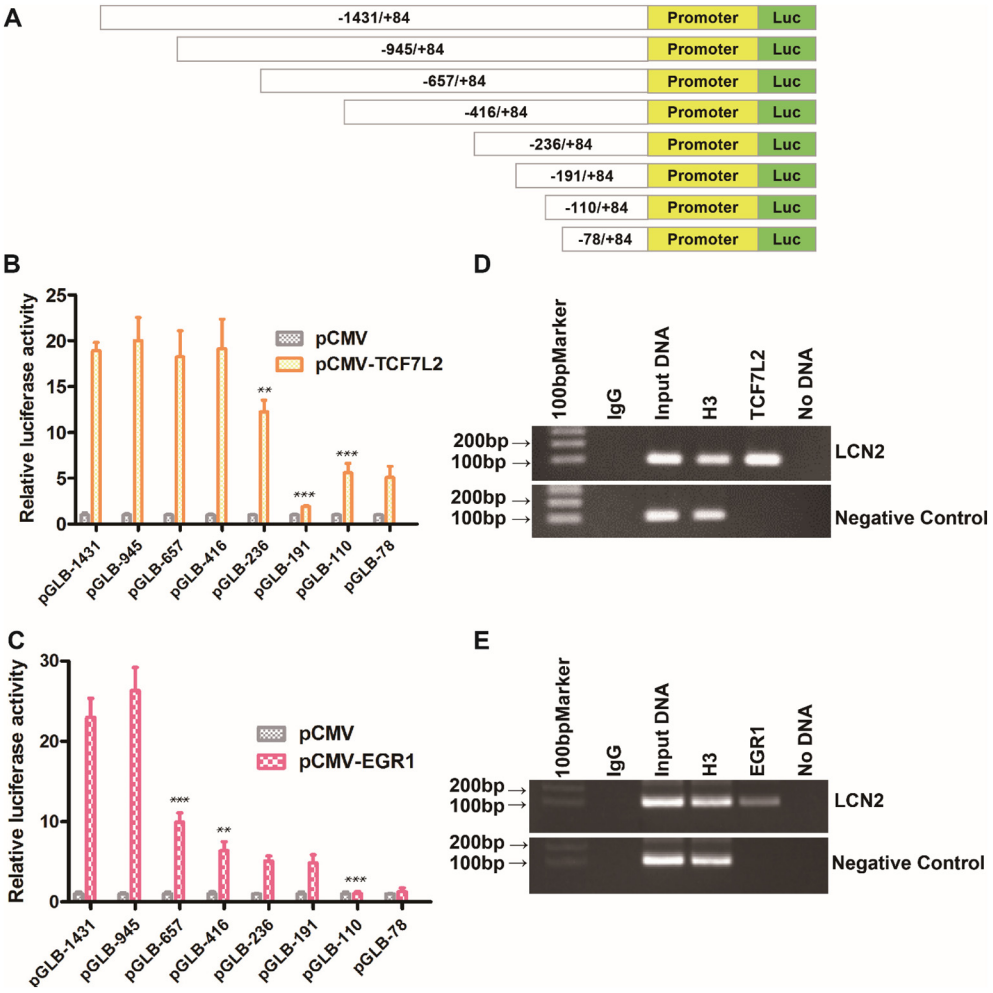

Fig. 2. Transcriptional regulation of the human *LCN2* 5′-flanking region in ESCC cells. (A) Schematic representation of LCN2 promoter 5′-deletion constructs used for transient transfections. The *LCN2* transcription initiation site (TIS) and ATG start codon (+69/+71) were contained in all fragments. (B-C) 5′-deletion constructs were co-transfected with pRL-TK and transcription factor-overexpressing plasmids into KYSE150 cells. Luciferase activity was normalized to Renilla luciferase activity and then shown relative to that of KYSE150 cells transfected with the pCMV plasmid negative control, which was set to 100%. Each value represents the mean ± S.D. of at least two independent experiments. Transfections were carried out in triplicate for each experiment. ***, $p < 0.001$. (D-E) Chromatin immunoprecipitation assays confirmed the binding of TCF7L2 and EGR1 to the human *LCN2* promoter in ESCC cells. Cross-linked chromatin isolated from KYSE150 cells was immunoprecipitated with anti-TCF7L2 or anti-EGR1 (lane 4). The associated chromosomal DNA fragments were amplified with a human LCN2 promoter-specific primer pair resulting in a 151 bp fragment. ChIP DNA with non-specific IgG (lane 1), chromosomal DNA input (lane 2, described in "Materials and methods"), positive control H3 (lane 3) and the control reaction (no DNA, lane 5) were subjected to the same PCR amplification. PCR products were separated on a 2% agarose gel containing ethidium bromide and detected via ultraviolet illumination.

