

LAMC1, Upregulated by TGF β in Tumor Cells, Contributed to The Formation of Inflammatory Cancer-Associated Fibroblasts Via NF- κ B/CXCL1/STAT3 in Esophageal Squamous Cell Carcinoma.

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Research

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Abstract

Background: The tumor microenvironment (TME) consists of a variety of cells that interact with each other through cytokines. As an important member of the TME, cancer-associated fibroblasts (CAFs) play an important role in the development of tumor cells, which is influenced by the heterogeneity of CAFs. Transforming growth factor β (TGF β) not only plays a dual role in the progression of tumor cells directly but also influences tumor cells by regulating the heterogeneity of CAFs.

Methods: we explored oncogenes regulated by TGF β , which were involved in signaling molecules and interactions in the TME. We analyzed sequencing data of TCGA and GSE53625, as well as ESCC cell lines with or without TGF β 1 stimulation, and then we focused on laminin subunit gamma 1 (LAMC1). The upregulation of LAMC1 after TGF- β 1 stimulation was examined by western blot (WB), quantitative real time PCR (qRT-PCR) and Chromatin immunoprecipitation (ChIP). We performed gain-of-function and loss-of-function assays to examine the effect of LAMC1 on proliferation and migration of ESCC cells. CAFs were isolated and cocultured with ESCC cells. And conditional medium of shLAMC1 ESCC cells and CAFs with different treatments were collected. RNA-seq of those cells were also performed. Luminex liquid suspension chip detection, ELISA, WB, qRT-PCR and rescue experiments were carried out to reveal the interaction of between ESCC cells and CAFs.

Results: LAMC1 was highly expressed in ESCC, affecting the prognosis of patients. Moreover, LAMC1 could be upregulated by TGF β 1 through SMAD4 and SP1 synergistic activation. Further experiments showed that LAMC1 would promote the proliferation and migration of tumor cells mainly via Akt/NF- κ B/MMP9 and MMP14. Additionally, LAMC1 would promote CXCL1 secretion mainly by activating NF- κ B. Tumor-secreted CXCL1 remodeled the formation of inflammatory CAFs (iCAFs) through CXCR2/pSTAT3. The conditioned medium of iCAFs promoted the proliferation and migration of tumor cells.

Conclusions: Our study identified the mechanism by which upregulation of LAMC1 by TGF β in tumor cells not only promoted ESCC progression but also indirectly induced carcinogenesis by stimulating CXCL1 secretion and promoting the formation of iCAFs. This suggests that LAMC1 could be a potential therapeutic target and prognostic marker for ESCC.

Background

Esophageal cancer is one of the most common cancers, 90% of which is Esophageal Squamous Cell Carcinoma (ESCC)²². ESCC is highly malignant, with a five-year survival rate between 15% and 25%¹. The tumor microenvironment (TME) is mainly composed of immune cells, fibroblasts, endothelial cells and extracellular matrix (ECM), which play an important role in promoting tumor proliferation, inhibiting tumor apoptosis and causing immune escape^{25, 30, 44}. As an important member of the TME, cancer-associated fibroblasts (CAFs) mainly secrete cytokines, modify the ECM, and interact with tumor cells and other cells^{14, 23, 42}. For example, CAFs can secrete VEGF to promote tumor cell angiogenesis⁴⁹, while CAFs-secreted IL6 and FAP increase proliferation and tumor recurrence^{11, 15, 31, 38}. CAFs have been used as a

therapeutic target in many previous studies, but the results were disappointing^{3, 9, 34}. An important reason may be the heterogeneity of CAFs^{16, 33}. Similar to tumor cells, different subgroups of CAFs exist among different tumors and within the same tumor^{5, 18, 29}. According to the different functions of CAFs in tumors, CAFs are divided into tumor-promoting CAFs, tumor-suppressing CAFs and neutral CAFs in gastrointestinal tumors¹⁸. In pancreatic ductal adenocarcinoma, CAFs are classified into distinct inflammatory CAFs (iCAF) and myofibroblasts (myCAF), which are not only different in their transcriptional profile but also significantly differ in their tumor distribution location and effect on tumors^{5, 33}. In breast cancer, the specific subgroup of CD10⁺ GPR77⁺ CAFs can maintain the stemness of tumor cells by secreting IL6 and IL8 and causing chemotherapy resistance. Targeting this specific CAFs subgroup can reverse chemotherapy resistance in breast cancer⁴⁰. The heterogeneity of CAFs is influenced by paracrine and other pathways in other cells, such as IL1-induced LIF expression and downstream JAK/STAT activation to generate iCAF^{5, 39}.

The regulation of CAFs heterogeneity by TGF β is controversial. The TGF β signaling pathway has both oncogenic and anticancer effects in ESCC^{25, 35, 36}. In the early stage, tumor growth is inhibited by reduced TGF β responsiveness. However, it later also promoted tumor invasion and metastasis. This "switch" in carcinogenesis may be related to the absence of adaptor proteins, such as β 2-spectrin²⁵. This contradictory effect of TGF β not only exists in tumor cells but also regulates the phenotype of CAFs. The exosomes secreted by tumor cells contain TGF β and induce the transformation of fibroblasts into activated CAFs in bladder cancer³⁹. In prostate cancer, bone marrow-derived mesenchymal stem cells secrete TGF β , which promotes the conversion of normal fibroblasts into CAFs with tumorigenicity⁴⁶. However, tumor cell-secreted TGF β antagonizes the activation of IL1 signaling and inhibits the transformation of myCAF into iCAF, which plays an anticancer role in pancreatic ductal adenocarcinoma⁵.

Overall, the tumorigenic effect of TGF β on tumor cells has dual roles: it can affect tumor cells directly and indirectly through TME cell cross-talk, especially between tumor cells and CAFs³⁷. We explored oncogenes regulated by TGF β 1, which are involved in signaling molecules and interactions in the TME. Through bioinformatics analysis, we hypothesized that laminin subunit gamma 1 (LAMC1) is an oncogene that is upregulated by TGF β 1 and participates in cell-to-cell signal transduction in the TME in ESCC. Many previous studies have shown that high expression of LAMC1 promotes tumor progression and can be used as a prognostic biomarker in many cancers, such as hepatocellular carcinoma, colorectal cancer, and endometrial cancer^{21, 52}. LAMC1 is involved in many carcinogenic effects within tumor cells. For example, miR-29b-3p negatively regulated LAMC1 to inhibit melanoma invasion⁴. LAMC1 can also promote the Warburg effect by upregulating PKM2 in hepatocellular carcinoma⁵⁰. This study explored the carcinogenic effect of LAMC1 in ESCC from two aspects: direct effect on tumor cells and indirect effect through the TME, especially CAFs. Overall, we hypothesized that high expression of LAMC1, upregulated by TGF β 1, affected the prognosis of ESCC patients, and LAMC1 was involved in signaling molecules and interaction pathways in the tumor microenvironment. This study focused on

exploring the tumorigenic mechanism of LAMC1 and its role in the microenvironment, especially in the interaction of tumor cells and CAFs.

Methods

Patients and tumor samples

A paraffin-embedded ESCC microarray containing 55 ESCC tissues and 50 adjacent tissues was purchased from Outdo Biotech (catalog no. HEsoS105Su01) for immunohistochemistry; 12 fresh ESCC tissues were obtained at our hospital for isolation of CAFs in 2018.

Isolation of CAFs and coculture system

As described in our previous study, homogeneous CAFs were isolated from fresh tumor tissue and were identified using the cellular immunofluorescence marker α SMA⁸. Homogeneous CAFs were obtained for further analysis. All CAF cells used in the experiment were grown for no more than 10 passages. For the coculture system, CAFs were seeded in a 24-well plate with or without SB225002 in the medium, and the ESCC cells were placed in the upper chamber with a 0.4 μ m pore size (Corning, USA). Cells were cocultured for 48 h, and then the RNA and proteins of CAFs were extracted.

Collection of conditioned medium (CM)

As previously described, the CM was collected after cells had been cultured in serum-free medium for 24 h and was centrifuged at $1,000 \times g$ for 5 min⁸. The CM was only concentrated 40-fold for western blot via Centricon Centrifugal filter (Millipore, USA). The CM used to stimulate cells was sterile filtered and diluted 1 time with the medium.

Drugs

For KYSE30 and KYSE450 cell treatments, recombinant TGF β 1 (R&D, USA) was used at a final concentration of 10 ng/ml unless otherwise specified. TNF α (PeproTech, China) was used at final concentrations of 10 ng/ml. Treatment periods were 24 h unless otherwise specified. We used 10 μ M SB505124 and 10 μ M JSH-23 (Selleck) to inhibit TGF β signaling and NF- κ B signaling, respectively. These inhibitors were administered to the cells 30 min before any other treatments. And 5 μ M MK-2206 2HCl(Selleck) was used to selectively inhibited Akt phosphorylation for 24 h. CAFs were treated with 10 ng/ml recombinant CXCL1 (PeproTech, China) for 24 h. SB225002 (Selleck, USA) was added 1 h prior to inhibiting CXCL1/CXCR2.

Tumor xenograft experiment

As previously described, 1×10^6 LAMC1 knockdown or overexpression KYSE30 cells and control cells were subcutaneously injected into the flanks of BALB/c nude mice to establish tumor xenografts (6 mice per group). In another experiment, MRC-5 cells were stimulated with 20 ng/ml TGF β 1 for 4–5 days before

further animal experiments. A total of 1×10^6 WT KYSE30 cells alone or 5×10^5 WT KYSE30 cells admixed with 5×10^5 MRC-5 cells (or rCXCL1-pretreated MRC-5 cells) were resuspended in equal 0.2 ml of PBS and then subcutaneously injected into the flanks of mice to establish tumor xenografts (6 mice per group). Subgroups of mice were treated with SB225002 (1 mg/kg) intraperitoneally once every two days when the tumors reached 5 mm in diameter. The tumor volume was calculated by the formula $V = (L \times W^2)/2$. Three or four weeks later, all BALB/c nude mice were sacrificed, and the tumors were excised and weighed.

