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CCN1 promotes APRIL/BAFF signaling in esophageal squamous cell carcinoma but attenuates it in esophageal adenocarcinoma

Lingling Xing[1,2,3,4], Zhenyu Jiang[1,2,3,4], Ruize Xu[1,2,3], Tong Dang[1,2,3], Jinbao Wu[1,2,3✉], Jianyuan Chai[1,2,3✉] & Xianmei Meng[1,2,3✉]

CCN1 is a matricellular protein highly expressed in esophageal squamous cell carcinoma (ESCC) but hardly detectable in esophageal adenocarcinoma (EAC). Expression of CCN1 in EAC cells leads to TRAIL-mediated apoptosis. Unlike TRAIL, which primarily triggers cell death, APRIL and BAFF promote cell growth via NFκB signaling. They become active ligands by Furin cleavage. This study found that CCN1 upregulated APRIL and BAFF expression in both ESCC and EAC cells but attenuated their signaling in the latter. CCN1 kept Furin stable in ESCC allowing APRIL/BAFF to signal through their common receptor BCMA properly. In EAC cells, however, expression of CCN1 lowered Furin activity and thus limited APRIL/BAFF cleavage. As a result, ESCC cells benefited from CCN1 while EAC cell viability was attenuated by it.

Keywords Esophageal cancer, CCN1, APRIL, BAFF, BCMA, Furin

Abbreviations

APRIL	A proliferation–inducing ligand
BAFF	B cell activating factor of the TNF family
BCMA	B cell maturation antigen
cAPRIL	Cellular APRIL residue
cBAFF	Cellular BAFF residue
CCN1	Cellular communication network 1
EAC	Esophageal adenocarcinoma
ESCC	Esophageal squamous cell carcinoma
GERD	Gastroesophageal reflux disease
pCCN1	pcDNA3.1–CCN1
rCCN1	Recombinant CCN1
rAPRIL	Recombinant APRIL
rBAFF	Recombinant BAFF
rFurin	Recombinant active Furin
sAPRIL	Soluble APRIL
sBAFF	Soluble BAFF
TACI	Transmembrane activator and calcium signal–modulating cyclophilin ligand interactor
TNFSF	Tumor necrosis factor superfamily
TNFRSF	Tumor necrosis factor receptor superfamily
TRADD	TNFR1 associated death domain protein

CCN1 (Cell Communication Network 1, or Cyr61) is a matricellular protein that mainly supports cell adhesion, proliferation, and migration, playing critical roles in various physiological processes, including angiogenesis, wound healing, and inflammatory management. Some cancers take this advantage to facilitate tumor growth

[1]Inner Mongolia Institute of Digestive Diseases, Baotou, China. [2]Inner Mongolia Engineering Research Center for Prevention and Treatment of Digestive Diseases, Baotou, China. [3]The Second Affiliated Hospital of Baotou Medical College, Inner Mongolia University of Science and Technology, 30 Hudemulin Rd, Baotou 014030, China. [4]Lingling Xing and Zhenyu Jiang contributed equally to this work. ✉email: wjb_2fy@163.com; Jianyuan.chai@gmail.com; mxm_2fy@163.com

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and metastasis, while others find CCN1 intolerable[1,2]. Esophageal cancer is a great example. Esophageal cancer is often diagnosed as either squamous cell carcinoma (ESCC) or adenocarcinoma (EAC). CCN1 was highly expressed in ESCC, benefiting tumor growth[3], but was hardly detectable in EAC. Overexpression of CCN1 in EAC cells led to TRAIL/DR5-mediated apoptotic cell death[4].

TRAIL and DR5 belong to TNFSF (tumor necrosis factor superfamily) and TNFRSF (tumor necrosis factor receptor superfamily) respectively, which contain 18 and 29 members correspondingly, and their products are involved in many biological processes including inflammation, immune response, programmed cell death, and cancer development. The members of TNFSF are type II transmembrane proteins whose C-terminal extracellular portion is constitutively cleaved off by convertases (such as Furin) becoming soluble functional cytokines. The members of TNFRSF, on the other hand, are either type I or type III transmembrane proteins (except OPG and DCR3), and their extracellular portions sometimes can also be shed by metalloproteinases producing soluble decoy receptors to interfere with the normal function of the ligands[5]. APRIL (A proliferation-inducing ligand) and BAFF (B cell activating factor of the TNF family) are TNFSF ligands that share many characteristics in both structure and function. Unlike TRAIL, which primarily triggers cell death, APRIL and BAFF promote cell growth via activating NFκB signaling. They have two common receptors, BCMA (B cell maturation antigen) and TACI (transmembrane activator and calcium signal-modulating cyclophilin ligand interactor). Like other TNFRSF receptors, BCMA responds to APRIL or BAFF trimers, but TACI only responds to oligomerized ligands, such as 60-mer BAFF, which only represent < 5% of the total APRIL or BAFF in a cell. The majority of cellular APRIL and BAFF exist as trimers[6]. BAFF also interacts with another receptor BAFFR exclusively. These three receptors are predominantly and successively expressed in B cells to support B cell maturation and differentiation. BAFFR is mostly seen in the early B cells, followed by TACI in memory B cells, and BCMA finally takes over in plasma cells[7]. APRIL and BAFF are initially synthesized as a 27kD and a 31kD peptide respectively. After post-translational modifications in the endoplasmic reticulum and the Golgi apparatus, they form homo-trimers inserted in the cell membrane where they are cleaved constitutively by Furin (a convertase) into functional ligands, soluble APRIL (17kD) and soluble BAFF (18kD) releasing to outside the cells. Sometimes, BCMA, TACI, and BAFFR also get cleaved by γ-secretase or ADAM10 or ADAM17, generating soluble decoy receptors to block APRIL/BAFF signaling. In addition to B cells, many cancer cells are found to express APRIL/BAFF/BCMA[8], including bladder carcinoma[9], breast cancer[10], glioblastoma[11], head/neck carcinoma[9], pancreatic adenocarcinoma[12], B cell lymphoma[13], Hodgkin lymphoma[14], lung carcinoma[15], and colon carcinoma[16].