3.2. TCF7L2 and EGR1 are key transcriptional activation proteins (KTAP) for LCN2 in ESCC

To identify the promoter regulatory regions, a series of 5′-deletion constructs were analyzed (Fig. 2A). In KYSE150 cells, the −945/+84 region conferred maximum luciferase activity. Deletion from −945/+84 to −416/+84 had considerably high reporter activity when overexpressing EGR1, whereas 5′-deletion from −416/+84 to −191/+84 caused a near 70% reduction of reporter activity when overexpressing TCF7L2 (Fig. 2B-C). These two regions coincide with the above bioinformatic prediction for the TCF7L2 and EGR1 binding sites. When compared with the two regions, the reporter activities in other promoter plasmids, including −1431/+84, −945/+84, −657/+84, and −416/+84, did not markedly change when overexpressing TCF7L2 (Fig. 2B). When overexpressing EGR1, sequence deletion from −945 to −657 caused an approximately 50% reduction in luciferase activity, whereas 5′-deletion from −416 to −191 nearly abolished the activity (Fig. 2C).

These data suggest that TCF7L2 and EGR1 are the KTAPs that regulate promoter activity of human LCN2 in KYSE150 cells. To investigate whether TCF7L2 and EGR1 directly bind to the LCN2 promoter in ESCC cells, we performed a chromatin immunoprecipitation (ChIP) assay. Immunoprecipitated chromosomal DNA was subjected to PCR using primers designed to amplify the LCN2 promoter region harboring the predicted TCF7L2 and EGR1 binding sites. TCF7L2 and EGR1 indeed bound to the LCN2 promoter region containing the predicted TCF7L2 and EGR1 sites (Fig. 2D-E). ChIP of acetylated histone H3, provided in the kit (see Materials and Methods), was used as a positive control confirming that the LCN2 promoter was transcriptionally active.

3.3. Function of TCF7L2 and EGR1 in ESCC

We next investigated the biological functions of TCF7L2 and EGR1 in ESCC cells. A scratch assay was applied to investigate the effect of TCF7L2 and EGR1 on migration in ESCC cells. Overexpression of either TCF7L2 or EGR1 strongly enhanced migration compared with the empty vector-transfected control. Cells showed greater migration following co-transfection of both TCF7L2 and EGR1, compared to transfection of a single plasmid, suggesting each gene separately influences the transcription of LCN2 (Fig. 3A). These phenomena were also demonstrated in the ECa109 cell line as well (data not shown). Then, we used transwell assays to detect the change in migration and invasion of the KYSE150 cell line. Results show that the numbers of migrated or invaded cells over a 48-h period were much higher than the control group. Similar to the scratch results, an additive effect of these two transcription factors was also observed (Fig. 3B). Actually, the number of invading cells in the pCMV control averaged 5.8 ± 1.9 per/field. On the other hand, the cell number in the invasion assay for TCF7L2, EGR1 and their co-transfection was 14.6 ± 3.4, 10.6 ± 2.7 and 19.8 ± 4.1 per/field, respectively. Statistical significances were found when the migrated or invade cell number of TCF7L2, EGR1 and their co-transfection compared to that of the pCMV control (Fig. 3B). These results indicate that both TCF7L2 and EGR1 independently facilitate the migration and invasion of ESCC cells. MTS assays on TCF7L2- or EGR1-overexpressing cells indicated there were no statistically significant differences in proliferation compared to control KYSE cells, although