Lung colonization assay

As described previously, cells were injected into female NOD-SCID mice through the tail vein. A total of 1×10^6 sh-1, sh-vec, LAMC1 and vector KYSE30 cells were injected (6 mice per group)²⁶. The mice were sacrificed seven weeks later, and the lungs were excised and fixed with 4% polysorbate, followed by embedding in paraffin for hematoxylin and eosin (H&E) staining. The number of lung surface metastatic nodes was calculated by gross and microscopic examination as previously described.

Statistical analysis

Prism GraphPad version 6.0, SPSS, GSEA, R script were used. Correlations between mRNA expression levels were analyzed using Pearson's correlation coefficient. A chi square test was performed to determine the relationship between clinicopathological variables and LAMC1 expression. Overall survival (OS) curves were analyzed by the Kaplan-Meier method and log-rank tests. The significant differences between different groups were analyzed using a two-tailed t-test. Data are presented as the mean \pm standard deviation (SD). Differences were considered significant at $P < 0.05$ and are indicated as **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ (ns, not significant)

Results

1. LAMC1 expression was upregulated by TGF β through synergistic activation of SMAD4 and SP1 and predicted a poor prognosis in ESCC.

Much of the previous research has established that TGF β plays an important role in the tumor microenvironment, especially in cell-to-cell signaling²⁵. We sought to identify genes regulated by TGF β 1, and those genes were found to be involved in signaling molecules and interaction pathways of the tumor microenvironment in ESCC. Furthermore, these genes themselves could influence the prognosis of patients with ESCC. We made these observations through the following process. First, by analysis of our mRNA microarray data (GSE53625), we found that 4130 genes were upregulated in cancer tissues compared with adjacent tissues ($\text{Log}_2\text{FC} > 0.5$, $\text{FDR} < 0.001$), and 238 genes were significantly ($p < 0.05$) associated with poor prognosis (Fig S1A). Second, by using Pearson's correlation analysis of GSE53625 and TCGA data, we found that 1609 genes were positively correlated with TGF β 1 in ESCC cancer tissues ($r > 0.15$, $\text{FDR} < 0.05$) (Fig. 1A, S1B). At the same time, in our previous study, RNA-seq was performed on

TGF β 1-treated and untreated ESCC cells²⁶, and we found that a total of 3084 genes were upregulated (Log₂FC > 0, FDR < 0.05) (Fig. 1B). Combining the two results, we found that a total of 625 genes were co-expressed with TGF β 1 and upregulated by TGF β 1 (Fig. 1C). Enrichment analysis of these genes (KOBAS)⁴⁷ (FDR < 0.0001) revealed that a total of 11 pathways were involved in environmental information processing, only two pathways of which, cytokine-cytokine receptor interaction and ECM-receptor interaction, were included in signaling molecules and interaction pathways (Fig. 1D, Fig S1C). A total of 31 genes were enriched in these two pathways. The 31 genes are positively regulated by TGF β 1 and are involved in signaling and cellular interactions in the TME. Overlap of the genes associated with prognosis showed that LAMC1 was the only one of the 31 genes that affected the prognosis of ESCC patients (Fig. 1E). Accordingly, at the protein level, LAMC1 was more highly expressed in cancer tissues than in para-cancer tissues, as demonstrated by IHC staining (Fig. 1F) and was also associated with low OS (Fig. 1G) and tumor stage (Supplementary Table 1). Therefore, LAMC1 can be used as an independent prognostic marker for ESCC.

Compared with that in the control group, the expression of LAMC1 was increased at the protein and mRNA levels in KYSE30 and KYSE450 cells after TGF β 1 treatment, which was time- and concentration-dependent in ESCC cells (Fig. 1H, Fig S2A, B). Additionally, to determine whether the TGF β signaling pathway is responsible for the expression of LAMC1, we used the TGF β receptor inhibitor SB505124 to eliminate the effect of TGF β 1 on LAMC1. The results showed that SB505124 could reverse TGF β 1-induced LAMC1 expression in KYSE30 and KYSE450 cells (Fig. 1I, J). These results suggest that TGF β signaling is responsible for the induction of LAMC1 transcription. By TRANFAC and JASPER database prediction, we speculated that the transcription factors SMAD4 and SP1 synergistically induced LAMC1 transcription. A ChIP assay was performed using anti-SMAD4 and anti-SP1 antibodies, and we found that TGF β 1 led to a significant increase in the enriched LAMC1 promoter sequence, suggesting that SMAD4 and SP1 were recruited to the promoter of the LAMC1 gene by TGF β 1 treatment (Fig. 1K). Additionally, by measuring proteins of chromatin fractions with antibodies against SP1 and SMAD4, we found that the expression of SMAD4 and SP1 were increased in each other's chromatin fraction (Fig. 1L). Furthermore, we conducted knockdown SP1 or SMAD4 ESCC cells respectively, and conducted ESCC cells that combined knockdown SP1 and SMAD4 (Fig. 1M, N, Fig S3 A, B). The expression of LAMC1 was decreased in those cells, which could not be rescued by TGF β 1 treatment (Fig. 1O-Q). This result suggested that the transcription factors SP1 and SMAD4 together induced the transcription of LAMC1. Taken together, these results showed that LAMC1 would be directly regulated by the TGF β /SMAD4-SP1 signaling pathway.

2. LAMC1 promoted the proliferation and migration of ESCC cells in vitro and in vivo.

To evaluate the tumorigenic effect of LAMC1 on ESCC, we constructed KYSE30 and KYSE450 cell lines with stable knockdown or overexpression of LAMC1 (Fig. 2A, B). shLAMC1 in KYSE30 and KYSE450 cells inhibited cell proliferation. Accordingly, overexpression of LAMC1 promoted cell proliferation (Fig. 2C). And shLAMC1 in KYSE30 and KYSE450 cells promoted apoptosis (Fig. 2D). Furthermore, we also found

that overexpression of LAMC1 could promote ESCC cell migration, while shLAMC1 inhibited migration (Fig. 2E).

In vivo, we established a xenograft tumor mouse model by subcutaneous inoculation or intravenous tail injection of KYSE30 cells transfected with shLAMC1, sh-vec, overexpression-LAMC1 and control vector. Consistent with the results of the in vitro experiments, the tumor volume and weight in the overexpressing LAMC1 group were significantly increased compared with those in the control group. The shLAMC1 group exhibited the opposite pattern (Fig. 2G-J). The number of pulmonary metastasis nodules in the groups showed similar results (Fig. 2K, L).

3. The positive effect of LAMC1 on the migration in ESCC cells mainly via the Akt/IKK α /NF- κ B/MMP9-MMP14 pathway.

We performed mRNA sequencing in KYSE30 and KYSE450 cells with sh-1 LAMC1 or sh-vec to explore downstream signaling pathways responsible for the aggressiveness of ESCC. Gene set enrichment analysis (GSEA) suggested that LAMC1 knockdown could affect the apoptosis pathway, NF- κ B pathway, and cytokine and chemokine pathways (Fig. 3A). We detected IKK α phosphorylation of Akt, IKK α and p65 levels in shLAMC1 and LAMC1-overexpressing ESCC cells and found that expression of LAMC1 was positive correlated with phosphorylation of Akt, IKK α and p65 (Fig. 3B, C). Matrix metalloproteinases (MMPs) play an important role in tumor cell invasion and metastasis and are common downstream regulators of NF- κ B-mediated cell metastasis¹⁷. We detected MMP2, MMP9, MMP10, MMP13, and MMP14. The results showed that the expression of MMP9 and MMP14 was in accordance with the phosphorylation of Akt, IKK α and p65 in shLAMC1-expressing and LAMC1-overexpressing ESCC cells (Fig. 3B, C). Furthermore, through cell immunofluorescence, we also found the expression of NF- κ B (p65) were decreased in the nucleus in knockdown LAMC1 cells than the controls (Fig. 3D). Additionally, TNF α , as an activator of the NF- κ B pathway, could restore the expression of phosphorylation of IKK α , NF- κ B, MMP9 and MMP14 in knockdown LAMC1 (Fig. 3E). Accordingly, the Akt phosphorylation selective inhibitor MK-2206 and the NF- κ B nuclear translocation inhibitor JSH-23 both could reversed the high expression of phosphorylation of Akt, IKK α , NF- κ B, MMP9 and MMP14 in overexpression LAMC1 cells (Fig. 3F). TNF α , also, reversed the inhibitory migration of shLAMC1 ESCC cells (Fig. 3G, S4 A). Accordingly, JSH-23 abrogated the promotion of migration in LAMC1-overexpressing ESCC cells (Fig. 3H, S4 B I).

