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Lipocalin 2 promotes the migration and invasion of esophageal squamous cell carcinoma cells through a novel positive feedback loop

Ze-Peng Du [a,b,e,1], Bing-Li Wu [a,c,1], Yang-Min Xie [a,d], Ying-Li Zhang [a,c], Lian-Di Liao [a,b], Fei Zhou [a,d], Jian-Jun Xie [a,c], Fa-Min Zeng [a,c], Xiu-E Xu [a,b], Wang-Kai Fang [a,c], En-Min Li [a,c,*], Li-Yan Xu [a,b,*]

[a] The Key Laboratory of Molecular Biology for High Cancer Incidence Coastal Chaoshan Area, Shantou University Medical College, Shantou 515041, China
[b] Institute of Oncologic Pathology, Shantou University Medical College, Shantou 515041, China
[c] Department of Biochemistry and Molecular Biology, Shantou University Medical College, Shantou 515041, China
[d] Department of Experimental Animal Center, Shantou University Medical College, Shantou 515041, China
[e] Department of Pathology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, Guangdong Province 515041, China

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ABSTRACT

Lipocalin 2 (LCN2) is a poor prognostic factor in esophageal squamous cell carcinoma (ESCC), however its functional roles and molecular mechanisms of action remain to be clarified. Here, we described the functions and signaling pathways for LCN2 in ESCC. Overexpression of LCN2 in ESCC cells accelerated cell migration and invasion *in vitro*, and promoted lung metastasis *in vivo*. Blocking LCN2 expression inhibited its pro-oncogenic effect. Either overexpression of LCN2 or treatment with recombinant human LCN2 protein enhanced the activation of MEK/ERK pathway, which in turn increases endogenous LCN2 to increase MMP-9 activity. The decreased p-cofilin and increased p-ERM induced by pERK1/2 cause the cytoskeleton F-actin rearrangement and alter the behavior of ESCC cells mediated by LCN2. As a consequence, activation of MMP-9 and the rearrangement of F-actin throw light on the mechanisms for LCN2 in ESCC. These results imply that LCN2 promotes the migration and invasion of ESCC cells through a novel positive feedback loop.

1. Introduction

Lipocalin 2 (LCN2), also named Neutrophil gelatinase-associated lipocalin (NGAL), a member of the lipocalin superfamily, is a 25 kDa protein containing a signal peptide that enables it to be secreted and form complexes with matrix metalloproteinase-9 (MMP-9) through disulfide bonds [1]. Similar to other lipocalin family members, LCN2 is involved in diverse cellular processes, including the transport of small hydrophobic molecules, protection of MMP-9 from proteolytic degradation, and cell signaling [2–4]. Furthermore, LCN2 can tightly bind to bacterial siderophore through a cell surface receptor, possibly serving as a potent bacteriostatic agent by sequestering iron, regulating innate immunity and protecting kidney epithelial cells from ischemia–reperfusion injury [5–7].

Elevated LCN2 has also been observed in a wide spectrum of solid tumor cells, including breast [8,9], colorectal [10,11], pancreatic [12], ovarian [13], thyroid [14], bladder [15], kidney [16] and gastric cancers [17], and gliomas [18], as well as esophageal squamous cell carcinomas (ESCC) [19–21]. 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) [17]. Our recent studies show that LCN2 is upregulated in ESCC tumor tissue and is significantly correlated with cancer cell differentiation and tumor invasion, and both elevated LCN2 and its receptor are independent prognostic factors for ESCC [19–21]. However, the functional role of LCN2 in cancer cells is not fully understood at present, even sometimes there are controversial conclusions dropped from different researches. LCN2 is able to facilitate gastrointestinal mucosal regeneration by promoting cell motility and invasion and decreases E-cadherin mediated cell–cell adhesion in colon cancer [10]. On the other hand, it has been suggested that LCN2 reduces adhesion/invasion partly by suppressing FAK activation and inhibits angiogenesis partly by blocking VEGF production in pancreatic cancer cells [12]. Hanai et al. also found LCN2 could convert 4T1-Ras-transformed mesenchymal tumor cells to an epithelial phenotype, increase E-cadherin expression, and suppress cell invasiveness *in vitro* and tumor growth and lung metastases *in vivo* [22]. Moreover, ectopic expression of LCN2 in human hepatocellular carcinoma cells significantly inhibits the growth of cancer cells *in vitro* and *in vivo*, reduces the invasive potential of cells, and inhibits the expression of matrix metalloproteinase 2 (MMP-2) by the suppression of the JNK and PI3K/Akt pathways [23]. Although the function of LCN2 appears to be cell-type-dependent, the

* Corresponding authors at: The Key Laboratory of Molecular Biology for High Cancer Incidence Coastal Chaoshan Area, Shantou University Medical College, Shantou 515041, China.
E-mail addresses: nmli@stu.edu.cn (E.-M. Li), lyxu@stu.edu.cn (L.-Y. Xu).
[1] These authors contributed equally to this work.

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exact role of LCN2 in ESCC cells remains to be elucidated. The purpose of this study was to investigate the effects of LCN2 on the metastasis and invasiveness of ESCC cells, and explore the underlying molecular mechanisms.

2. Materials and methods

2.1. Cell culture and reagents

Several human ESCC cell lines were utilized in this study. The EC109 cell line was purchased from the Chinese Academy of Science. SHEE and SHEEC cell lines were isolated in our laboratory [24]. The EC8712 cell line was provided by professor Sai-Wah Tsao (Department of Anatomy, University of Hong Kong, China), KYSE150 and KYSE180 cell lines were generous gifts from Professor Dong Xie (Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China), and the TE3 cell line was a gift from Professor Mingzhou Guo (Department of Gastroenterology & Hepatology, Chinese PLA General Hospital, China). KYSE150, KYSE180 and TE3 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (HYCLONE, USA) with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mg/l streptomycin). EC109, EC8712, SHEE and SHEEC cells were cultured in 199 medium (HYCLONE, USA) with 10% new bovine serum and antibiotics (100 U/ml penicillin and 100 mg/l streptomycin). All cells were grown in a humidified 5% CO_2 atmosphere at 37 °C. All these human ESCC cell lines in this study