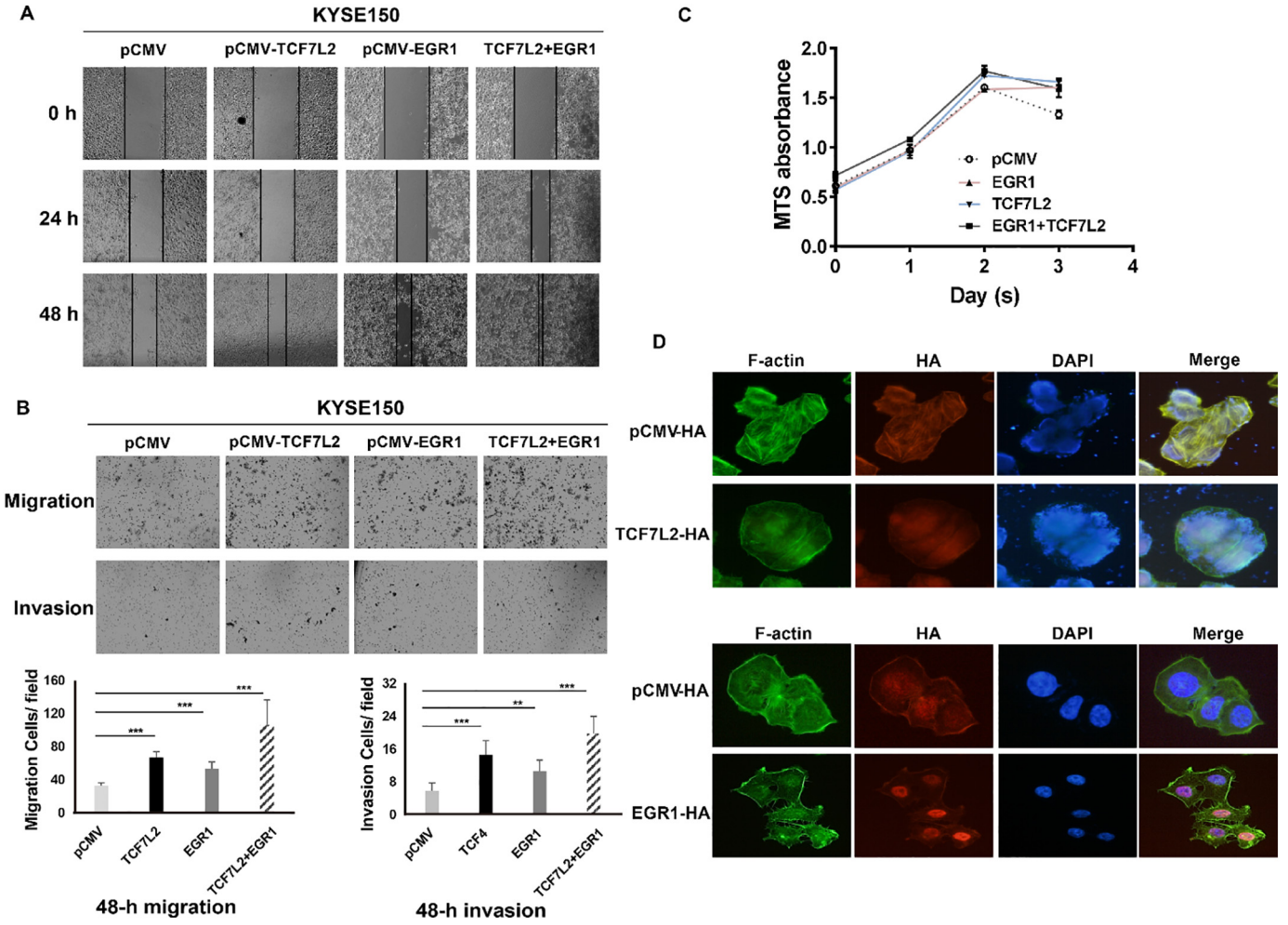

Fig. 3. TCF7L2 and EGR1 promote migration of ESCC cells. (A) Analyses of ESCC cell migration by scratch assay in vitro. KYSE150 cells were transfected with the TCF7L2 or EGR1 plasmid individually or in combination, the migration distance is shown at indicated times after scratching. Images were acquired at 0 h, 24 h and 48 h. The lines define the areas lacking cells. (B) Migration and invasion assays for TCF7L2- or EGR1-overexpressing ESCC cells. The number of migrated or invaded cells is represented the means ± SD from 10 random fields (lower panel). For the migration and invasion assay, each experiment was carried out in triplicate. (C) MTS assay of KYSE150 cells after overexpression of TCF7L2, EGR1 and both TCF7L2 and EGR1. Experiments were repeated three times with similar results. (D) TCF7L2 or EGR1 overexpression mediated actin cytoskeleton rearrangement. Stress fibers in TCF7L2-overexpressing ESCC cells were more disordered, and filopodia were increased and prolonged in EGR1-overexpressing ESCC cells than control, and with the F-actin cytoskeleton being radially arranged in the periphery following TCF7L2 or EGR1 overexpression in ESCC cells.

there was a trend toward enhancement (Fig. 3C).

We presumed that TCF7L2 or EGR1 promotion of metastasis and invasion via the change of cell morphology. Therefore, we used phalloidin to stain F-actin. Following TCF7L2 overexpression stress fiber formation was dispersed and suppressed in KYSE150 cells (Fig. 3D, upper panel). On the other hand, the stress fibers did not change significantly, but filopodia were increased and elongated in KYSE150-EGR1 cells compared with the control (Fig. 3D, lower panel). These results show that upregulation of TCF7L2 inhibits stress fiber formation, and EGR1 enhances filopodia extension in KYSE150 cells, suggesting that TCF7L2 or EGR1 overexpression mediates actin cytoskeleton rearrangement to promote tumor cell migration.