4. LAMC1 inhibited apoptosis mainly through the Akt/NF- κ B/caspase9-caspase3-PARP cascade.

Cleaved caspase-9, cleaved caspase-3, and cleaved PARP levels increased in shLAMC1 KYSE30 and KYSE450 cells compared with the control group after cisplatin treatment (Fig. 4A), while they decreased in LAMC1-overexpressing cells compared with vector only cells (Fig. 4B). Furthermore, previous studies have shown that the NF- κ B pathway can regulate cell anti-apoptosis via caspase¹⁹. We found that cleaved caspase9, cleaved caspase-3, and cleaved PARP expression in shLAMC1 cells was decreased by TNF α stimulation (Fig. 4C). Accordingly, after treatment with Akt phosphorylation selective inhibitor MK-2206 and the NF- κ B nuclear translocation inhibitor JSH-23, LAMC1-overexpressing ESCC cells showed

the opposite results in proliferation and expression of cleaved caspase and PARP (Fig. 4D). Furthermore, JSH-23 also restored the positive effect on proliferation of overexpression LAMC1 cells (Fig. 4E), and TNF α could restore the negative effect on proliferation and the positive effect on apoptosis of shLAMC1 KYSE30 and KYSE450 cells (Fig. 4F, G).

5. CXCL1 would be regulated by LAMC1 mainly via NF- κ B activation.

Increasing attention has been paid to the role of the TME in solid tumors. Cytokines and chemokines, as tumor-promoting factors, often play a role in intercellular signaling. By GSEA enrichment analysis of mRNA-seq data of shLAMC1 and sh-vec ESCC cells, knockdown of LAMC1 affected the cytokine and chemokine signaling pathways (Fig. 3A). Moreover, enrichment analysis of RNA-seq data of ESCC tissues (GSE53625) revealed the same effect of LAMC1 (Fig. 5A). We hypothesized that LAMC1 is involved in the regulation of signaling molecules and interactions in the TME via enrichment analysis of genes positively regulated by TGF β 1. All the above findings suggest that LAMC1 may be involved in regulating the secretion of cytokines or chemokines. First, we detected a total of 48 cytokines and chemokines in the conditioned medium (CM) of shLAMC1 cells using the Bio-Plex Pro Human Chemokine Panel 48-plex kit. We found that the expression of CXCL1, IL8, and MIF increased in sh-vec cells compared with sh-1 cells at a higher concentration (Fig. 5B). We also detected the expression of the three cytokines in concentrated CM and found that only CXCL1 was regulated by LAMC1 both in KYSE30 and KYSE450 cells (Fig. 5C). Considering those results, we speculate that LAMC1 may upregulate CXCL1, and it was confirmed in knockdown LAMC1 and overexpression LAMC1 cells by ELISA (Fig. 5D). Thus, CXCL1 could be identified as a downstream target of LAMC1.

As predicted on the Cistrome website⁵³, NF- κ B can upregulate CXCL1 through transcriptional activation (Fig. 5E). In addition, we determined that LAMC1 may activate the NF- κ B pathway, so we speculated that LAMC1 upregulates CXCL1 through NF- κ B transcriptional activation. To verify whether NF- κ B is responsible for CXCL1 expression, we used ELISA and WB to detect CXCL1 secretion of knockdown LAMC1 cells with or without TNF α stimulation and that of overexpression LAMC1 cells with or without MK-2206 2HCl and JSH-23. As expected, lower CXCL1 secretion by LAMC1 knockdown cells could also be increased by TNF α , and MK-2206 2HCl and JSH-23 could reverse the high expression of overexpression LAMC1 cells (Fig. 5F, G) And at the mRNA levels, expression of CXCL1 in shLAMC1 cells also could be reversed by TNF α (Fig. 5H).

In vivo, the expression of LAMC1 was associated with that of CXCL1 at the RNA level (Fig. 5I), and the expression of CXCL1 was higher in cancer tissues than in adjacent tissues (Fig. 5J), but it did not affect the prognosis of patients, especially OS (Fig S4E).

6. CXCL1 secreted by ESCC tumor cells promoted the transformation of CAFs into inflammatory CAFs.

CAFs are heterogeneous cells with different subtypes, such as iCAFs and myCAFs³³. The two subgroups not only have significant differences in their transcriptional profiles but also have different effects on tumor cells. myCAFs are contractile and can remodel the stroma, while iCAFs are characterized by a

secretory phenotype and regulate tumor cells and other cells in a paracrine manner. CAFs were isolated from fresh tumor tissue and cultured *in vitro* and were identified by α SMA expression by cell immunofluorescence detection (Fig S5A). To explore whether ESCC tumor cell secreted CXCL1, upregulated by LAMC1, influences CAF heterogeneity, we performed mRNA-seq in the following cells: CAFs treated with PBS, CAFs with recombinant CXCL1 (rCXCL1) treatment and CAFs with sh-vec CM treatment. We found that in CAFs treated with rCXCL1 or sh-vec CM, some gene clusters of iCAFs, including cytokines (CSF2, VEGF, etc.), chemokines (CXCL2, CXCL3, CXCL5, etc.) and interleukins (IL6, IL7, etc.), were upregulated than CAFs with PBS stimuli. In addition, gene clusters of myCAFs, such as COL1A1 and COL4A1, were downregulated (Fig. 6A). Moreover, compared with analysis of the controls, GSEA of CAFs with rCXCL1 or sh-vec CM treatment confirmed the upregulation of the cytokine/chemokine signaling and the regulation of STAT cascade, especially phosphorylation of STAT3 pathway. But the smooth muscle contraction pathway was downregulated (Fig. 6B, C).

In order to verify the above sequencing results, we detected iCAF markers (IL1, IL6, LIF, CSF3) and myCAF markers (Acta2, Ctgf) in CAFs treated with CM from shLAMC1 ESCC cells, CAFs cocultured with shLAMC1 ESCC cells, and controls. Since inflammatory markers are secreted, the expression of these proteins was detected in the concentrated CM of CAFs. At the protein and mRNA level, we found that CAFs cocultured with sh-vec ESCC cells or treated with the CM of sh-vec ESCC cells had higher expression of inflammatory markers than CAFs cocultured with shLAMC1 ESCC cells (Fig. 6D, E) or treated with CM from shLAMC1-1 ESCC cells (Fig. 6F, G). But the myCAF markers (Acta2, Ctgf) had lower expression in CAFs cocultured with sh-1 ESCC cells or treated with the CM of sh-1 ESCC cells than controls at protein level, which were not obvious at mRNA level (Fig. 6G). To verify whether CXCL1 is the main driver of this effect, we directly treated CAFs with rCXCL1 and obtained a similar result (Fig. 6H, I).

7. Tumor-secreted CXCL1 induced iCAF formation via phosphorylation of STAT3.

The CXCL1 common receptor is CXCR2, and combined with the above results of GSEA, we suggest that the CXCR2/pSTAT3 pathway may be responsible for this effect. We found that CXCR2 and pSTAT3 expression in CAFs was upregulated at the RNA and protein levels after rCXCL1 treatment (Fig. 7A, S5B). Additionally, SB225002, an inhibitor of CXCR2, reversed the upregulation of the inflammatory markers, CXCR2 and pSTAT3 and the downregulation of α SMA by rCXCL1 in CAFs (Fig. 7D-F). In addition, SB225002 decreased the changes in these proteins, especially inflammatory markers, in CAFs cocultured with shLAMC1 ESCC cells and CAFs treated with CM from shLAMC1 ESCC cells (Fig. 7B-C, G-L). All the above results suggest that CXCL1, regulated by LAMC1 and secreted by tumor cells, promotes the transition of CAFs into iCAFs via CXCR2/pSTAT3.

8. iCAFs, induced by CXCL1, promoted ESCC progression *in vivo* and *in vitro*.

Furthermore, to explore the effect of iCAFs induced by CXCL1 on the proliferation of tumor cells, we compared the proliferation of tumor cells after coculture with CAFs stimulated by PBS or rCXCL1 together with or without SB225002, and we found that CAFs with rCXCL1 promoted WT ESCC proliferation, which could be reversed by SB225002 (Fig. 8A). Additionally, to explore the influence of iCAFs on migration, WT

ESCC cells with different treatments were divided into four groups: the control group (WT ESCC cells treated with PBS) and WT ESCC cells treated with CM secreted by CAFs that were pretreated with rCXCL1 or PBS (CM-CAFs-PBS, CM-CAFs-pretreatCXCL1) in the presence or absence of SB225002. We found that both CM-CAFs-pretreatCXCL1 and CM-CAFs-PBS could promote the proliferation and migration of ESCC cells, and CM-CAF-pretreatCXCL1 had a stronger promoting effect than CM-CAFs-PBS, while SB225002 could also reverse the effect (Fig. 8B, C). In vivo, the tumor volume and weight of WT KYSE30 cells mixed with rCXCL1-pretreated CAFs were larger than those of cells mixed with CAFs without rCXCL1 pretreatment. SB225002 also weakened this effect (Fig. 8D, E). The migration markers MMP9 were measured by IHC and western blot in xenograft tumor tissue. Tumor with rCXCL1 pre-treated CAFs had higher expression of MMP9 than that of tumor with PBS pre-treated CAFs, which could be decreased by SB225002 (Fig. 8F, G).

Discussion

As in other types of tumors, high expression of LAMC1 can promote ESCC tumor cell proliferation and migration and has a poor prognosis, which can be used as a biomarker. We found that TGF β 1 regulates the expression of LAMC1 via SMAD4/SP1 synergic activation. Previous studies have shown that LAMC1 is regulated by SP1 in hepatocellular carcinoma^{13, 20, 32}. The mechanism by which SMADs and SP1 synergistically activate genes has also been reported previously⁴⁸.