This study aimed to determine how CCN1 affects APRIL/BAFF signaling in esophageal cancer cells.

Results

CCN1 promotes mRNA expression of APRIL, BAFF, and BCMA in both ESCC and EAC cells

Initially, we were interested to know how CCN1 affects TNFSF/TNFRSF expression in the context of esophageal cancer. So we treated both ESCC (KYSE150 and KYSE410) and EAC (OE19 and OE33) cells with a recombinant CCN1 protein at various concentrations, namely, in the range of 0.005–50 µg/ml for 2, 6, and 12 h, and assessed cell viability afterward. It was found that 1.0 µg/ml was the minimal effective concentration for the recombinant CCN1 and 6-hour treatment was the most effective time point for these cells. Then we decided to use a 6-hour treatment with 1.0 µg/ml of the recombinant CCN1 for our investigation. RNA was isolated for RT-PCR analysis using a cDNA array containing all the TNFSF/TNFRSF genes plus some other mediators involved in their signaling. One of the things that caught our attention was the effect of CCN1 on the APRIL/BAFF system. As shown in Table 1, CCN1 upregulated APRIL, BAFF, and BCMA but downregulated TACI and BAFFR expression in both ESCC and EAC cells ($P < 0.01$).

In ESCC, CCN1 not only promotes APRIL/BAFF protein expression but also promotes their release to the extracellular environment

Based on our earlier studies[4,17], CCN1 has distinctly opposite effect on ESCC and EAC. It facilitates ESCC growth but induces apoptosis in EAC. We wondered why CCN1 promoted gene expression of APRIL/BAFF/BCMA in both types of cancer, knowing that these genes mainly support cell growth. We understand that an upregulation of mRNA does not necessarily lead to more translation of the protein. Therefore, we decided to examine the protein expression of these five genes. As shown in Fig. 1, in response to CCN1 treatment, APRIL and BAFF were upregulated in ESCC and EAC cells by 3.5- and 4.8-fold respectively ($P < 0.05$). Of notice, both APRIL and BAFF bands appeared higher than predicted in the Western blots due to post-translational modification. To ensure the observed bands represent the molecules we were interested in, we pre-incubated the antibodies with the recombinant peptides of their targeted molecules before they were applied to the Western blots. The bands

Symbol	Common name	OE19	OE33	KYSE150	KYSE410
TNFSF13	APRIL	338.6 ± 11.5*	256.6 ± 17.5*	1640.2 ± 35.5*	501.8 ± 21.9*
TNFSF13B	BAFF	46.1 ± 9.0*	184.3± 12.5*	820.1 ± 20.2*	252.3 ± 19.5*
TNFRSF17	BCMA	170.5 ± 13.8*	64.6 ± 7.7*	1.7 ± 0.5	0.7 ± 0.2
TNFRSF13B	TACI	5.3 ± 1.5	0.3 ± 0.0	0.8 ± 0.5	0.2 ± 0.1
TNFRSF13C	BAFFR	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.0 ± 0.0