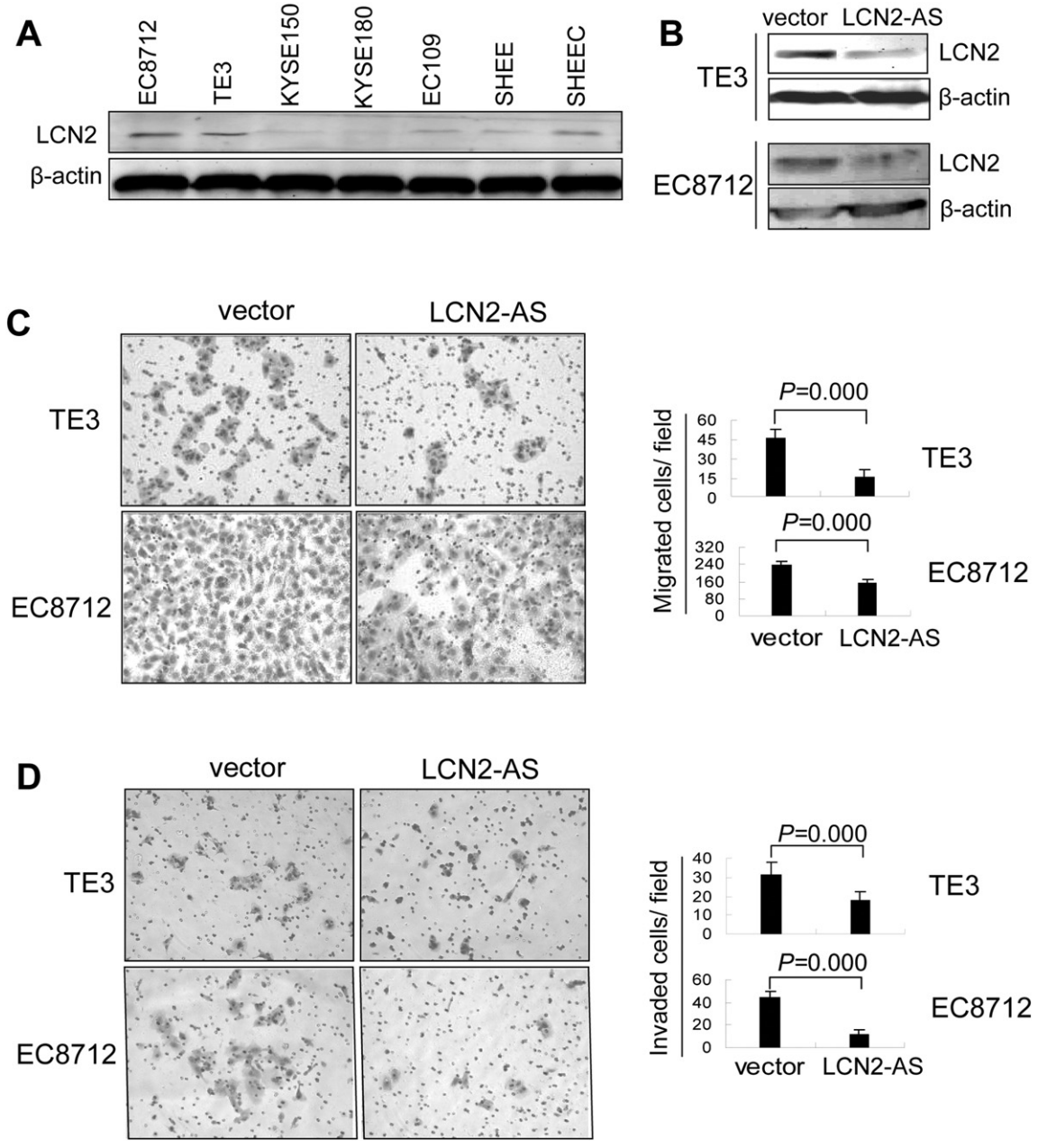

Fig. 1. Anti-sense-mediated inhibition of LCN2 expression reduces migration and invasiveness of ESCC cells *in vitro*. (A) Western blot analysis of LCN2 protein levels in human esophageal carcinoma cell lines. SHEE is an immortalized esophageal epithelial cell line. SHEEC, EC8712, EC109, TE3, KYSE150 and KYSE180 are esophageal squamous carcinoma cell lines. β-actin was the internal control. (B) LCN2 protein levels in anti-sense LCN2-expressing and control esophageal carcinoma cells. Vector, cells transfected with pcDNA3.0; LCN2-AS, cells transfected with pcDNA-LCN2(−). (C) Migration assays for LCN2-anti-sense-expressing ESCC cells. (D) Invasion assays for LCN2- anti-sense-expressing ESCC cells. For migration and invasion assay, each experiment was carried out in triplicate, and results represent the Sum ± SD of three experiments.