The positive effect of LAMC1 on the phosphorylation Akt mediated NF- κ B activation in ESCC, could promote the downstream processes of anti-apoptosis, pro-migration and secretion of CXCL1. However, the specific mechanism of the positive effect of LAMC1 on phosphorylation Akt mediated NF- κ B activation remains to be further studied. Many previous studies also confirmed the high expression of NF- κ B in ESCC26. NF- κ B expression was increased in mouse models of ESCC with p120-catenin knockdown. NF- κ B signals are activated by regulating upstream mediators, such as upregulating the transcription factor Id-1 or downregulating the tumor suppressor Nkx2-825. And TGF β -induced long noncoding RNAs repressed NF- κ B signals²⁶. We found that shLAMC1 ESCC cells had higher expression of cleaved caspase 9, caspase 3, and PARP. The apoptosis pathways are mainly divided into endogenous and exogenous pathways, and the activation of caspase 9 and caspase 8, which are apoptotic initiators, represent endogenous and exogenous apoptosis initiation. After being cleaved and activated, caspase enzymes, such as caspase 3, are activated in a cascade manner, and cytoskeletal and nuclear proteins, such as PARP, are cleaved to promote apoptosis. LAMC1 could promote ESCC migration mainly by upregulating MMP9 and MMP14 downstream of NF- κ B. In ESCC, some MMPs are upregulated to promote invasion, such as MMP2, MMP7, and MMP9^{12, 17}. In addition, LAMC1 and MMPs are both components of the ECM and are related to ECM remodeling. ECM remodeling plays an important role in the development of tumors, especially in invasion⁶. Other components of the ECM have also been reported in ESCC, such as fibronectin, proteoglycan dermatan sulfate and hyaluronan⁴³. LAMC1 could promote CXCL1 secretion through the transcriptional activation of NF- κ B. As a transcription factor, NF- κ B can regulate cytokine production⁴¹. Activation of the NF- κ B pathway is often considered an important link

between the inflammatory microenvironment and tumor development⁴¹. The cytokines downstream of NF- κ B in ESCC have been reported to be IL-8 and IL1^{2,10,24}. IL1 promotes tumor invasion, tumor-mediated immunosuppression, and tumor stem cell self-renewal²⁸. IL1 also plays a role in inducing iCAFs⁵.

Many previous studies of LAMC1 have indicated that highly expressed LAMC1 can serve as a biomarker for a variety of tumors²¹, but less attention has been paid to the effect of LAMC1 on the heterogeneity of CAFs. We determined that ESCC tumor cells secrete CXCL1 and promote iCAF activation. In addition, we verified that TGF β 1 upregulated LAMC1. Furthermore, we found that CXCL1 expression was increased with TGF β 1 treatment, which could be eliminated by TGF β receptor SB (Fig S2A-C). And TGF β 1 was positively associated with CXCL1 based on GSE53625 data (Fig S2D). Consistent with our findings, in previous studies, CXCL1 expression increased in cocultures of CAFs and oral squamous cell carcinoma cells with IL1 β , promoting tumor invasion and CAF activity⁴⁵. In addition, CAF-secreted CXCL1 can induce the progression of ESCC tumor cells⁵¹. It is worth mentioning that CXCR2 inhibitors showed a better response to rCXCL1 on CAFs than tumor CM, suggesting that LAMC1 may also influence other factors contributing to the formation of iCAFs. Whether LAMC1 influences the heterogeneity of CAFs by affecting other factors or exosome secretion in tumor cells may require further study. Furthermore, CXCL1 increased the expression of inflammatory markers in CAFs and decreased the expression of myofibroblast markers. In accordance with previous studies, iCAFs stimulated by rCXCL1 and sh-vec CM also presented a secretory phenotype that can interact with other cells in a paracrine manner and have tumor-promoting functions³³. Due to its secretory phenotype, we speculate that iCAFs induced by CXCL1 may be involved in cancer-associated systemic effects³³. CXCL1 activates phosphorylation of STAT3 in CAFs, which leads to an increase in many inflammatory mediators. Similarly, in pancreatic ductal cell carcinoma, tumor cell-secreted IL1 induces the formation of iCAFs in a cascade involving increased LIF expression and activation of JAK/STAT signaling⁵. It has also been confirmed that CAFs can reprogram cytokines secretion by other cells³⁹. Moreover, it is worth mentioning that CAFs stimulation by either rCXCL1 or tumor CM did not induce proliferation (Fig S4A). This further indicated that the carcinogenic effect of CAFs was not through their own proliferation but indirectly regulated the phenotype of tumor cells through paracrine signaling^{14,31}.

We confirmed that TGF β 1 acted on tumor cells, causing a series of changes: upregulation of LAMC1, phosphorylation of NF- κ B, secretion of CXCL1, phosphorylation of STAT3 in CAFs, and finally induction of the formation of iCAFs, that is, tumor-promoting CAFs. Previous studies have shown that TGF β signaling plays a dual role in CAFs⁴⁶. It can activate CAFs, such as through the conversion of NFs into CAFs and fibroblasts into tumor-promoting CAFs^{39,46}. However, TGF β 1 secreted by tumor cells inhibits iCAF formation by antagonizing IL1 signaling activity and prevents myCAF conversion to iCAFs by blocking the JAK/STAT pathway⁵. The reason for these dual functions may be due to the balance between TGF β signaling and STAT signaling in CAFs^{5,7,27}.

Conclusions

Overexpression of LAMC1, upregulated by TGFβ1 via SMAD4/SP1 synergic activation, would promote proliferation and migration of tumor cells mainly via NF-κB/MMP9-MMP14. Additionally, LAMC1 facilitated CXCL1 secretion via NF-κB. Tumor-secreted CXCL1 remodeled the formation of iCAFs through CXCR2/pSTAT3. The CM of iCAFs promoted the proliferation and migration of tumor cells.

Abbreviations

TME: The tumor microenvironment; CAFs: cancer-associated fibroblasts; iCAFs: inflammatory CAFs; myCAFs: myofibroblasts; TGFβ: Transforming growth factor β; LAMC1: laminin subunit gamma 1; ChIP: Chromatin immunoprecipitation; ESCC: Esophageal Squamous Cell Carcinoma; ECM: extracellular matrix; CM: conditioned medium; GSEA: Gene set enrichment analysis; MMPs: Matrix metalloproteinases; rCXCL1: recombinant CXCL1.

Declarations

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Availability of data and materials

All data during our work are included in this published article and its additional files.

Authors' contributions

LL.F., N.S., and J.H. conceived the ideas and designed the experiments. LL.F., Y.C., CQ.Z., JB.H., YY.L. and ZL.L. performed the experiments. Y.C. and N.S. provided the clinical samples. LL.F. analyzed study data. LL.F. wrote the paper. J.H. reviewed the manuscript. All authors read and approved the final manuscript.

Competing Interests

This work was not related with any competing financial interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All patients from whom CAFs isolated signed informed consent. Our study was approved by the Committee for the Ethics Review of Research Involving Human Subjects of the Cancer Hospital of the Chinese Academy of Medical Sciences. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