Table 1. The effect of CCN1 on gene expression of APRIL/BAFF system (fold change). Cells were treated with a recombinant CCN1 protein at 1.0 µg/ml for 6 h. gene expression was analyzed by quantitative RT-PCR. *Statistical significance ($P < 0.05$).
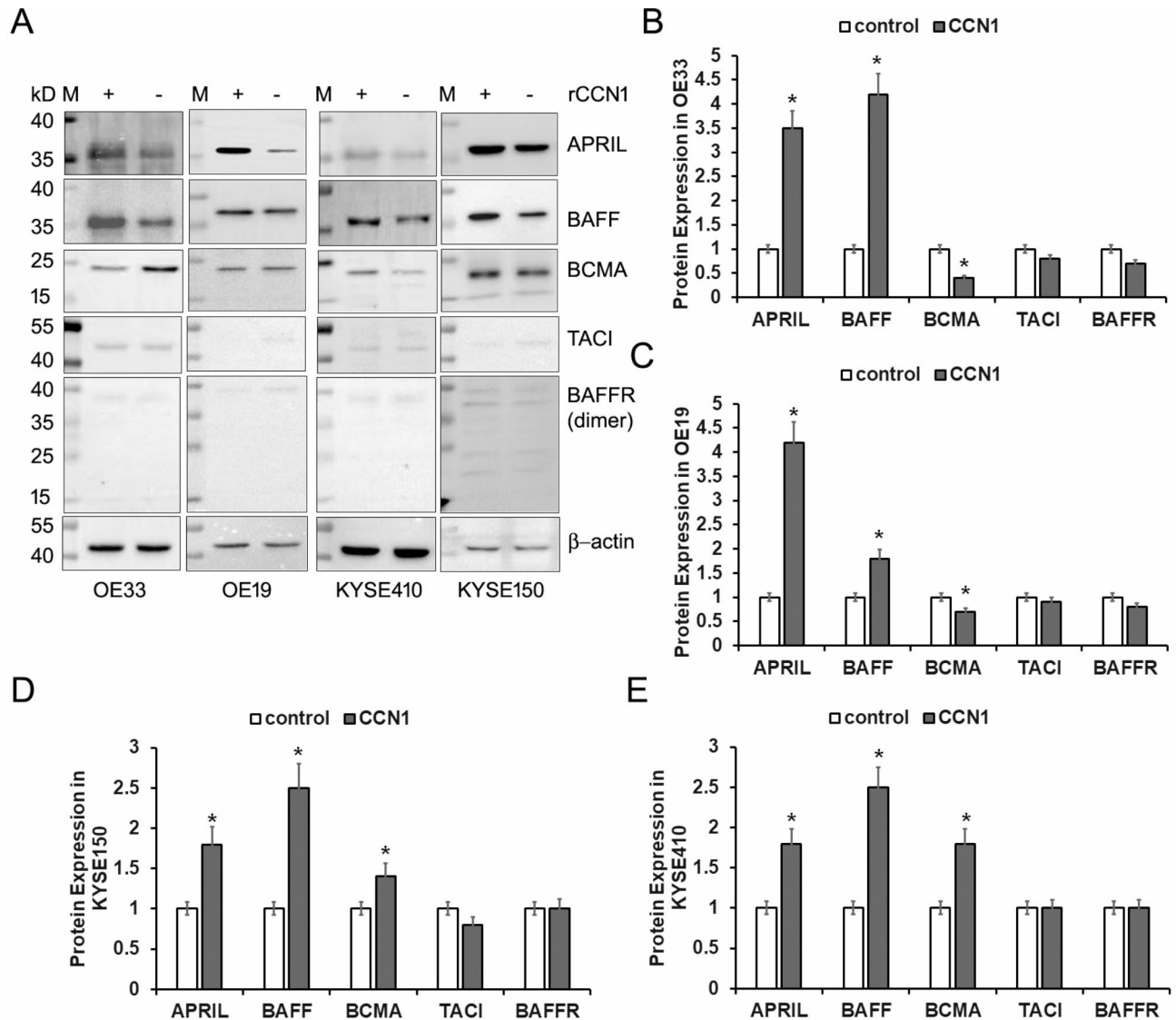
Fig. 1. The effect of CCN1 treatment on protein expression of APRIL/BAFF system. (**A**) Western blot analyses show that treatment with recombinant CCN1 (rCCN1) at 1 µg/ml for 6 h promotes APRIL and BAFF expression in both ESCC (KYSE150 and KYSE410) and EAC (OE19 and OE33). (**B**) Quantitative analysis of protein expression of the APRIL/BAFF system in OE33 based on at least 3 replicates. (**C**) Quantitative analysis of protein expression of the APRIL/BAFF system in OE19 based on at least 3 replicates. (**D**) Quantitative analysis of protein expression of the APRIL/BAFF system in KYSE150 based on at least 3 replicates. (**E**) Quantitative analysis of protein expression of the APRIL/BAFF system in KYSE410 based on at least 3 replicates. *Statistical significance. M – molecular weight ladder.

were confirmed to be what we intended to see (Supplementary File 1). BCMA (20kD), on the other hand, was upregulated in ESCC but downregulated in EAC. TACI (45kD) and BAFFR (19kD) expressed weakly and did not respond to CCN1 treatment significantly. To ensure the observed bands are TACI and BAFFR, we included their recombinant proteins in the Western blots as references. Their identities were confirmed (Supplementary Files 2 and 3). Thus, we decided to focus on APRIL, BAFF, and BCMA in the rest of our investigation.

To further characterize the effect of CCN1 on APRIL/BAFF/BCMA signaling, we transfected the cells with pcDNA3.1-CCN1 plasmid. As shown in Fig. 2, transfection increased CCN1 protein expression by 5-fold in two ESCC cell lines, and by 18- and 13-fold in OE19 and OE33 cells respectively ($P < 0.01$). Because of the flag tag, the plasmid CCN1 (pCCN1) was easily distinguishable from the endogenous CCN1 in ESCC cells. EAC cells, on the other hand, barely express CCN1, hence, the plasmid CCN1 was the only visible CCN1 band in Western blots.