3.4. Possible mechanism of TCF7L2 and EGR1 enhancement of LCN2 expression

To explore the molecular mechanism of TCF7L2 and EGR1 on LCN2 expression, KYSE150 cells were transfected or co-transfected with pCMV-TCF7L2 and/or pCMV-EGR1. Total protein was extracted and the expression levels of TCF7L2, EGR1, phospho-ERK1/2 (p-ERK1/2), ERK1/2 and LCN2 were analyzed by western blotting. Interestingly, overexpression TCF7L2/EGR1 increased p-ERK1/2 and upregulated endogenous LCN2 in both transfectants and co-transfectants. Similar results were obtained in ECa109 cells (data not shown). These data suggest that TCF7L2 and EGR1 upregulate LCN2 expression in ESCC cells through the ERK pathway. Compared with transfections involving single TCF7L2 or EGR1 plasmids, endogenous LCN2 expression increased substantially by co-transfection with TCF7L2/EGR1, strongly suggesting a combined effect of TCF7L2 and EGR1 in the activation of LCN2 transcription (Fig. 4A). In addition, treatment with recombinant human LCN2 (rhLCN2) in ESCC cells enhanced the activation of the ERK1/2/p-ERK1/2 pathway to facilitate endogenous LCN2 expression. Consistent with our positive feedback model, rhLCN2 also increased the expression of endogenous TCF7L2 and EGR1 (Fig. 4B). To confirm the role of ERK pathway in the transcription upregulation, the MEK inhibitor U0126 was added to the TCF7L2- or EGR1-overexpressing cells. U0126 clearly inhibited the activation of p-ERK1/2 in KYSE150 cells. In addition, endogenous LCN2, as well as TCF7L2 and EGR1, were reduced after the treatment of U0126 (Fig. 4C). The expression of TCF7L2 or EGR1 indeed increased after transfection, but also decreased with the treatment of U0126. These suggested that the treatment of U0126 partially inhibited the transcription activation of LCN2 by TCF7L2 and