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Figures

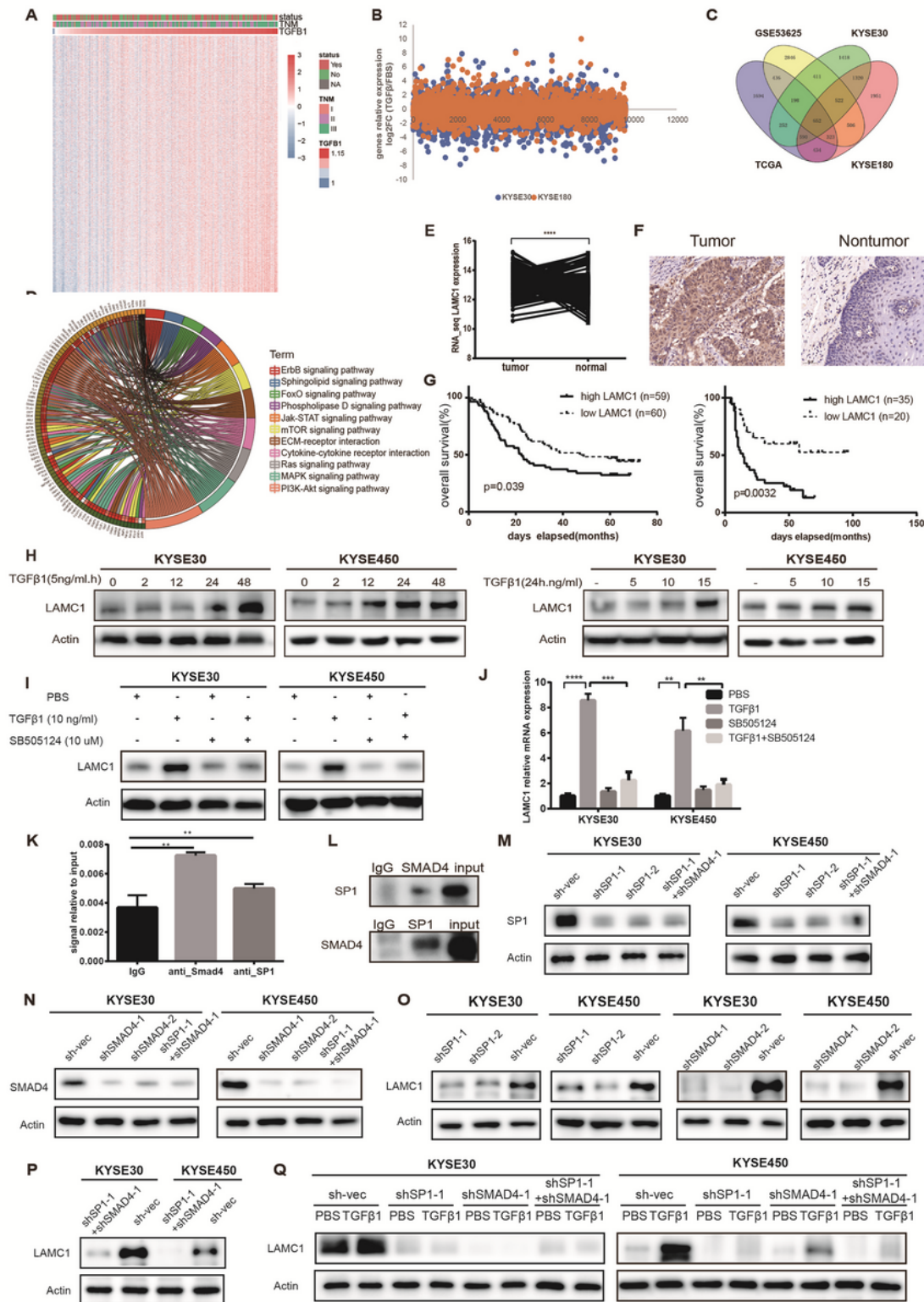


Figure 1

Overexpression of LAMC1 was upregulated by TGFβ1 via Smad4 and SP1 synergistic activation and had a poor prognosis in ESCC. (A). Heatmap of the RNA-seq data listing genes positively correlated with TGFβ1 in ESCC of GSE53625 ($r > 0.15$, $p < 0.05$). (B). 3084 genes of KYSE30 and KYSE180 were upregulated after TGFβ1 treatment using RNA-seq data (fold change > 1). (C). Venn diagram of 652 genes overlapping based on positive regulation by TGFβ1 in ESCC. (D) The 31 genes of 652 genes were

enriched into 11 pathways involved in the tumor microenvironment. (E-F) LAMC1 was more highly expressed in cancer than in para-cancer at the RNA and protein levels. Representative immunohistochemical (IHC) images of LAMC1 staining in ESCC tumor tissues and nontumor tissues (original magnification: 200x). (G). Kaplan–Meier survival analysis of overall survival (OS) based on high (n=89) and low (n=90) LAMC1 expression in GSE53625 (left) and based on high (n=35) and low (n =20) LAMC1 expression (by IHC, right). (H-I). As verified by western blot and RT-qPCR, TGFβ1 would upregulate LAMC1 expression in a concentration- (at different concentration for 24h) and time-(at 5 ng/ml for different times) dependent manner in KYSE30 and KYSE450 cells (H) which could be reversed by TGFβR1 inhibitor SB505124 (10 μM) at protein (I) and RNA levels (J). (K-L) ChIP assay and western blot showed that the Smad4 and SP1 complex localizes to the LAMC1 promoter in KYSE30 cells treated with TGFβ1(5 ng/ml) for 30 min. (M-N) Knockdown efficiency of SP1 combined with or without SMAD4 in ESCC cells was verified by western blot. (O-Q) The expression levels of LAMC1 decreased in knockdown SP1 combined with (P) or without (O) Smad4 ESCC cells, which could not be rescued by TGFβ1 treatment (Q). Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Student's t test).

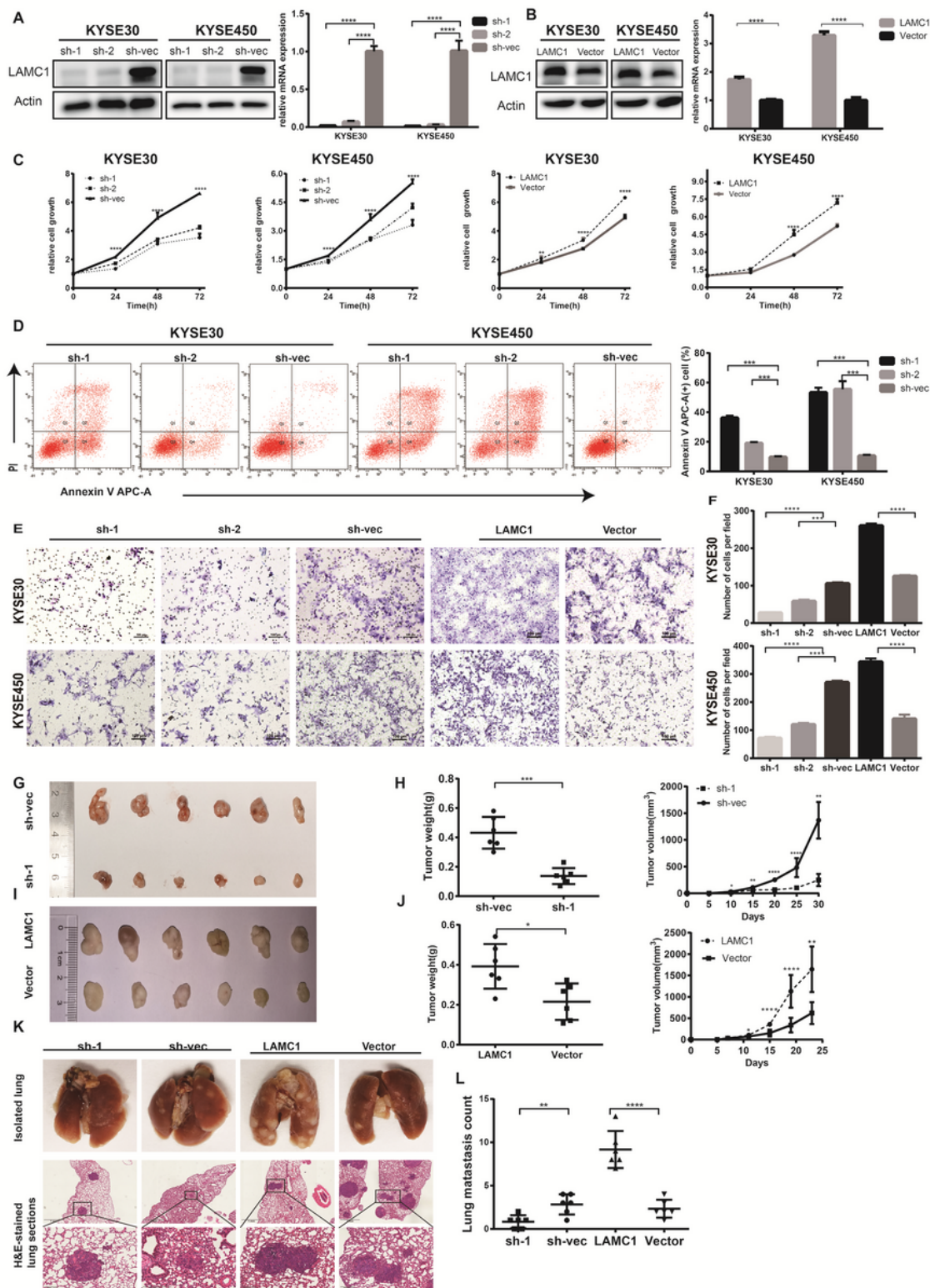


Figure 2

LAMC1 promoted the proliferation and metastasis of ESCC cells in vivo and in vitro. (A-B) Knockdown or overexpression efficiency of LAMC1 in ESCC cells was verified by western blot and qRT-PCR. (C) Rate of cell growth of KYSE30 and KYSE450 cells treated with shLAMC1 or overexpressing LAMC1 and vector control cells were measured by CCK8 assay. (D) Apoptosis of shLAMC1-1, -2 and sh-vec KYSE30 and KYSE450 cells was analyzed by the Annexin V APC Apoptosis Detection Kit. (E-F) The migration ability of

shLAMC1-1, -2, sh-vec, LAMC1, and vector KYSE30 and KYSE450 cells was detected by chamber assay. The numbers of migrating cells were compared between the groups. (G-J) Representative images of tumor formation in nude mice subcutaneously inoculated with KYSE30 cells stably expressing LAMC1 shRNA or negative control and expressing LAMC1 or mock-vehicle control (G, I), and tumor weights and volumes for groups (H, J). (K-L) Representative images of lung tissues isolated from mice injected with 1×10^6 shLAMC1, sh-vec, LAMC1, or vector KYSE30 cells via the tail vein and hematoxylin and eosin-stained images (200 \times) of such tissues, and quantification of lung metastasis (scale bars, 1 mm and 200 μm for the top and bottom rows of photomicrographs, respectively). Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Student's t test).