Next, we examined how ESCC cells responded to CCN1 transfection. As shown in Fig. 3A (middle panel), overexpression of CCN1 not only upregulated the full-length APRIL protein but also increased its cleaved products, 17kD soluble APRIL (sAPRIL) and 10kD cellular residual (cAPRIL). However, they appeared weak in the cell lysates likely due to sAPRIL release and cAPRIL degradation. We precipitated APRIL from the conditioned cell culture media to confirm this speculation using an antibody against APRIL. As shown
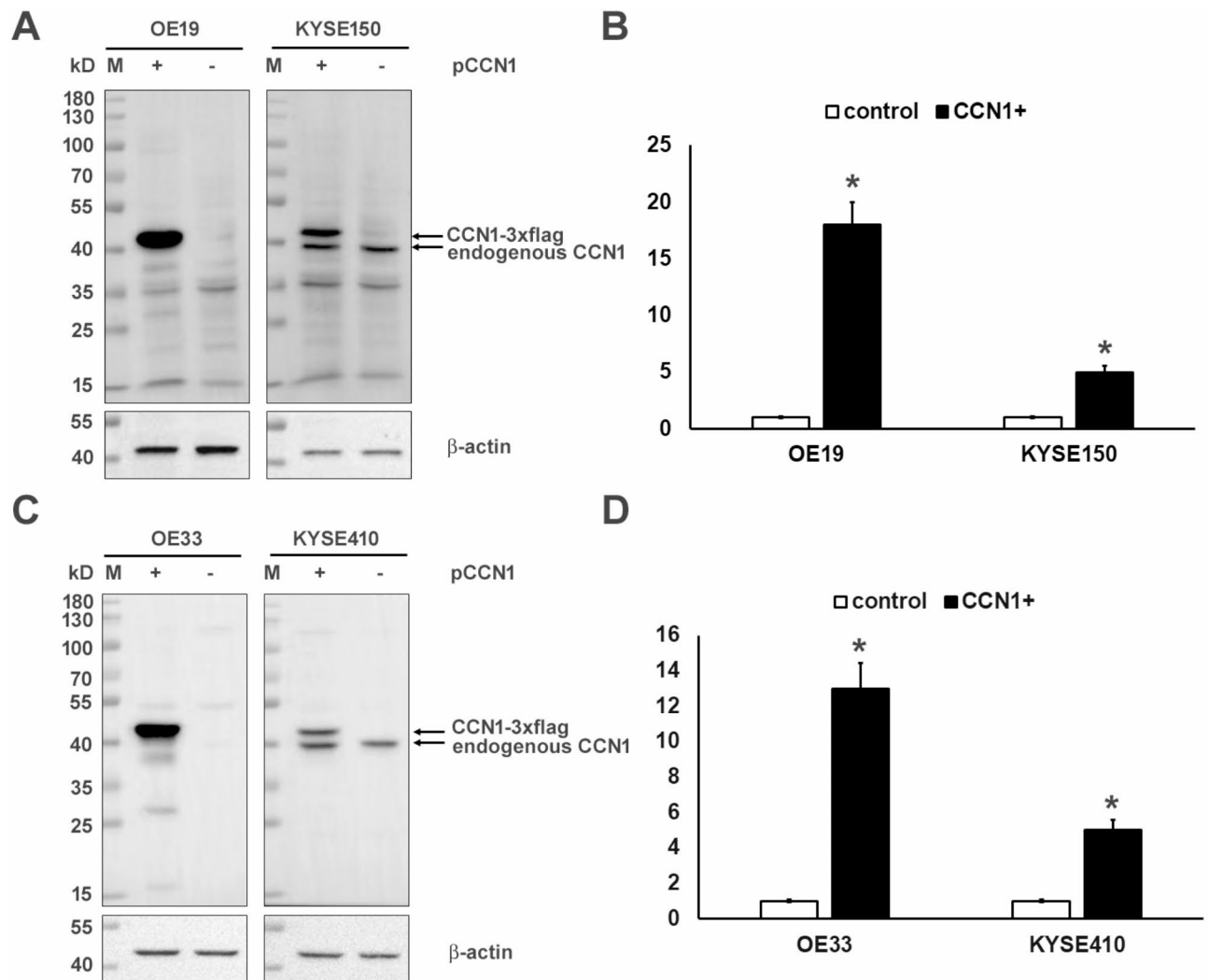
Fig. 2. CCN1 transfection efficiency. (**A**) Western blot analyses show CCN1 protein expression levels in OE19 and KYSE150 cells after transfection with pcDNA3.1-CCN1 (pCCN1) compared to vector-transfected control. (**B**) Quantitative analysis of CCN1 expression in OE19 and KYSE150 in response to cell transfection, based on at least 3 replicates. (**C**) Western blot analyses show CCN1 protein expression levels in OE33 and KYSE410 cells after transfection with pcDNA3.1-CCN1 (pCCN1) compared to vector-transfected control. (**D**) Quantitative analysis of CCN1 expression in OE33 and KYSE410 in response to cell transfection, based on at least 3 replicates. *Statistical significance. M – molecular weight ladder.