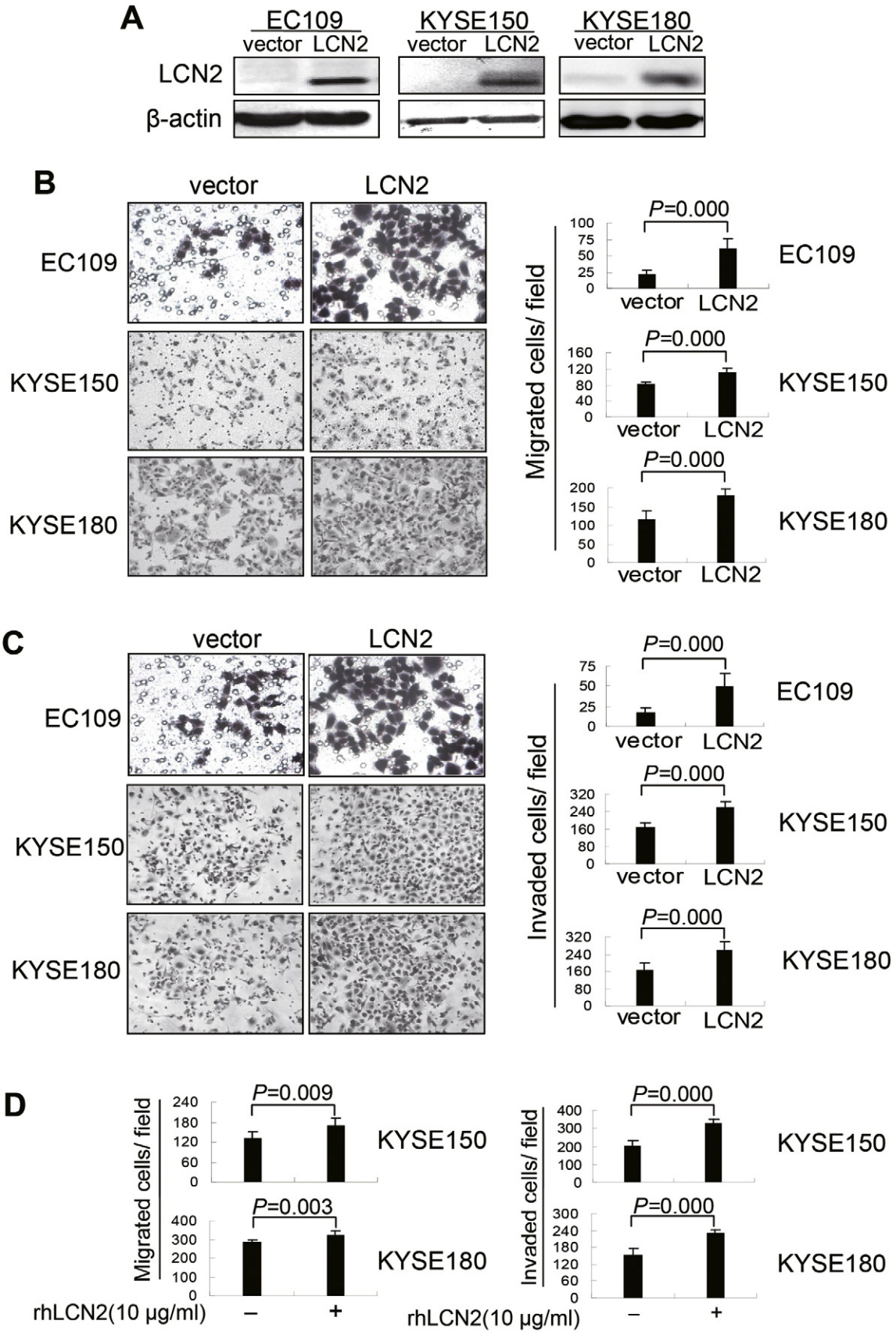

Fig. 2. LCN2 overexpression promotes migration and invasiveness of ESCC cells *in vitro*. (A) LCN2 overexpression was detected by western blot. Vector, cells transfected with pcDNA3.0; LCN2, cells transfected with pcDNA-LCN2(+). (B–C) Migration and invasion assays for LCN2-overexpressing ESCC cells. (D) Migration and invasiveness of ESCC cells promoted by the stimulation of rhLCN2. For migration and invasion assay, each experiment was carried out in triplicate, and results represent the Sum ± SD of three experiments.

have been tested and authenticated by short tandem repeat (STR) DNA profiling (Land Huagene Biosciences Co., LTD), and the authentication results are shown in Fig. S1.

Recombinant human LCN2 protein (rhLCN2, 10222-H08H) was purchased from Sino Biological Inc. of China. An Actin Reorganization Antibody Sample Kit (9967S), including antibodies against cofilin, p-cofilin (Ser3) and p-ERM (T567), was from Cell Signaling Technology Inc. (Danvers, MA). ERK (sc-94) and p-ERK1/2 (sc-7383) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against beta-actin (Sigma-Aldrich, St. Louis, USA, A2228) and LCN2 (R&D SYSTEMS, INC. Minneapolis, USA, MAB1757) were also used. The following secondary antibodies were used for western blot analysis: goat anti-rabbit IgG-HRP (sc-2030, Santa Cruz), goat anti-mouse IgG-HRP (sc-2005, Santa Cruz) and goat anti-rat IgG-HRP (sc-2032, Santa Cruz). The MEK inhibitor U0126 was purchased from Promega (Madison, USA, V1121).

2.2. Expression constructs

pcDNA-LCN2(+) and pcDNA-LCN2(−) plasmids were constructed as follows: briefly, the full-length coding region of human LCN2 (NM_005564.3) was amplified by RT-PCR from SHEEC cells using the primers as follows: Forward: 5′-GTGGATCCTTCCTCGGCCCTGAAATC ATG-3′, reverse: 5′-GGGAATTCTCAGCCGTCGATACACTGGTC-3′. The PCR product was subcloned into the *Eco*RI site of pcDNA3.0(+) vector (Invitrogen), resulting in constructs pcDNA-LCN2(+) (referred as LCN2) and pcDNA-LCN2(−) (referred as LCN2-AS), the latter transcribing an antisense RNA to complement mature LCN2 mRNA. All relevant regions of the final constructs were confirmed by DNA sequencing.

2.3. Establishment of stable LCN2-overexpressing or LCN2 knockdown cell lines

EC109, KYSE150, KYSE180, TE3 and EC8712 cells were cultured in basic medium condition and plated (2×10^5 cells/35-mm dish) 12 h prior to transfection with SuperFect transfection reagent (QIAGEN, USA) according to the manufacturer's recommendations. After 48 h, cells were selected with the medium containing 400 μg/ml G418. The plasmid pcDNA-LCN2(+) was transfected into EC109, KYSE150 or KYSE180 cells, and referred to henceforth as the LCN2 group. The plasmid pcDNA-LCN2(−) was transfected into TE3 or EC8712 cells, and referred to henceforth as the LCN2-AS group. Empty vector pcDNA3.0 was transfected into each cell line as a control, and referred to henceforth as the vector group. A single clone of transfected cells from each group was passaged and used for all studies.

2.4. Western blotting

Western blotting was performed as described previously [20]. Whole-cell protein extracts were obtained by using RIPA buffer, and then subjected to 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore, USA). The membranes were blocked in 5% nonfat milk for 1 h, followed by the addition of primary antibody for 2 h. The membranes were then washed and incubated with secondary antibody coupled to horseradish peroxidase for 2 h. Antigen-antibody complexes were detected by Western blot luminol reagent (Santa Cruz Biotechnology, USA). Photography and quantitative analyses of related-immunoreactive bands were performed using a FluorChem™ IS-8900 (Alpha Innotech, USA).

2.5. Cell migration and invasion assays in vitro

Migration and invasion assays for stably-transfected cells were performed using transwell chambers as described as we described before [25]. For invasion assays, 1×10^5 cells were seeded onto the top chamber of a 24-well matrigel-coated membrane with 8-μm pores (Millipore, USA), and the bottom chamber was filled with medium with 10% fetal or new bovine serum (for the migration assay, cells were directly plated on an uncoated chamber). After 24 h (invasion assays) or 48 h (migration assays), the membranes were fixed and stained by Giemsa reagent. The cell numbers were quantified by counting 10 random fields under a light microscope (400×). The mean value was calculated from data obtained from 3 separate chambers. To investigate the effects of rhLCN2 or U0126 on migration and invasion assays, cells were treated with 10 μg/ml rhLCN2 or 40 μM/ml U0126 after plating in the top chamber and quantified by the same methods.

2.6. Tumor cell injection in SCID mice

Two age-matched groups of SCID mice (7 weeks-old) were injected with EC109-vector cells or EC109-LCN2 cells (1.2×10^7) in 0.2 ml serum-free 199 medium *via* the tail vein. These mice were raised under pathogen-free (SPF) conditions. The use of animals complied with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1985) and current Chinese law on the protection of animals. Each group contained six SCID mice (three males, three females). At 87 days after cell inoculation, five EC109-vector cell-injected mice were still alive, while all of the EC109-LCN2 cell-injected mice were dead.

In a separate study, to examine the effect of LCN2 on tumor metastasis *in vivo*, another two groups of SCID mice (7 weeks-old) were injected with EC109-vector cells and EC109-LCN2 cells. These mice were kept 28 days under the same conditions until they were euthanized. Lungs of the SCID mice were removed and stored in 4% paraformaldehyde for examination by hematoxylin–eosin (H&E) staining.