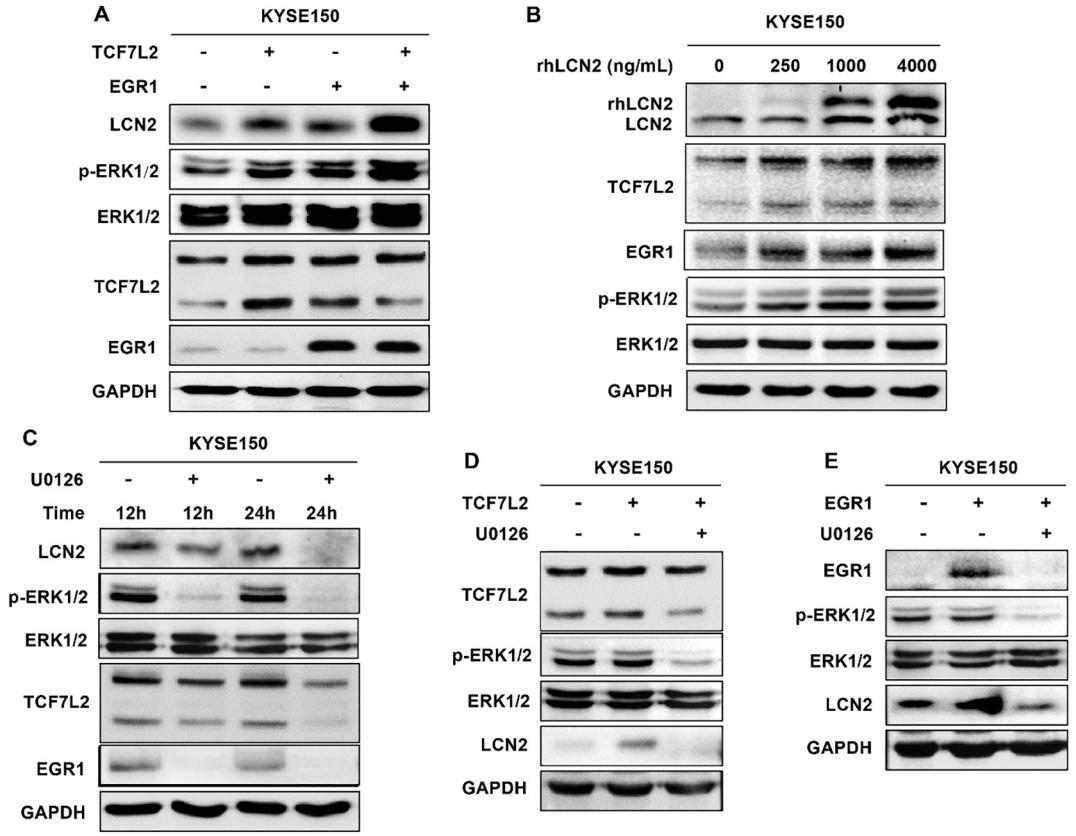

Fig. 4. TCF7L2 and EGR1 are involved in the transcription activation of LCN2 via an ERK1/2-dependent pathway. (A) Transient expression of TCF7L2, EGR1, or both TCF7L2 and EGR1 up-regulate the expression level of LCN2 and phospho-ERK1/2 (p-ERK1/2) in ESCC cells. (B) Treatment of 0, 0.25, 1, or 4 μg rhLCN2 enhances activation of the ERK pathway, which enhances endogenous LCN2 expression, as well as increases the protein levels of TCF7L2 and EGR1. The 75 kDa TCF7L2 isoform is the main band when considering its role in the regulation of LCN2 expression according to the previous report [24]. (C) Inhibitory effect of U0126 on ERK1/2 signaling cascade. Using anti-p-ERK1/2 antibody, which recognizes the active phosphorylated ERK1/2, it was shown that U0126 inhibits the activation of p-ERK1/2 in KYSE150 cells at both 12 h and 24 h. In addition, the expression of endogenous LCN2, TCF7L2 and EGR1 were inhibited. The amount of total ERK1/2 showed little change with the various treatments. (D) Inhibitory effect of U0126 on ERK1/2 signaling cascade in the presence of TCF7L2/EGR1. U0126 inhibits the activation of p-ERK1/2 in KYSE150 cells while endogenous LCN2 was inhibited. The expression of TCF7L2 or EGR1 indeed increased after their transfection, but decreased with the treatment of U0126. The amount of total ERK1/2 shows little change with the various treatments. GAPDH is shown as a loading control. Experiments were repeated two to three times with similar results.

EGR1 overexpression, respectively (Fig. 4D-E). Thus, we favor a transcription regulation model in which TCF7L2 and EGR1 are the KTAPs that the expression of LCN2, whereas the transcription of these three genes are regulated via an ERK1/2-dependent pathway (Fig. 5).

4. Discussion

LCN2, a secreted glycoprotein, is elevated in many cancers. The molecular mechanism of LCN2 appears to be cell type-dependent, especially the regulation of its expression at the transcriptional level. Several studies show that LCN2 plays an important role in enhancing proliferation and invasion by protecting MMP-9 from degradation to promote malignancy in tumor progression [25]. Leng et al. have demonstrated that LCN2 is induced by the HER2/phosphoinositide 3-kinase/AKT/NF-κB signaling cascade, which is a critical factor in enhancing tumor formation and progression of breast cancer [26]. Our previous study indicated that the ERK1/2 pathway can enhance LCN2 expression in ESCC, and might serve as a self-recruiting pathway for LCN2 expression regulation.

TCF7L2, also named TCF4, is a member of the LEF/TCF (lymphoid enhancer factor/T cell factor) family of transcription factors [27]. TCF7L2, as a β-catenin transcriptional partner, plays a vital role in the Wnt/β-catenin signaling pathway [28]. TCF7L2 as an indicator of poor prognosis in ESCC has been suggested by Ishiguro et al., who found TCF7L2 is expressed in 57% (45/79) of ESCC patients, and patients who are TCF7L2-positive have a significantly lower survival rate than those negative for TCF7L2 [29]. Although TCF7L2 is activated by β-catenin to influence the Wnt signaling pathway [30], expression of beta-catenin is decreased in 69.8% (74/106) of ESCC patients, and the level of protein expression correlates only with histologic grade [31]. As a member of the TCF family, the relationship between TCF7L1 and LCN2 has been reported to be independent of β-catenin [32]. Interestingly, TCF7L2, has been predicted to be one of the most probable KTAPs of LCN2, and localizes to the nucleus without β-catenin [33]. Miao et al. found TCF3, another member of the TCF family, can promote cell migration through LCN2 [34]. These studies led us to explore the function of TCF7L2 in ESCC. Zhou et al. found overexpression of human pituitary tumor transforming gene (hPTTG) is regulated by the β-catenin/TCF7L2 pathway in ESCC [35]. These results suggest that TCF7L2 is an important molecule in ESCC. ERK pathway involvement in TCF7L2-mediated gene regulation has not been reported. Our results indicate TCF7L2 activity could be enhanced by the ERK pathway to subsequently activate transcription of its target genes, including LCN2.