in Fig. 3A (upper panel), sAPRIL was increased by 3.3-fold in the CCN1-transfected KYSE150 ($P < 0.05$). A similar increase was seen in KYSE410 (data not shown), suggesting that CCN1 may facilitate APRIL signaling in ESCC cells (Fig. 3A and B). Compared to APRIL, BAFF expression was upregulated even more prominently by CCN1 transfection (Fig. 3C and D). The full-length BAFF and its soluble form sBAFF (18kD) were increased by 2.3- and 3.5-fold respectively ($P < 0.05$). BCMA was seen as monomers (20kD) and trimers (60kD), both were augmented moderately in response to CCN1 transfection (Fig. 3E and F). BCMA cleavage was not observed, suggesting that BCMA was fully available to support APRIL and BAFF signaling in ESCC.

Expression of CCN1 upregulates APRIL/BAFF in EAC as in ESCC but suppresses their cleavage and release

Next, we performed the same experiments using the transected EAC cells as we did with the ESCC cells. As shown in Fig. 4A and B, the full-length APRIL was upregulated in the presence of CCN1, but its cleavage was too weak to see in the cellular extracts until the blots were over-exposed. Moreover, the immunoprecipitation of the conditioned cell culture media convinced us that sAPRIL release was truly reduced in EAC cells in response to CCN1 transfection ($P < 0.05$). A similar expression pattern was seen for BAFF (Fig. 4C and D). That is to say, in EAC cells CCN1 did not downregulate APRIL/BAFF expression but suppressed their cleavage and release. In addition, BCMA was downregulated moderately by CCN1 transfection (Fig. 4E and F). Again, we did not see BCMA cleavage in EAC cells regardless of CCN1 expression. Taken together, it appears that CCN1 may attenuate APRIL/BAFF/BCMA signaling in EAC.
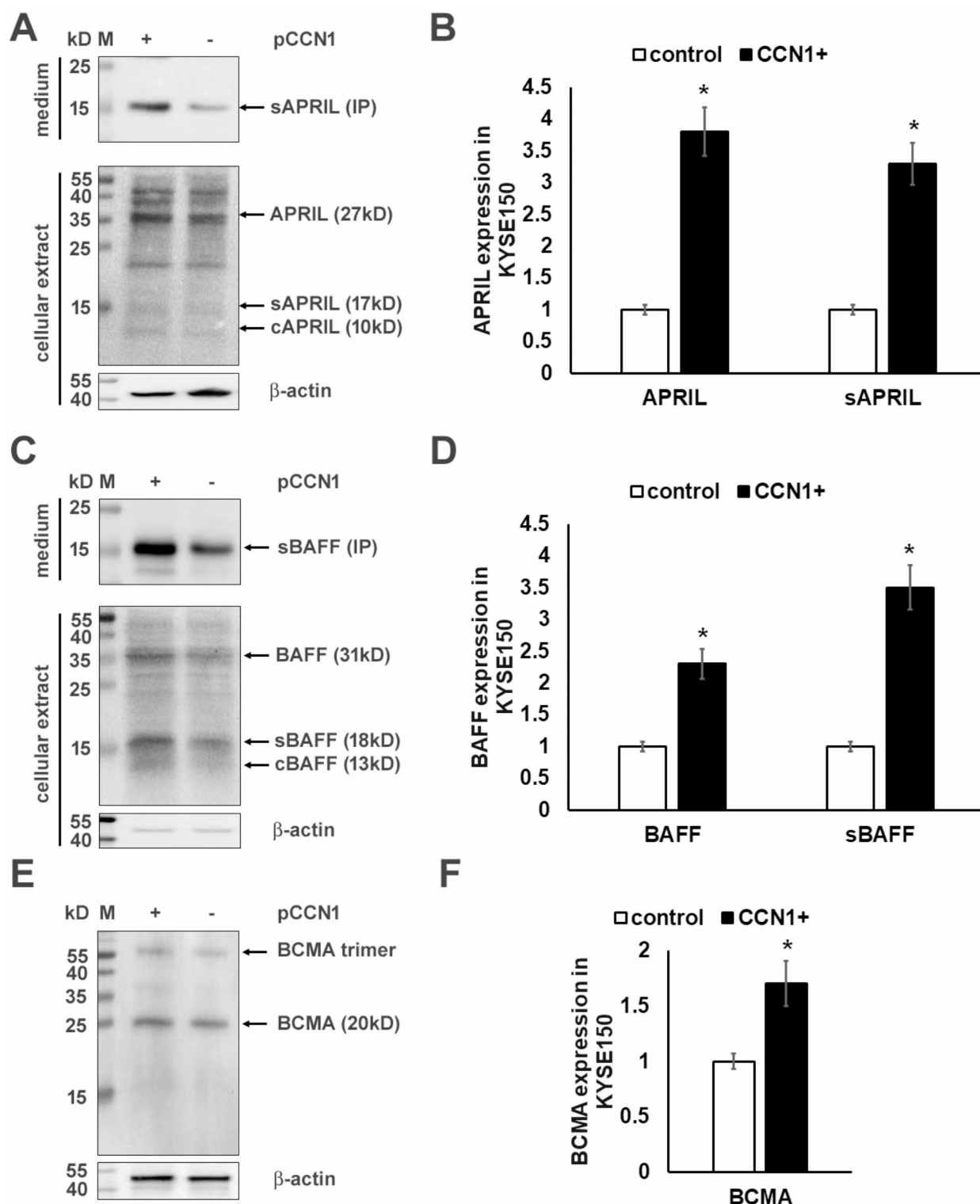