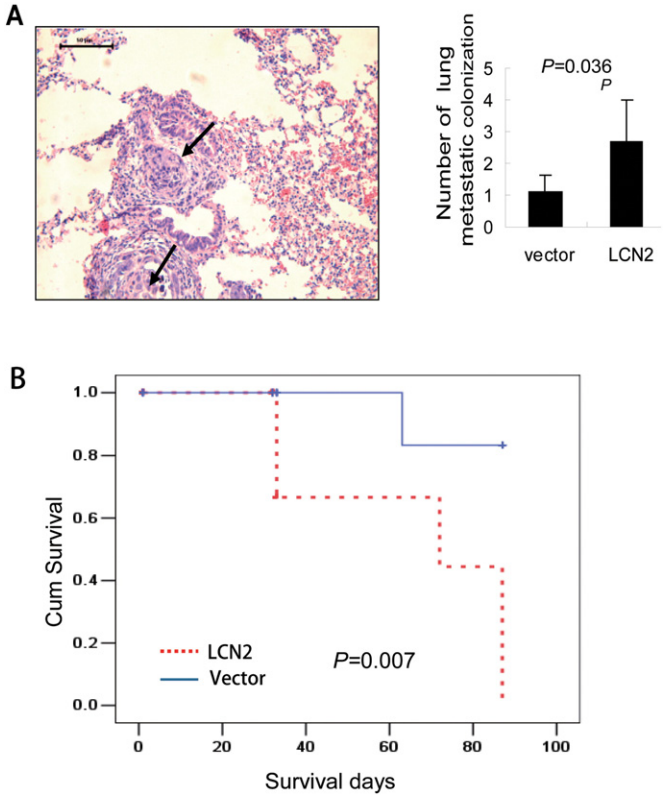

Fig. 3. LCN2 overexpression leads to increased lung metastasis and a shorter survival time. (A) Lung metastasis observed in nude mice following tail vein injection with stably-transfected EC109-LCN2 cells. H&E staining of lung tissue (left panel); a significant difference was found between nude mice injected with EC109-vector and EC109-LCN2 (right panel) cells. (B) Kaplan–Meier analysis showed LCN2 overexpression was negatively correlated with nude mice survival ($P = 0.007$).

2.7. Immunofluorescence

Stably-transfected KYSE150 cells were grown on slides in 24-well plates, which were pretreated with 10 μg/ml fibronectin at 37 °C for 2 h. When cells are 80%–90% confluent, medium was aspirated and cells were rinsed 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. The coverslips were put on ice for less than 10 min and washed again as described above. 30 μl rabbit monoclonal anti-Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (41A3) antibody (1:500, CST, #3149) was added to each coverslip and incubated overnight at 4 °C in the dark. After washing, coverslips were stained

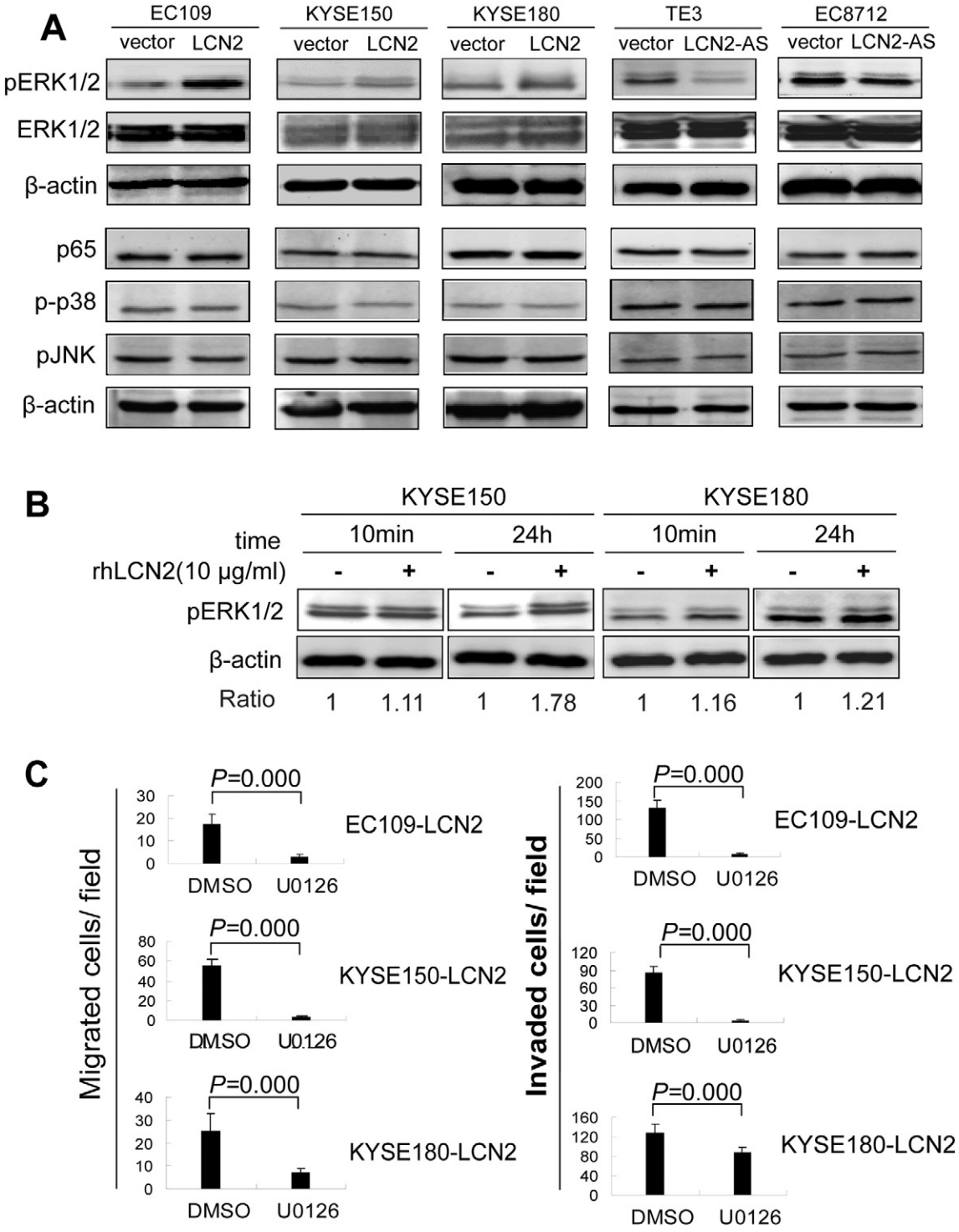

Fig. 4. Signal pathway molecules involved in LCN2-overexpressing ESCC cells. (A) pERK1/2 was elevated in ESCC cells overexpressing LCN2 and total ERK1/2 did not change. Molecules in NF-κB and MAPK signaling pathways, such as p65, p-p38 and p-JNK remained unchanged. Vector, cells transfected with pcDNA3.0; LCN2, cells transfected with pcDNA-LCN2(+). β-actin was used as a loading control. (B) pERK1/2 was elevated in serum-starved KYSE150 or KYSE180 cells stimulated for 10 min or 24 h with 10 μg/ml rhLCN2. (C) LCN2-induced cell migration and invasion is ERK-dependent. LCN2-overexpressing cells, pre-incubated for 24 h in RPMI 1640 medium without serum, were treated with or without the MEK inhibitor U0126 (40 μM), in a migration assay (left panel) and invasion assay (right panel). U0126 abolished the enhancement of migration and invasiveness by LCN2 overexpression in EC109, KYSE150 and KYSE180 cells. Data are representative of three separate experiments.

with Acti-stain™ 488 phalloidin (Cytoskeleton, US, #PHDG1) and Dylight 594-conjugated AffiniPure Donkey anti-rabbit IgG (H + L) (Jackson, #99212) for 1 h. After staining with DAPI for 10 min, the coverslips were mounted with antifade mounting medium (Beyotime, China, #P0126) on slides, and cells were observed with a confocal microscope (FV1000, OLYMPUS).