EGR1 belongs to the EGR family of C2H2-type zinc-finger proteins, and is a nuclear protein that functions as a transcriptional regulator mediating growth, proliferation, differentiation and apoptosis [36]. Though EGR1 was not identified as being upregulated in the Oncomine database, previous reports have found overexpression of EGR1 mRNA and its proteins in dysplasia and ESCC [37]. Wu et al. found EGR1 is a downstream factor regulated by GROβ, whose inhibition can decrease

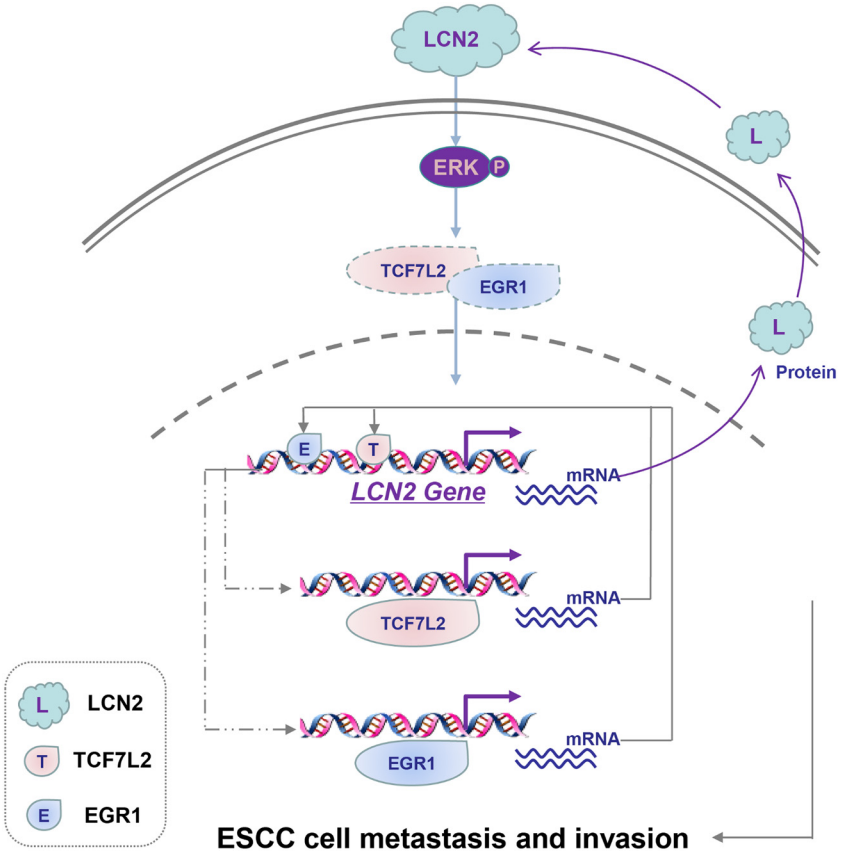

Fig. 5. Schematic representation of the molecular mechanism through which LCN2 promotes the metastasis and invasiveness of ESCC cells by regulating EGR1 and TCF7L2 via an ERK1/2-dependent pathway.

cisplatin-induced apoptosis in ESCC cells, suggesting EGR1 may be a potential radiation response gene marker for ESCC [38], although silencing expression of EGR1 can also decrease cisplatin-induced apoptosis in esophageal cancer cells [39]. Nevertheless, few targets of EGR1 and the biological functions of EGR1 in ESCC have been reported. That the relationship between EGR1 and MMP-2, through PAK5-ERG1-MMP2 signaling, affects migration and invasion has been reported for breast cancer [40]. Combined with our luciferase reporter and ChIP results, this study confirms that LCN2 is a direct target of EGR1. We believe that the EGR1-mediated activation of LCN2, is one of the numerous pathways through which EGR1 participates in ERK pathway activity to form an important axis in tumor progression. Zwang et al. demonstrated an ERK-EGR1 threshold mechanism that digitizes graded external signals into an all-or-none decision obligatory for S phase entry [41]. Thus, combined with the results described above, LCN2 is the target gene through which EGR1, as well as TCF7L2, promotes the migration and invasion of ESCC cells. This study also is the first report of the biological function of TCF7L2 and EGR1 in ESCC.

In summary, we found TCF7L2 and EGR1 are important transcription factors for LCN2 expression via an ERK1/2-dependent pathway in ESCC. TCF7L2 and EGR1 are also increased in the loop to further enhance the expression of LCN2, forming a continually activated "LCN2 → MEK/ERK → TCF7L2/EGR1 → LCN2" path in the promotion of migration and invasion of ESCC cells.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81672473, No. 81502138), the Science and Technology Program of Guangdong (No. 2014A030310390, No. 2017A030313181).

Declarations of interest

None.