Fig. 3. Expression of APRIL, BAFF, and BCMA in KYSE150 in response to CCN1 transfection. (**A**) Upper panel: Immunoprecipitation (IP) of soluble APRIL (sAPRIL) from the cell culture media. Middle panel: Western blot analyses of cell extracts show APRIL precursor (27kD) and its cleaved products: soluble APRIL (sAPRIL) and cellular residue (cAPRIL). (**B**) Quantitative analysis of APRIL expression and cleavage in KYSE150 in response to CCN1 transfection, based on at least 3 replicates. (**C**) Upper panel: Immunoprecipitation (IP) of soluble BAFF (sBAFF) from the cell culture media. Middle panel: Western blot analyses of cell extracts show BAFF precursor (31kD) and its cleaved products: soluble BAFF (sBAFF) and cellular residue (cBAFF). (**D**) Quantitative analysis of BAFF expression and cleavage in KYSE150 in response to CCN1 transfection, based on at least 3 replicates. (**E**) Western blot analyses of cell extracts show BCMA expression in KYSE150. (**F**) Quantitative analysis of BCMA expression in KYSE150 in response to CCN1 transfection, based on at least 3 replicates.* Statistical significance. M – molecular weight ladder.
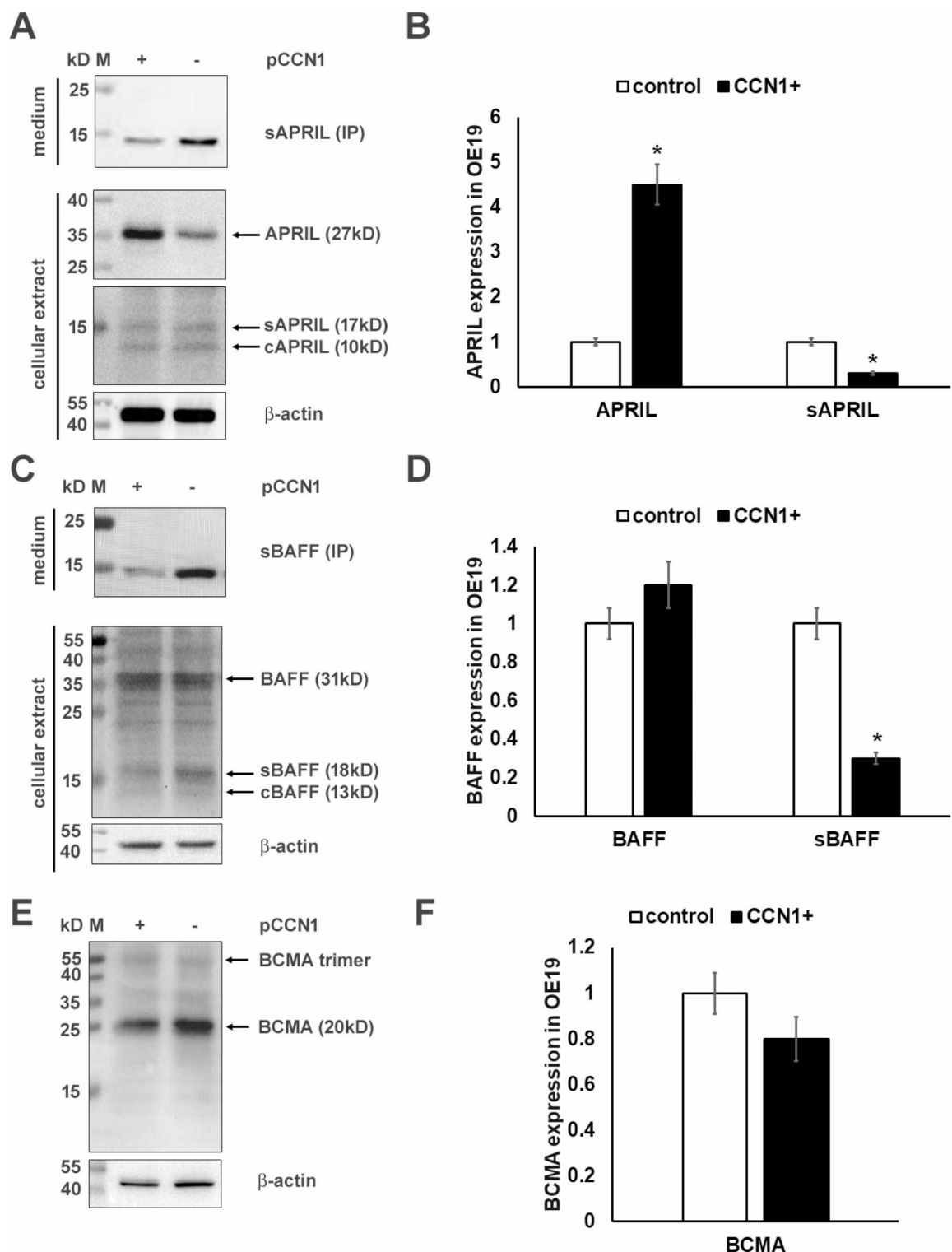
Fig. 4. Expression of APRIL, BAFF, and BCMA in OE19 in response to CCN1 transfection. (**A**) Upper panel: Immunoprecipitation (IP) of soluble APRIL (sAPRIL) from the cell culture media. Middle panel: Western blot analyses of cell extracts show APRIL precursor (27kD) and its cleaved products: soluble APRIL (sAPRIL) and cellular residue (cAPRIL). (**B**) Quantitative analysis of APRIL expression and cleavage in OE19 in response to CCN1 transfection, based on at least 3 replicates. (**C**) Upper panel: Immunoprecipitation (IP) of soluble BAFF (sBAFF) from the cell culture media.Middle panel: Western blot analyses of cell extracts show BAFF precursor (31kD) and its cleaved products: soluble BAFF (sBAFF) and cellular residue (cBAFF). (**D**) Quantitative analysis of BAFF expression and cleavage in OE19 in response to CCN1 transfection, based on at least 3 replicates. (**E**) Western blot analyses of cell extracts show BCMA expression in OE19. (**F**) Quantitative analysis of BCMA expression in OE19 in response to CCN1 transfection, based on at least 3 replicates.* Statistical significance. M – molecular weight ladder.