2.8. Live-cell imaging

Stably-transfected KYSE150 cells were plated in fibronectin-coated 24-well plates. Cells were transiently transfected with the GFP-LifeAct (kindly provided by Prof. Brian Stramer, University of Manchester, UK) using Lipofectamine 2000 reagent (Invitrogen, USA). After 12 h, cells

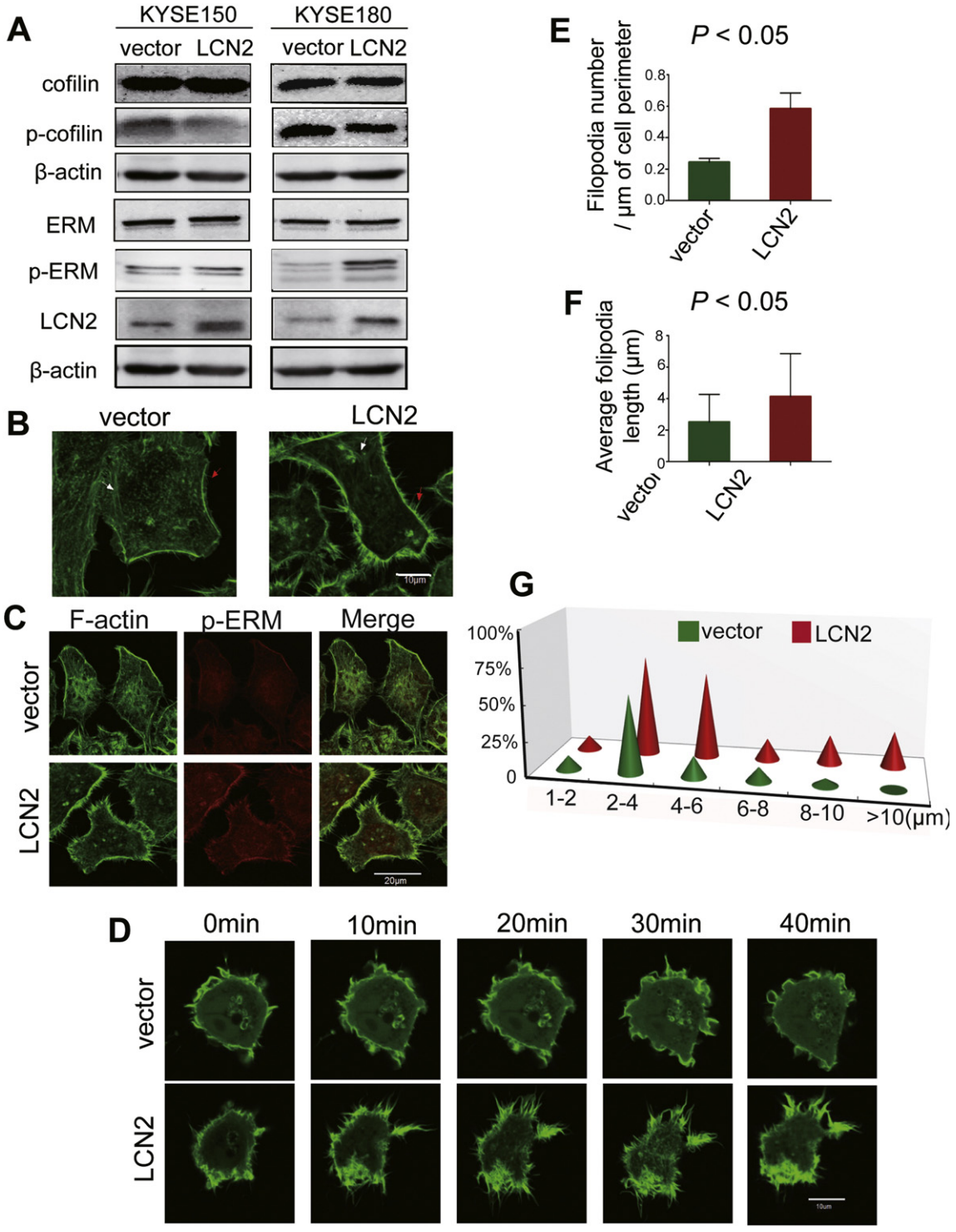

Fig. 5. LCN2 overexpression mediates actin cytoskeleton rearrangement. (A) Cofilin, an actin disassembly protein, was detected in LCN2-overexpressing ESCC cells. Inactive p-cofilin was decreased with unchanged total cofilin protein. The protein level of activated p-ERM (T567) was also increased in both LCN2-elevated KYSE150 and KYSE180 cells. (B) Filopodia in KYSE150-LCN2 cells were dramatic increased compared with the control. The stress fiber was indicated by a white arrow. Scale bar, 10 μm. (C) Increased p-ERM also co-localized with F-actin, especially in extended filopodia. Scale bar, 20 μm. (D) The number and length of filopodia were greater in LCN2-overexpressing cells shown in a time-lapse series. Scale bar, 10 μm. (E–G) The per cell filopodia counts, length and length range were analyzed from three KYSE150-LCN2 or KYSE150-vector cells.

were digested with trypsin, transferred to a 35 mm Petri-dish, and allowed to attach for 1 h before live-cell imaging. The treated cells were visualized using a confocal microscope (FV1000, OLYMPUS). We recorded one frame per 1 min. Filopodia number and length were measured and analyzed from two independent experiments using NIH ImageJ software.

2.9. Real-time reverse transcription polymerase chain reaction

Total cellular RNA was extracted with Trizol reagent (Invitrogen) from KYSE180 cells treated with 10 μg/ml rhLCN2 and reverse-transcribed to cDNA using the Reverse Transcription System (Promega, USA). Real-time RT-PCR was carried out using the Rotor-Gene 6000 system (Corbett Life Science, Australia) using SYBR®Premix Ex Taq™ (TaKaRa, Japan) according to the manufacturer's instruction. All PCRs were done in triplicate. The absolute LCN2 mRNA level was normalized to β-actin.

2.10. Statistical analysis

The differences in cell migration and invasiveness *in vitro* and the number of lung metastases in the SCID mice were analyzed by the Student's *t*-test. Lifespans of the mice were analyzed using the Kaplan–Meier method. A two-tailed *P*-value less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS 13.0 for Windows software (SPSS INC., Chicago, US).