References

[1] W. Chen, R. Zheng, P.D. Baade, et al., Cancer statistics in China, 2015, CA Cancer J. Clin. 66 (2) (2016) 115–132.
[2] H. Ding, Y. He, K. Li, et al., Urinary neutrophil gelatinase-associated lipocalin (NGAL) is an early biomarker for renal tubulointerstitial injury in IgA nephropathy, Clin. Immunol. 123 (2) (2007) 227–234.
[3] D.A. Jaques, L. Spahr, G. Berra, et al., Biomarkers for acute kidney injury in decompensated cirrhosis: a prospective Study, Nephrology (Carlton) (2018) (Epub ahead of print).
[4] O.L. Barbarash, I.S. Bykova, V.V. Kashtalap, et al., Serum neutrophil gelatinase-associated lipocalin has an advantage over serum cystatin C and glomerular filtration rate in prediction of adverse cardiovascular outcome in patients with ST-segment elevation myocardial infarction, BMC Cardiovasc. Disord. 17 (1) (2017) 81.
[5] S.S. Kang, Y. Ren, C.C. Liu, et al., Lipocalin-2 protects the brain during inflammatory conditions, Mol. Psychiatry 23 (2) (2018) 344–350.
[6] H. Zhang, L. Xu, D. Xiao, et al., Up-regulation of neutrophil gelatinase-associated lipocalin in esophageal squamous cell carcinoma: significantly correlated with cell differentiation and tumor invasion, J. Clin. Pathol. 60 (5) (2007) 555–561.
[7] J. Yang, D.R. Bielenberg, S.J. Rodig, et al., Lipocalin 2 promotes breast cancer progression, Proc. Natl. Acad. Sci. U. S. A. 106 (10) (2009) 3913–3918.
[8] J.M. Ruiz-Morales, R. Dorantes-Heredia, O. Arrieta, et al., Neutrophil gelatinase-associated lipocalin (NGAL) and matrix metalloproteinase-9 (MMP-9) prognostic value in lung adenocarcinoma, Tumor Biol. 36 (5) (2015) 3601–3610.
[9] F.J.G.M. Kubben, C.F.M. Sier, L.J.A.C. Hawinkels, et al., Clinical evidence for a protective role of lipocalin-2 against MMP-9 autodegradation and the impact for gastric cancer, Eur. J. Cancer 43 (12) (2007) 1869–1876.
[10] M.F. Liu, Y.Y. Hu, T. Jin, et al., Matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex activity in human glioma samples predicts tumor presence and clinical prognosis, Dis. Markers 2015 (2015) 138974.
[11] L. Roli, V. Pecoraro, T. Trenti, Can NGAL be employed as prognostic and diagnostic biomarker in human cancers? A systematic review of current evidence, Int. J. Biol. Markers 32 (1) (2017) 53–61.
[12] Z.P. Du, Z. Lv, B.L. Wu, et al., Neutrophil gelatinase-associated lipocalin and its