Expression of CCN1 promotes APRIL/BAFF binding to BCMA in ESCC cells but suppresses it in EAC cells

For either APRIL or BAFF to function, it has to bind to its membrane receptors, in this case, BCMA. To determine how CCN1 affects APRIL or BAFF binding to BCMA, we precipitated BCMA from the cellular extracts using a specific antibody and then probed for APRIL and BAFF. As shown in Fig. 5A and C, more APRIL and BAFF came down with BCMA in ESCC cells, while the results in EAC cells were just the opposite ($P < 0.05$), indicating that CCN1 facilitates APRIL/BAFF/BCMA signaling in ESCC but attenuates it in EAC. Consistent with the results of immunoprecipitation, immunofluorescence cell staining also showed that in response to CCN1 transfection, more APRIL and BAFF were released from ESCC cells to the extracellular environment, which interacted with the membrane-bound BCMA actively, while in EAC cells both ligands mostly withdrew to inside the cells, particularly near the cell membrane (Fig. 5D).

CCN1 reduces active furin levels in EAC cells

As mentioned at the beginning, both APRIL and BAFF are cleaved by Furin to become active ligands. Furin is synthesized initially as a 96kD protein containing 794 amino acids, including the signal peptide (1–24), prodomain (25–107), and the catalytic unit (108–794). It becomes active after removing the first 107 amino acids from the C-terminus by auto-cleavage. The active Furin is expected to be 67kD but can show up in the range of 58-85kD under reducing conditions[18]. Based on our Western blot analyses (Fig. 6A and B), expression of CCN1 in OE19 cells resulted in a 60% reduction in active Furin ($P < 0.05$), as reflected by the amount of the cleaved
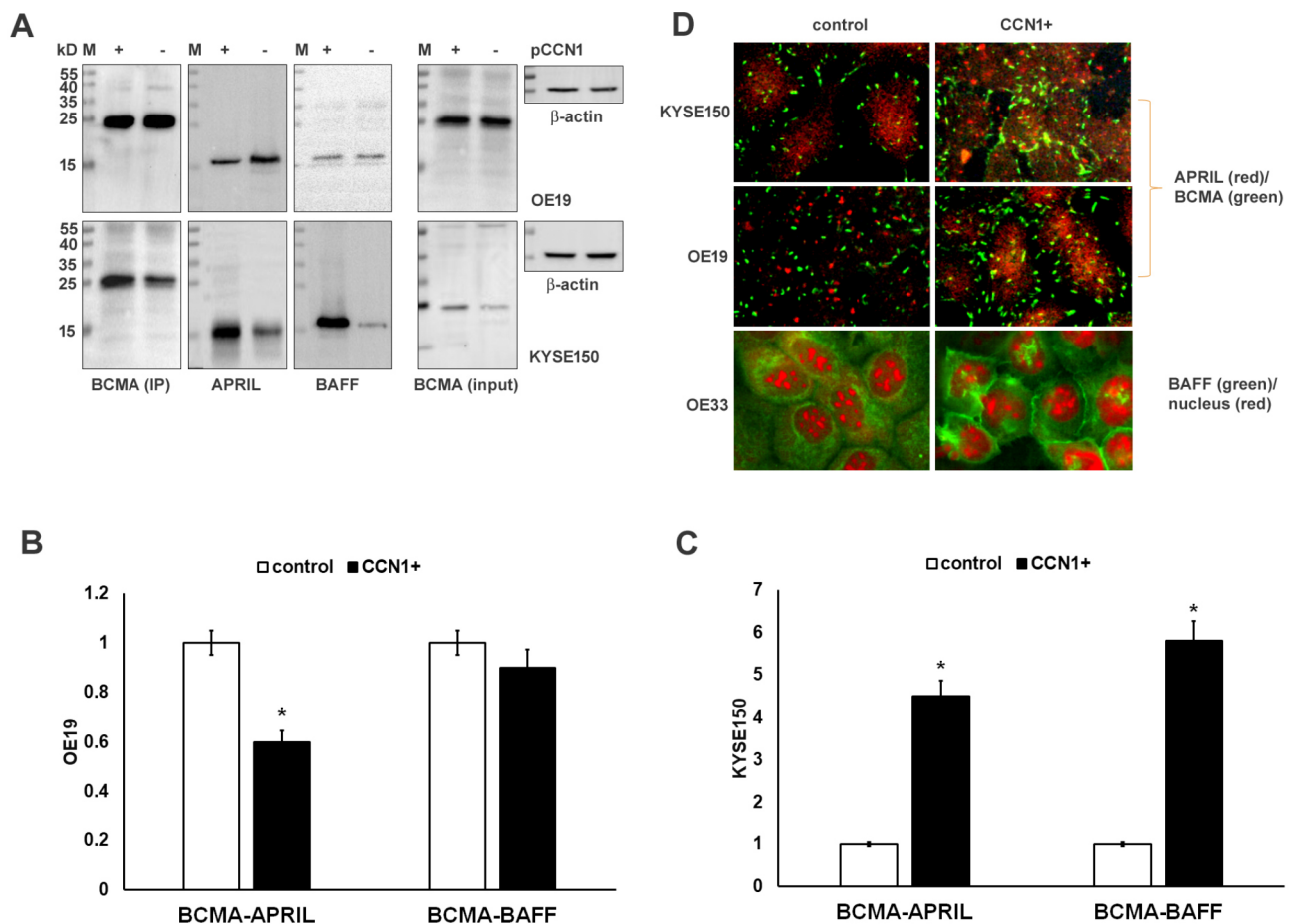