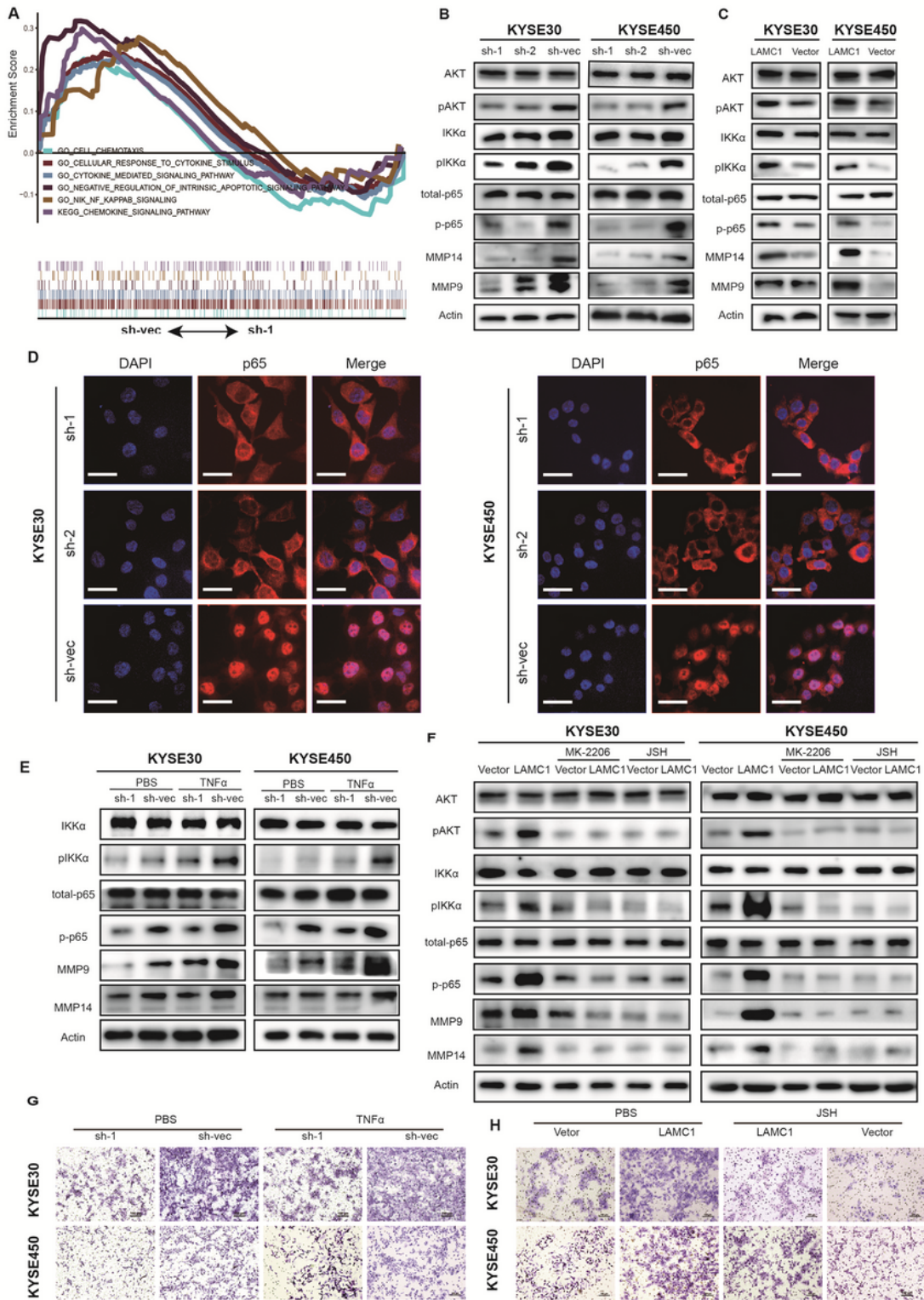


Figure 3

The positive effect of LAMC1 on the migration in ESCC cells would be mainly via the Akt/IKK α /NF- κ B/MMP9-MMP14 pathway. (A) Anti-apoptosis, NF- κ B and cytokine-related pathways enriched by sh-vec using GSEA. (B-C) Expression level of the phosphorylation of Akt, NF- κ B, IKK α , MMP9, and MMP14 in KYSE30 and KYSE450 cells stably expressing LAMC1 shRNA or negative control and expressing LAMC1 or mock-vehicle control. (D) Immunofluorescence staining of p65 in shLAMC1-1, shLAMC1-2, sh-vec

KYSE30 and KYSE450 cells. Nuclei were visualized with DAPI staining (blue). Representative images are shown. Scale bar, 34 μm . (E-F) Phosphorylation of IKK α , p65, MMP9, and MMP14 was also detected in shLAMC1 KYSE30 and KYSE450 cells treated with or without TNF α stimulation(D), and overexpressing LAMC1 with or without 5 μM Akt phosphorylation selective inhibitor MK-2206 2HCl and 10 μM NF- κ B JSH-23stimulation (E). (G-H) Representative images of migration of these subgroups, shLAMC1 with or without TNF α (10 ng/ml) stimulation, overexpression LAMC1 with or without JSH-23 stimulation, measured by chamber assay. Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Student's t test).

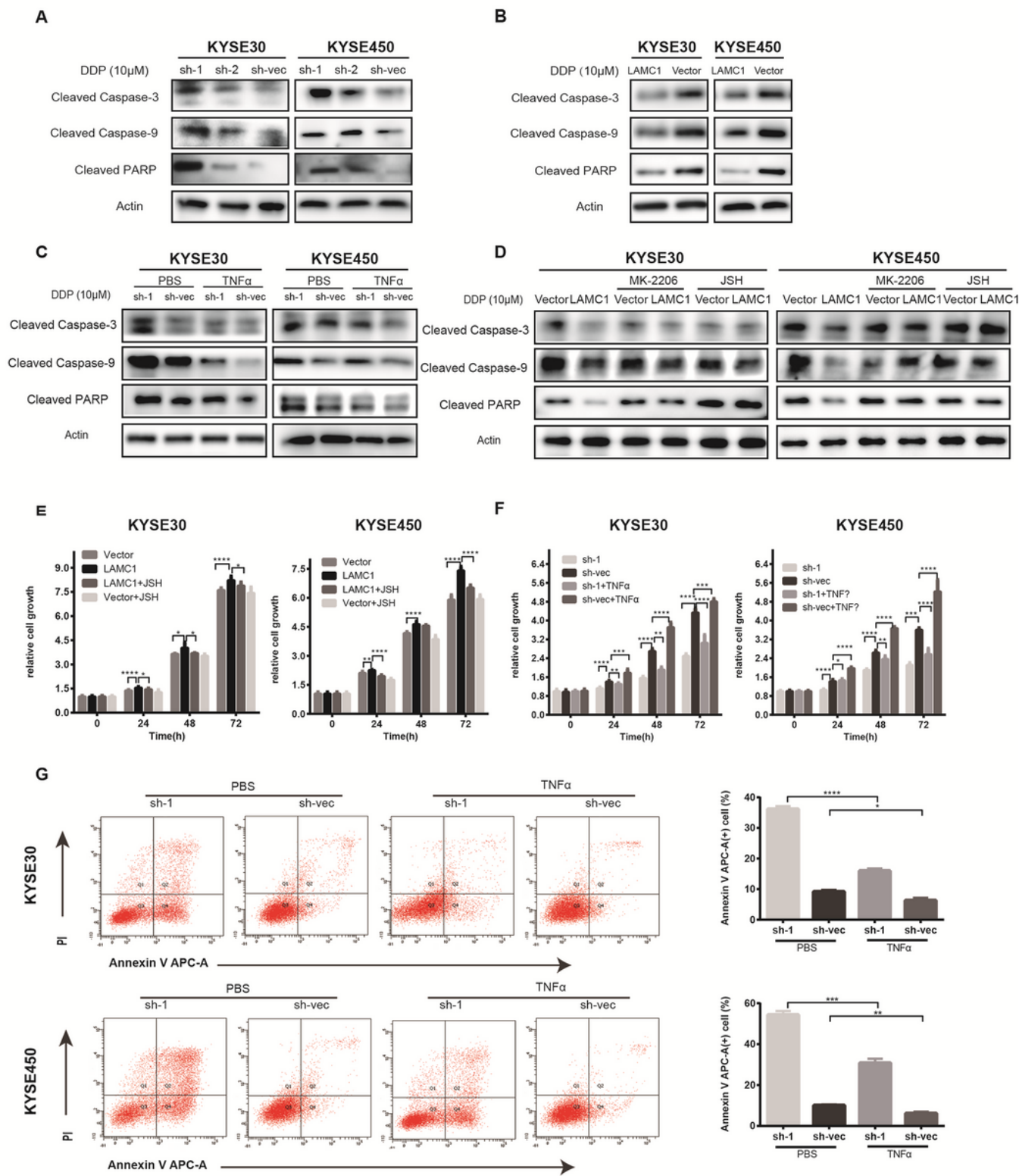


Figure 4

LAMC1 inhibited apoptosis possibly through the Akt/NF- κ B/caspase9-caspase3-PARP cascade. (A, B) Western blot was conducted to detect the protein levels of cleaved caspase-3, caspase-9, and PARP in KYSE30 and KYSE450 cells stably expressing LAMC1 shRNA or negative control and expressing LAMC1 or mock-vehicle control, which were induced by 10 μ M cisplatin for 24 h. (C-E) Proliferation and anti-apoptosis of KYSE30 and KYSE450 cells stably expressing LAMC1 shRNA or negative control and

expressing NKILA or mock-vehicle control with or without TNF α or JSH-38 (JSH), respectively stimulation, as measured by CCK8 assay. (F-G) After cisplatin induction, cleaved caspase-3, caspase-9, and PARP were detected in KYSE30 and KYSE450 cells treated with shLAMC1 with or without TNF α stimulation (F) or overexpressing LAMC1 with or without 5uM MK-2206 2HCl and 10 μ M NF- κ B nuclear translocation inhibitor JSH-23 stimulation (G). Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Student's t test).