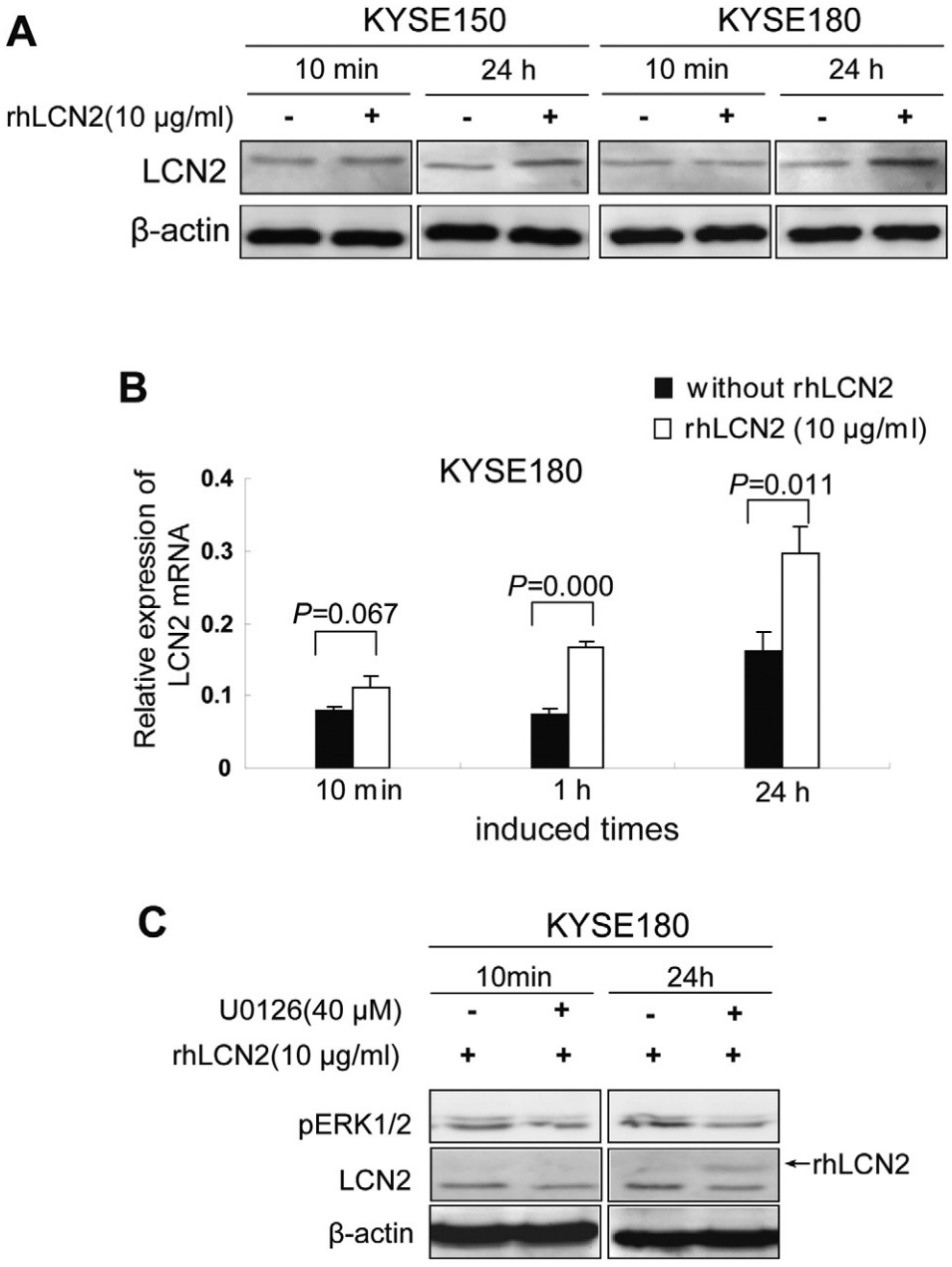

Fig. 6. A positive LCN2 feedback loop is mediated by the MEK/ERK signaling pathway. (A) LCN2 was elevated in KYSE150 or KYSE180 cells stimulated for 10 min or 24 h with 10 μg/ml rhLCN2. (B) LCN2 mRNA levels in cells induced by rhLCN2 at different time points were analyzed by qRT-PCR. β-actin was the internal control. (C) U0126 reduced endogenous LCN2 protein levels and abolished the effect of LCN2 on ERK. These results show that U0126 blocked the LCN2 positive feedback loop involving MEK/ERK. Cell lysates were obtained from KYSE180 cells stimulated for 10 min or 24 h with 10 μg/ml rhLCN2 with or without U0126 (40 μM) pretreatment for 1 h. Changes in pERK1/2 and endogenous LCN2 protein levels were detected.

3. Results

3.1. LCN2 over-expression increases the migration and invasiveness of ESCC cells in vitro

To study the expression pattern of the LCN2 in ESCC cell lines, we initially examined the LCN2 protein level in a panel of six ESCC cell lines (Fig. 1A). Low LCN2 protein levels were observed in EC109, KYSE150 and KYSE180 cells, which were selected for LCN2 over-expression, while the TE3 and EC8712 cell lines displayed high LCN2 protein levels and were selected for LCN2 knockdown in subsequent function studies. Since tumor cell motility is an important characteristic contributing to metastatic tumor cell spread, to explore the roles of LCN2 in migration and invasiveness, pcDNA-LCN2(−) or empty vector (pcDNA3.0) were stably transfected into the TE3 and EC8712 cell lines. Decreased expression of LCN2 protein was confirmed by western blotting (Fig. 1B). Then, we evaluated the migration and invasiveness of the transfected cells *in vitro*. As shown in Fig. 1C and D, the migration and invasiveness of both LCN2-AS ESCC cell lines was significantly lower than those of the control group.

On the other hand, when pcDNA-LCN2(+) was stably transfected into the EC109, KYSE150 and KYSE180 cell lines to over-express LCN2 protein (Fig. 2A), the migration and invasiveness of ESCC cells was significantly increased compared to empty vector-transfected cells (Fig. 2B and C). Since LCN2 is a secreted protein, it was of interest to understand the effects of the secreted LCN2 protein on ESCC cells. Migration and invasion assays were also performed with KYSE150 and KYSE180 cells that were stimulated by 10 μg/ml recombinant human LCN2 protein (rhLCN2) after inoculating in transwell chambers. Interestingly, migration and invasion of KYSE150 and KYSE180 cells was also greatly enhanced after rhLCN2 stimulation (Fig. 2D).

3.2. LCN2 overexpression promotes lung metastasis and decreases survival in tumor-bearing mice

To explore the role of LCN2 in the progression of ESCC, EC109 cells were stably transfected with pcDNA-LCN2(+) (EC109-LCN2) or empty vector pcDNA3.0 (EC109-vector) (Fig. 2A). Then, both cell lines were separately injected into SCID mice *via* the tail vein. The effect of LCN2 on tumor metastasis to the lung was addressed by observing metastatic colonizations on the lung surface, following H&E staining, at 28 days after tumor cell injection (Fig. 3A). Lung colonization by EC109-LCN2 cells was significantly greater than EC109-vector cells, suggesting that over-expression of LCN2 promotes cancer metastasis *in vivo* (Fig. 3A).