receptor: independent prognostic factors of oesophageal squamous cell carcinoma, J. Clin. Pathol. 64 (1) (2011) 69–74.
[13] Z.P. Du, H.M. Yuan, B.L. Wu, et al., Neutrophil gelatinase-associated lipocalin in gastric carcinoma cells and its induction by TPA are controlled by C/EBPβ, Biochem. Cell Biol. 89 (3) (2011) 314–324.
[14] P. Zhao, C.M. Elks, J.M. Stephens, The induction of lipocalin-2 expression in vivo and in vitro, J. Biol. Chem. 289 (9) (2014) 5960–5969.
[15] J. Conde, M. Otero, M. Scotece, et al., E74-like factor 3 and nuclear factor-κB regulate lipocalin-2 expression in chondrocytes, J. Physiol. 594 (21) (2016) 6133–6146.
[16] J. Stallhofer, M. Friedrich, A. Konrad-Zerna, et al., Lipocalin-2 is a disease activity marker in inflammatory bowel disease regulated by IL-17A, IL-22, and TNF-α and modulated by IL23R genotype status, Inflamm. Bowel Dis. 21 (10) (2015) 2327–2340.
[17] Z.P. Du, B.L. Wu, Y.M. Xie, et al., Lipocalin 2 promotes the migration and invasion of esophageal squamous cell carcinoma cells through a novel positive feedback loop, Biochim. Biophys. Acta 1853 (10) (2015) 2240–2250.
[18] L.Y. Xu, E.M. Li, W.J. Cai, et al., Constructing and identifying the firefly luciferase report gene vectors for 5' flanking regulated elements of NGAL gene, Carcinog. Teratog. Mutagen. 16 (1) (2004) 5–8.
[19] J. Casper, A.S. Zweig, C. Villarreal, et al., The UCSC genome browser database: 2018 update, Nucleic Acids Res. 46 (D1) (2018) D762–D769.
[20] A. Khan, O. Fornes, A. Stigliani, et al., JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework, Nucleic Acids Res. 46 (D1) (2018) D260–D266.
[21] D.R. Rhodes, S. Kalyana-Sundaram, V. Mahavisno, et al., Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles, Neoplasia 9 (2) (2007) 166–180.
[22] K. Aoyagi, K. Minashi, H. Igaki, et al., Artificially induced epithelial-mesenchymal transition in surgical subjects: its implications in clinical and basic cancer research, PLoS ONE 6 (4) (2011) e18196.
[23] S.Y. Gao, E.M. Li, L. Cui, et al., Sp1 and AP-1 regulate expression of the human gene VIL2 in esophageal carcinoma cells, J. Biol. Chem. 284 (12) (2009) 7995–8004.
[24] G. He, X. Guan, X. Chen, et al., Expression and splice variant analysis of human TCF4 transcription factor in esophageal cancer, J. Cancer 6 (4) (2015) 333.
[25] S. Candido, S.L. Abrams, L.S. Steelman, et al., Roles of NGAL and MMP-9 in the tumor microenvironment and sensitivity to targeted therapy, Biochim. Biophys. Acta 1863 (3) (2016) 438–448.
[26] X. Leng, T. Ding, H. Lin, et al., Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis, Cancer Res. 69 (22) (2009) 8579–8584.
[27] D. Hrckulak, M. Kolar, H. Strnad, et al., TCF/LEF transcription factors: an update from the internet resources, Cancers (Basel). 8 (7) (2016) 70.
[28] J.P. Morris IV, S.C. Wang, M. Hebrok, KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma, Nat. Rev. Cancer 10 (10) (2010) 683–695.
[29] H. Ishiguro, T. Wakasugi, Y. Terashita, et al., Nuclear expression of TCF4/TCF7L2 is correlated with poor prognosis in patients with esophageal squamous cell carcinoma, Cell. Mol. Biol. Lett. 21 (1) (2016) 5.
[30] T. Jin, Current understanding on role of the Wnt signaling pathway effector TCF7L2 in glucose homeostasis, Endocr. Rev. 37 (3) (2016) 254–277.
[31] X.J. Zhao, H. Li, H. Chen, et al., Expression of e-cadherin and β-catenin in human esophageal squamous cell carcinoma: relationships with prognosis, World J. Gastroenterol. 9 (2) (2003) 225–232.
[32] A.T. Ku, T.M. Shaver, A.S. Rao, et al., TCF7L1 promotes skin tumorigenesis independently of β-catenin through induction of LCN2, elife 6 (2017) e23242.
[33] J. Kudo, T. Nishiwaki, N. Haruki, et al., Aberrant nuclear localization of β-catenin without genetic alterations in β-catenin or Axin genes in esophageal cancer, World J. Surg. Oncol. 5 (1) (2007) 21.
[34] Q. Miao, A.T. Ku, Y. Nishino, et al., Tcf3 promotes cell migration and wound repair through regulation of lipocalin 2, Nat. Commun. 5 (2014) 4088.
[35] C. Zhou, S. Liu, X. Zhou, et al., Overexpression of human pituitary tumor transforming gene (hPTTG), is regulated by β-catenin/TCF pathway in human esophageal squamous cell carcinoma, Int. J. Cancer 113 (6) (2005) 891–898.
[36] J.I. Pagel, E. Deindl, Early growth response 1—a transcription factor in the crossfire of signal transduction cascades, Indian J. Biochem. Biophys. 48 (4) (2011) 226–235.
[37] M.Y. Wu, C.X. Zhuang, H.X. Yang, et al., Expression of Egr-1, c-fos and cyclin D1 in esophageal cancer and its precursors: an immunohistochemical and in situ hybridization study, World J. Gastroenterol. 10 (4) (2004) 476–480.
[38] M.Y. Wu, X.Y. Wu, Q.S. Li, et al., Expression of Egr-1 gene and its correlation with the oncogene proteins in non-irradiated and irradiated esophageal squamous cell carcinoma, Dis. Esophagus 19 (4) (2006) 267–272.
[39] Q. Dong, Zhang J, Hendricks Dt, Et Al., GROβ and its downstream effector EGR1 regulate cisplatin-induced apoptosis in WHCO1 cells, Oncol. Rep. 25 (4) (2011) 1031–1037.
[40] X.X. Wang, Q. Cheng, S.N. Zhang, et al., PAK5-Egr1-MMP2 signaling controls the migration and invasion in breast cancer cell, Tumour Biol. 34 (5) (2013) 2721–2729.
[41] Y. Zwang, A. Sas-Chen, Y. Drier, et al., Two phases of mitogenic signaling unveil roles for p53 and EGR1 in elimination of inconsistent growth signals, Mol. Cell 42 (4) (2011) 524–535.