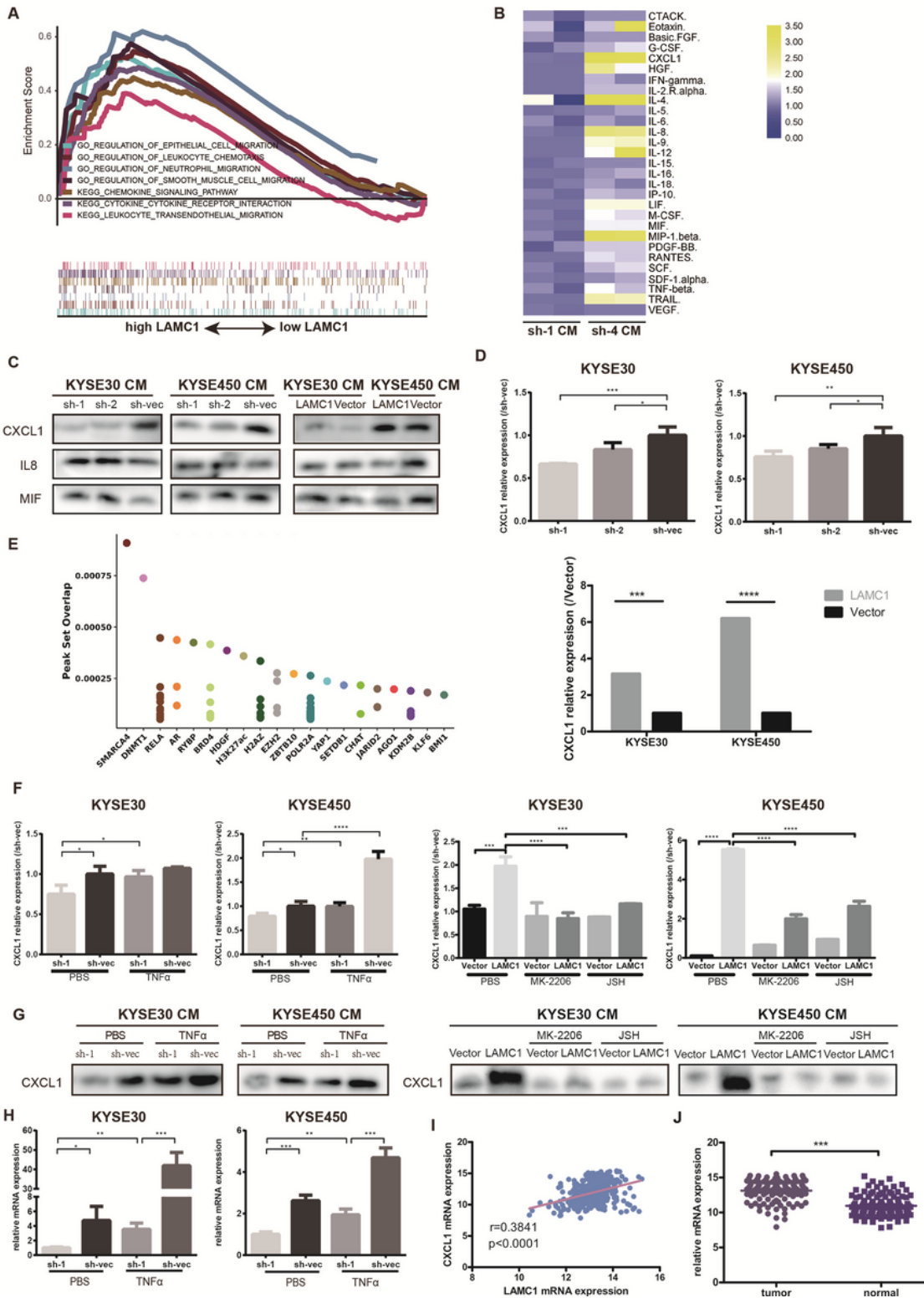


Figure 5

LAMC1 would promote CXCL1 secretion mainly by activating NF- κ B. (A) LAMC1 affected the cytokine and chemokine signaling pathways according to GSE53625 data. (B) A 48-cytokine panel was detected in the CM of shLAMC1 and sh-vec KYSE450 cells. (C) Expression of 3 cytokines in the CM of KYSE30 and KYSE450 cells stably expressing LAMC1 shRNA or negative control and expressing LAMC1 or mock-vehicle control by western blot. (D) CXCL1 expression in the CM of shLAMC1 and overexpression LAMC1 KYSE30 and KYSE450 cells, as measured by ELISA. (E) Top 20 predicted transcription factors of CXCL1 in the Cistome network. (F, G) Western blotting and ELISA were conducted to detect CXCL1 expression in the CM of shLAMC1 and sh-vec cells with or without TNF α treatment, and of overexpression LAMC1 cells with or without 5 μ M MK-2206 2HCl and 10 μ M NF- κ B nuclear translocation inhibitor JSH-23 stimulation. (H) RT-qPCR was conducted to detect CXCL1 expression in shLAMC1 and sh-vec cells with or without TNF α treatment. (I) LAMC1 was positively associated with CXCL1 based on GSE53625 data. (J) CXCL1 was upregulated in tumor tissue compared with adjunct tissue in the GSE53625 dataset. Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean \pm SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 (Student's t test).

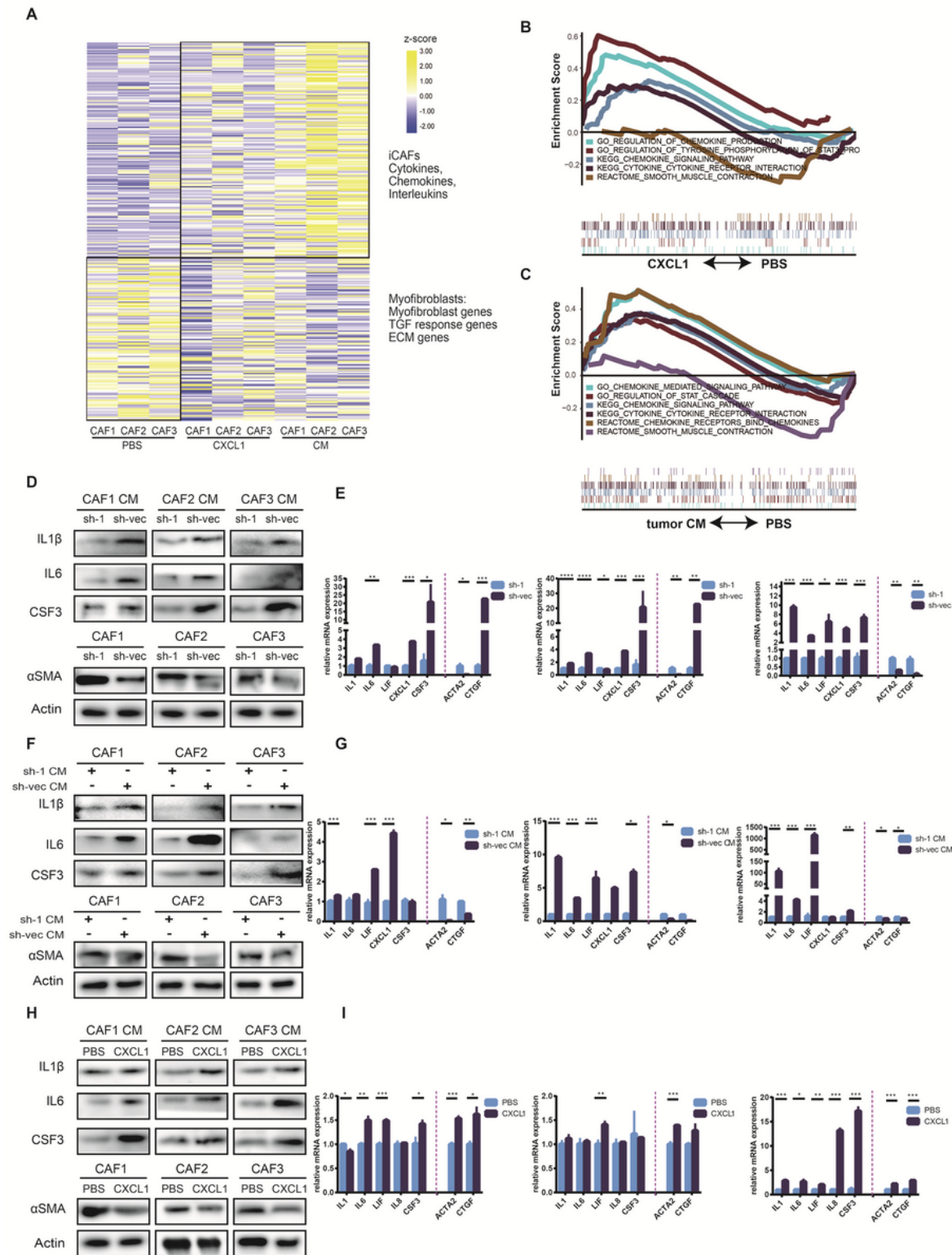


Figure 6

Tumor-secreted CXCL1 promoted the formation of iCAFs. (A) Transcriptional profile of CAFs with or without treatment with rCXCL1 and sh-vec CM. (B-C) rCXCL1 or sh-vec CM promoted activation of cytokine and chemokine pathways and phosphorylation of STAT3, as demonstrated by GSEA. (D-I) Western blot and RT-qPCR detection of markers of iCAFs and myfibroblasts, respectively, in CM of CAFs cocultured with shLAMC1 or sh-vec ESCC cells (D, E), treated with the CM of shLAMC1 or sh-vec ESCC

cells (F, G), or treated with rCXCL1 (H, I). Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Student's t test).