Effect of LCN2 overexpression on the survival of the nude mice was also analyzed. EC109-LCN2 and EC109-vector cells were injected into SCID mice *via* tail veins. The investigation was suspended when all of the EC109-LCN2 mice died, and then survival rates were evaluated using Kaplan–Meier survival curves and the log-rank test. As shown in Fig. 3B, the survival rate of LCN2-overexpressing cell-bearing mice was significantly lower than that of the control group ($P = 0.007$).

3.3. Increased LCN2 activates pERK1/2

There are several mechanisms for LCN2 involves in the metastasis and invasion of cancer cells, including MMP-9 [4,20], E-cadherin [10], and NF-κB [26]. To investigate whether overexpression of LCN2 altered the status of MMP-9 in ESCC, the zymography analyses were performed and showed that the activity of MMP-9 in LCN2 over-expressing cells was obviously elevated (Fig. S2), suggesting LCN2 could enhance tumor cell metastasis and invasiveness through enhancing the activity of MMP-9 in ESCC cells, which is consistent with the previous reports.

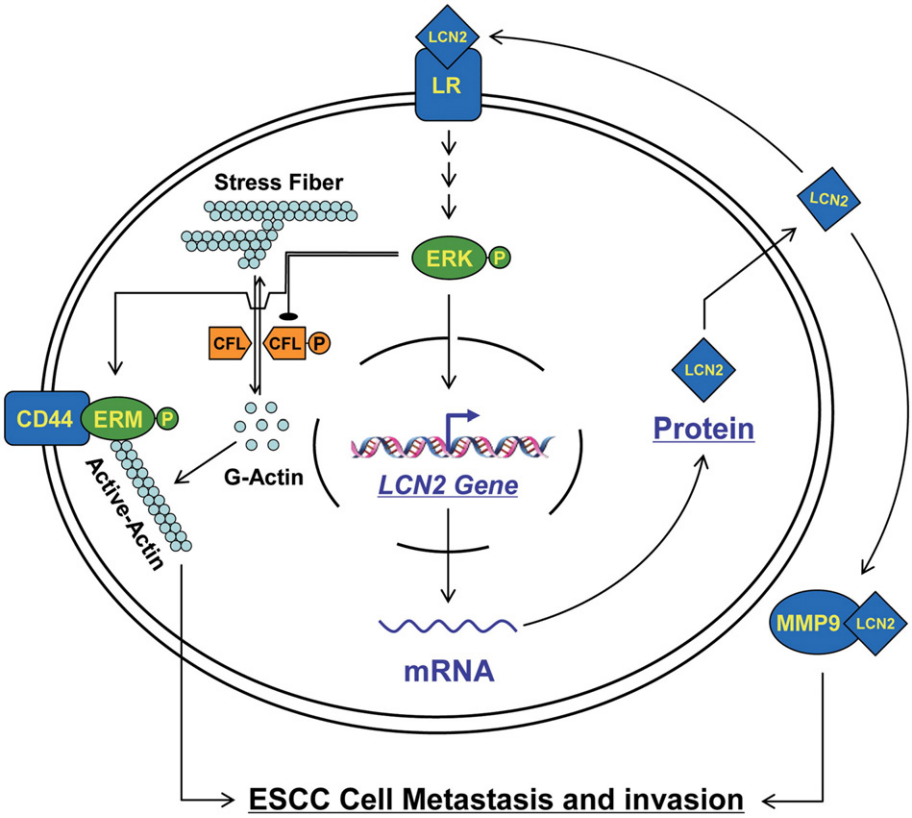

Fig. 7. Schematic representation of molecular mechanisms through which LCN2 promotes the invasiveness of ESCC cells by a positive feedback loop. LCN2 not only protects MMP-9 to facilitate cancer cell migration and invasion, but also increases the level of pERK1/2, which in turn leads to the increase of LCN2 at both the mRNA and protein levels. Elevated pERK1/2 also increases p-ERM, but decreases p-cofilin, causing rearrangement of the actin cytoskeleton to promote the migration and invasion of ESCC cells. LR, LCN2 receptor; CFL, cofilin; ERM, ezrin/radixin/moesin protein family.

We further investigated the effect of LCN2 on E-cadherin. It showed that the E-cadherin protein level did not alter obviously after the treatment of rhLCN2 (Fig. S3). These results indicated that some new mechanism underlies LCN2 promotion of metastasis and invasion, except the known protection of MMP-9 from degradation. To examine the impact of LCN2 on other signaling pathways, ERK1/2, pERK1/2, NF-κB, p-p38, p-JUN and p-AKT were also detected. Nevertheless, NF-κB, p-p38, p-JUN and p-AKT did not change significantly, suggesting that these molecules did not involve in the LCN2 promotion of metastasis and invasion in ESCC cell (Fig. 4A, Fig. S4). To our interest, the pERK1/2 level was significantly higher in LCN2-overexpression cells than that in the control cells, while pERK1/2 was decreased in the cells in which LCN2 expression was blocked (Fig. 4A). Intriguingly, addition of 10 μg/ml rhLCN2, which promoted ESCC cell migration and invasion, also resulted in the elevation of pERK1/2, with peak levels occurring at 24 h (Fig. 4B). To determine whether LCN2-mediated ERK activation was essential for cell migration and invasion, three LCN2-overexpressing cell lines were treated with the MEK inhibitor U0126 after inoculating cells in a transwell chamber. U0126 treatment completely abolished LCN2-mediated cell migration and invasion (Fig. 4C).

3.4. LCN2 overexpression-mediated actin cytoskeleton rearrangement

It has been reported that MEK/ERK promotes dephosphorylation of cofilin and subsequent the disassembly of stress fibers by interrupting the Rho-ROCK-LIMK cascade [27,28]. Cofilin, a small ubiquitous protein (~19 kDa) that is able to bind to both G-actin and F-actin, is closely associated with actin cytoskeletal rearrangement and tumor invasion, and which is inhibited by its phosphorylation (p-cofilin) [29]. It has previously been shown that ERK1/2 is upstream of ezrin/radixin/moesin (ERM) protein family and enhances its activation (phospho-ERM, p-ERM) [30]. The ERM protein family, which cross-links actin filaments with plasma membrane proteins, is able to enhance the assembly of stress fibers and thus filolodia after its phosphorylation modification [31,32]. Based on these evidences, we proposed whether LCN2 promotion of metastasis and invasion *via* the activation of ERK pathway, which induces its downstream effectors to cause the rearrangement of F-actin. Therefore, the protein levels of p-cofilin and p-ERM in these cell lines were detected by western blotting. The results showed that p-cofilin protein level was significantly decreased, but p-ERM (T567) was obviously increased after LCN2 overexpression (Fig. 5A). Inspired by the change of p-cofilin and p-ERM after LCN2 was overexpressed, we therefore examined the role of LCN2 in stress fiber and filopodia formation. Using phalloidin staining, we found that stress fiber formation was dispersed and suppressed in KYSE150-LCN2 cells. On the other hand, filopodia in KYSE150-LCN2 cells were significantly increased compared with the control (Fig. 5B). Moreover, we found that the stress fibers in LCN2-overexpressing EC109 cells were more disordered than the control, with F-actin cytoskeleton was radially rearranged in the periphery of LCN2-overexpressing cells (Fig. S5). These results suggest that upregulation of LCN2 inhibits stress fiber formation and enhances filopodia extension. Since p-ERM is enhanced after LCN2 overexpression, we next performed immunofluorescence staining experiments to evaluate the subcellular localization of p-ERM. We found increased p-ERM co-localization with F-actin, especially in the extended filopodia (Fig. 5C).