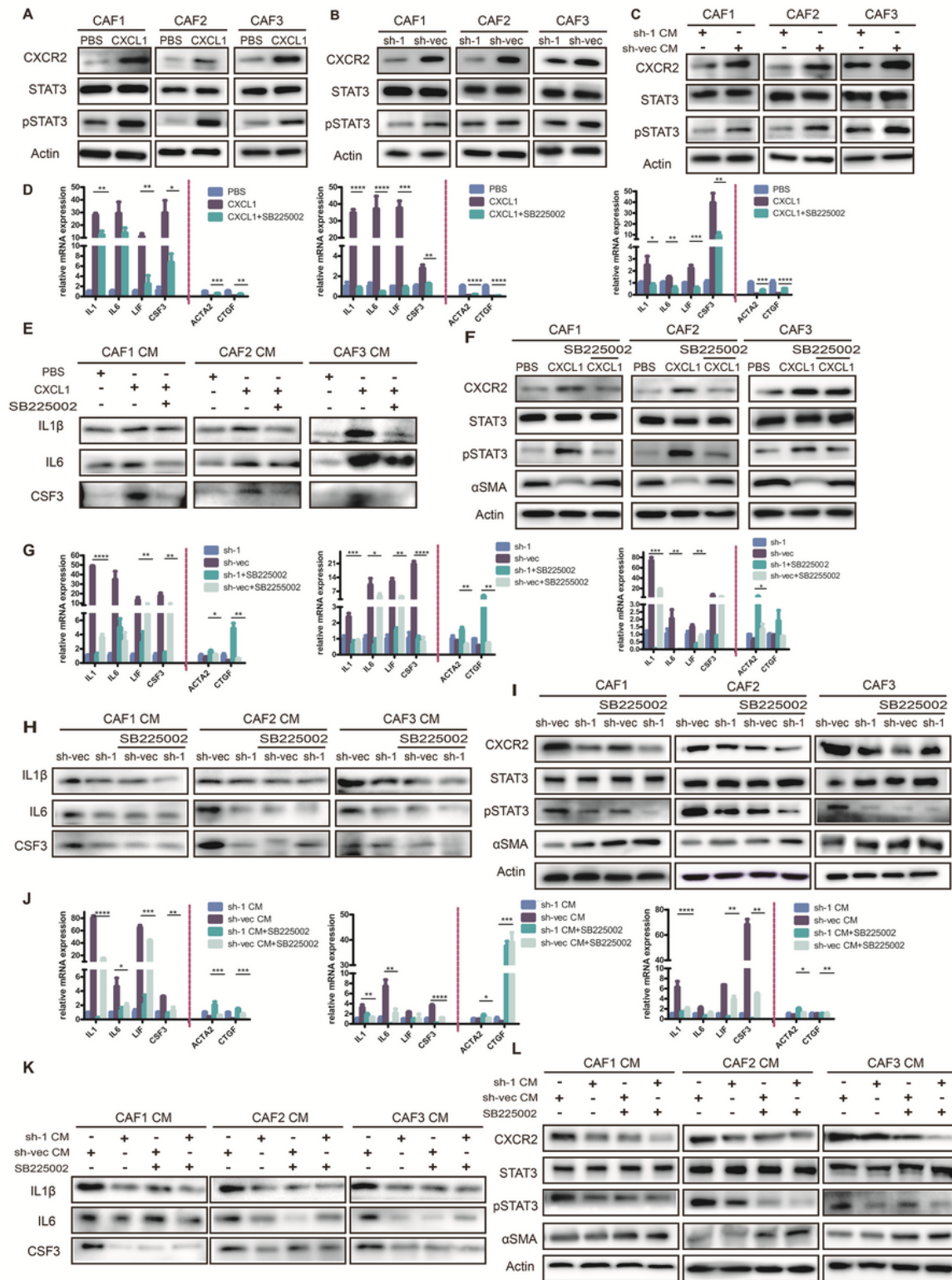


Figure 7

CXCL1 induced inflammatory CAF formation via phosphorylation of STAT3. (A-C) Expression of pSTAT3 and CXCR2 was detected in CAFs with three different treatments: induced with rCXCL1 (A), cocultured

with shLAMC1 and sh-vec KYSE30 cells (B), or stimulated by CM from these tumor cells (C), as demonstrated by western blot. (D-I) IL1 β , IL6, and CSF3 were detected by western blot and RT-qPCR in concentrated CM of CAFs with three treatments with or without the CXCR2 inhibitor SB225002 (D, E, G, H, J, K), and α SMA, pSTAT3 and CXCR2 were detected in the total protein of cells (F, I, L). Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean \pm SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 (Student's t test).

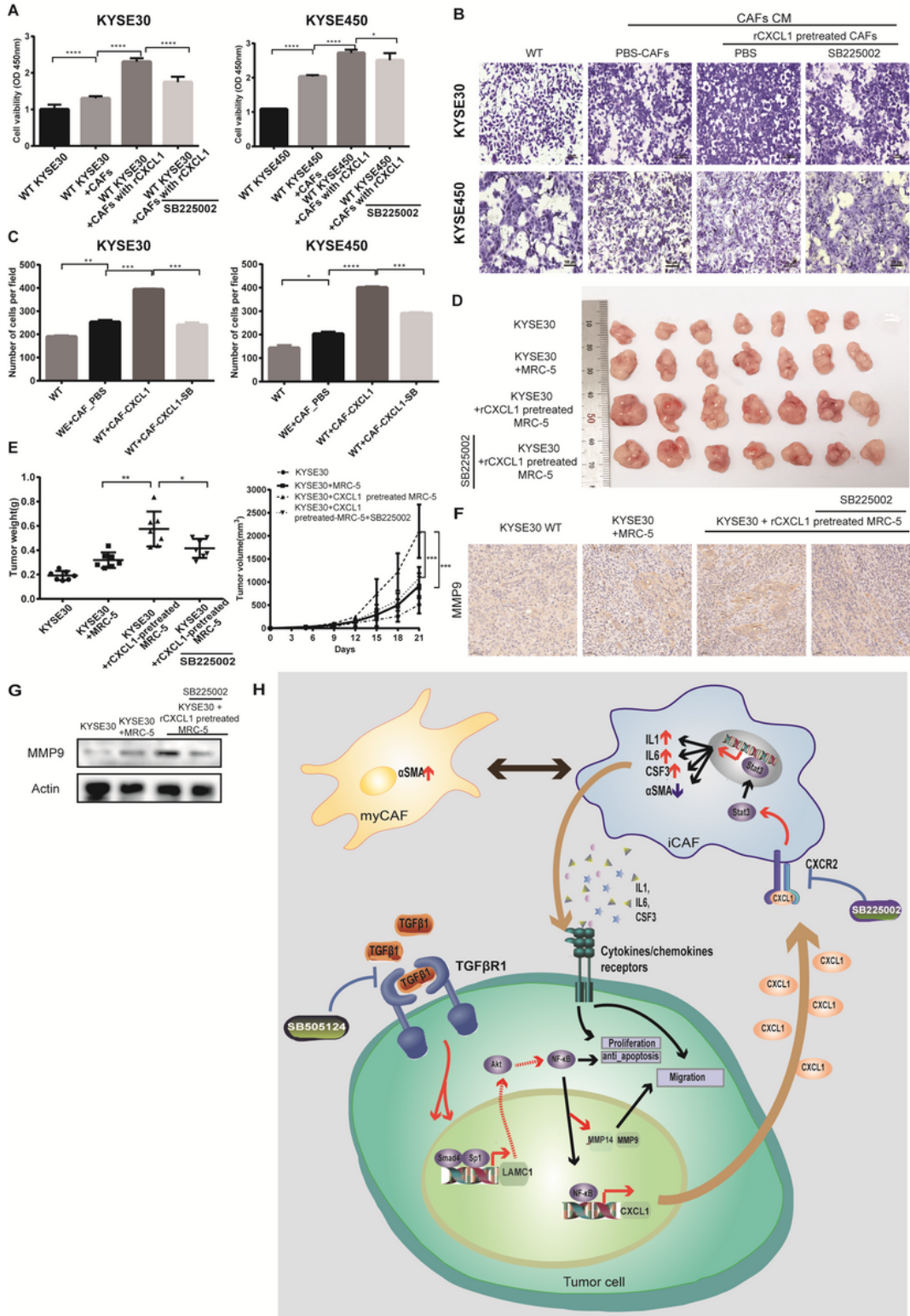


Figure 8

Inflammatory CAFs, induced by CXCL1, promoted the proliferation and migration of ESCC in vitro and in vivo. (A) Detection of the viability of WT KYSE30 and KYSE450 cells cocultured for 48 h with or without PBS-treated CAFs, CXCL1-induced CAFs, or with SB225002, as demonstrated by CCK8 assay. (B, C) Migration of WT KYSE30 cells with different treatments: with or without CM of CAFs that were or were not pre-induced with rCXCL1 combined with SB225002, as measured by chamber assay. (D, E) Comparison of tumor volume and weights of subcutaneous tumor xenografts established from co-implantation of KYSE30 cells and CXCL1-pretreated or not MRC-5 cells in the presence or absence of SB225002 treatment. Three biological replicates were performed for in vitro assays. Data in bar charts are presented as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Student's t test). (F) Representative immunohistochemical (IHC) images of MMP9 staining in xenograft tumor tissue of WT KYSE30 cells admixed with implanted CAFs with different pretreatments (original magnification: 200x). (G) Expression of MMP9 in xenograft tumor tissue of WT KYSE30 cells admixed with implanted CAFs with different pretreatments were detected by western blot. (H) Research summary: TGF β 1 upregulates expression of LAMC1 via SMAD4/SP1 synergic activation, which can be abrogated by TGF β R1 inhibitor (SB505124). LAMC1 promotes proliferation and migration of tumor cells via Akt/NF- κ B/MMP9-MMP14. LAMC1 also facilitates CXCL1 secretion via NF- κ B activation. Tumor-secreted CXCL1 can remodel the formation of iCAFs through CXCR2/pSTAT3 which can be blocked by CXCR2 inhibitor (SB225002). iCAFs promotes progression of ESCC cells through secreting cytokines and chemokines.

Supplementary Files

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