To further confirm our findings, we performed live-cell imaging analyses for KYSE150-transfected cells to observe the dynamic activity of filopodia. As shown in Fig. 5D, the number and length of filopodia was more obvious in the LCN2-overexpressing cells in a time course. The progression of extension and formation of filopodia is also shown in Movie S1 and Movie S2 for LCN2-overexpressing KYSE150 cells and its control, respectively. We also analyzed the per-cell filopodia counts, length and length range from three KYSE150-transfected cells. The results indicated that LCN2 overexpression leads to a greater number of filopodia, and filopodia that are longer on average (Fig. 5E–G).

3.5. ERK-mediated LCN2 regulations positive feedback

Because pERK1/2 was elevated after stimulation with rhLCN2 (Fig. 4B), we examined the impact of ERK signaling on the expression of LCN2. To our surprise, the endogenous LCN2 protein level was increased after stimulation with rhLCN2 protein in both KYSE150 and KYSE180 cells (Fig. 6A). The expression pattern of LCN2 was similar to the activation of pERK1/2, with levels of both peaking at 24 h. To test whether this regulation occurred at the transcriptional level, we analyzed LCN2 mRNA levels by qRT-PCR. Consistently, the results showed that at 10 min, 1 h and 24 h post-stimulation, the LCN2 mRNA level was elevated 1.40-fold, 2.24-fold and 1.83-fold, respectively (Fig. 6B). Analysis of the activity of the LCN2 promoter (−1431 to +81), in stably-transfected KYSE150 cells, using a dual-luciferase reporter system, also indicated that the promoter activity was higher in LCN2-overexpressing cells than that in the control cells (Fig. S6). Moreover, the MEK inhibitor U0126 could diminish the stimulation of endogenous LCN2 protein and pERK1/2 in KYSE180 cells induced by rhLCN2 (Fig. 6C).

4. Discussion

LCN2, a small molecular weight secreted glycoprotein, is upregulated in a variety of cancers, including ESCC [8–21]. Recent studies suggest that LCN2 is a key player in different cancer types [10,12,23]. However, its importance in the progression of cancers remains inconsistent and puzzling. The data presented in our study suggest that an oncogenic effect of LCN2 is as an enhancer of ESCC metastasis. This is supported by at least two lines of experimental evidence. First, ectopic expression of LCN2 in ESCC cell lines increased metastatic and invasive ability both *in vitro* and *in vivo*. In contrast, blocking LCN2 in ESCC cell lines with high endogenous LCN2 protein levels decreased the ability to metastasize and invade *in vitro*. Second, treatment with recombinant LCN2 enhanced the metastatic and invasive abilities of ESCC cell lines with low endogenous LCN2 expression.

Our data is consistent with our previous report that overexpression of LCN2 is associated with the tumor invasion depth in ESCC tissue [20]. In accordance with our results, mounting evidence has demonstrated that elevated LCN2 expression promotes the invasion and progression of tumors, such as breast cancer and colon cancer [10,11]. Conversely, several lines of evidence demonstrate that LCN2 is a suppressor of cancer cell metastasis and invasion, such as pancreatic cancer [12]. Two possibilities could explain this complex biological phenomenon. One is the distinct molecular mechanisms of LCN2 in different cell types. LCN2 has been reported to increase cell motility and invasion by decreasing E-cadherin mediated cell–cell adhesion in colon carcinoma cells [12]. But in our LCN2-overexpressing cell lines, E-cadherin did not show the typical decrease following LCN2 overexpression (Fig. S2). A second possibility is the different activated signaling pathway(s) involving LCN2. This is supported by a previous study showing that the HER2/phosphoinositide 3-kinase/AKT/NF-κB signal cascade induces LCN2 expression to facilitate breast tumorigenesis and metastasis [26]. However, we show here that LCN2 expression is largely dependent on the ERK1/2 pathway in ESCC.

Accumulated evidences suggest the importance role for ERK1/2 in the regulation of cytoskeleton rearrangement through its downstream effectors, such as cofilin and p-ERM [33,34]. Our results in this study also suggested that ERK1/2 might be a hub for the role of LCN2 in ESCC. Thus, we proposed that there is the positive feedback loop of LCN2 in ESCC, whereby LCN2 acts as a tumorigenic effector by stimulating the MEK/ERK signaling pathway through its membrane receptor (Fig. 7) [5,21,35]. In this instance, ectopic expression LCN2 activates pERK1/2, which in turn increases endogenous LCN2. The elevated LCN2 stabilized MMP-9 by forming heterodimer complexes to facilitate motility and invasiveness of ESCC cells. On the other hand, the decreased of p-cofilin and increased p-ERM induced by pERK1/2 cause the cytoskeleton F-actin rearrangement and alter the behavior of ESCC

cells mediated by LCN2. As a consequence, activation of MMP-9 and the rearrangement of F-actin throw light on the role of LCN2 in promoting migration and invasion in ESCC. To our knowledge, this is the first report that LCN2 activates MEK/ERK signaling in a feedback loop in cancer. Similar to our results, it has been reported the interaction of CTGF and β-catenin-TCF/Lef form a positive feedback loop, which could contribute to the tumorigenicity of ESCC [36]. These results indicate that feedback loops widely exist and regulate the behavior of ESCC cells. LCN2 plays an important role in ESCC cell biology by promoting ESCC cell invasion and metastasis, but evidence is still lacking about how it actives MEK/ERK to mediate this positive feedback loop and other key biological events in neoplastic progression.

In summary, our results suggest that LCN2 and MEK/ERK signaling pathway form a positive feedback loop. LCN2 can activate MEK/ERK signaling pathway and thus contribute to ESCC malignancy. Furthermore, MEK/ERK signaling also positively regulates the expression of LCN2, which further promotes the progression of ESCC. These findings not only provide insight into the molecular mechanism underlying LCN2 in cancer development, but also expand the regulatory network of the MEK/ERK-LCN2 signaling pathway in the development and progression of ESCC.

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Conflict of interest

The authors report no conflict of interest.

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