Fig. 5. Analyses of APRIL and BAFF binding to BCMA in EAC versus ESCC cells in response to CCN1 transfection. (**A**) Immunoprecipitation (IP) of BCMA from the cell extracts of OE19 and KYSE150 and Western blot analyses for its association with APRIL and BAFF. (**B**) Quantitative analysis of APRIL/BAFF binding to BCMA in OE19, based on at least 3 replicates. (**C**) Quantitative analysis of APRIL/BAFF binding to BCMA in KYSE150, based on at least 3 replicates. *Statistical significance. M – molecular weight ladder. (**D**) ESCC and EAC cells were transfected with pcDNA3.1-CCN1 or the empty vector (control) and then stained with the specific antibody for either APRIL, BAFF, or BCMA. Upper and middle panel: KYSE150 and OE19 cells were double-stained with a mouse-anti-APRIL antibody and a rabbit-anti-BCMA antibody. The fluorescence signal was generated using a Texas Red-conjugated goat-anti-mouse antibody (red) and a FITC-conjugated goat-anti-rabbit antibody (green). Lower panel: OE33 cells were first stained with a mouse-anti-BAFF antibody and then incubated with a FITC-conjugated goat-anti-mouse antibody (green). The nuclei were counterstained with propidium iodide (red).

Furin prodomain (38kD); while in KYSE150, Furin levels remained essentially unchanged, suggesting that CCN1 suppresses APRIL/BAFF release in EAC cells likely by lowering Furin activity. To verify this speculation, we treated the CCN1-transfected OE19 cells with a recombinant active Furin protein at 0.2 μg/ml overnight. Then we pulled down BCMA and examined its association with APRIL and BAFF. As shown in Fig. 6C and D, the addition of active Furin improved both APRIL and BAFF binding to BCMA in OE19 cells, indicating that CCN1 attenuates APRIL/BAFF release by reducing Furin activation. Similar results were obtained with transfected OE33 cells (data not shown).

APRIL and BAFF promote both ESCC and EAC cell growth but CCN1 promotes ESCC only

Finally, we compared the effect of APRIL and BAFF with CCN1 on the growth of ESCC and EAC cells. We treated the cells with either recombinant CCN1 (1 μg/ml), recombinant APRIL (20ng/ml), recombinant BAFF (2ng/ml), or recombinant CCN1 (1 μg/ml) plus recombinant active Furin (0.2 μg/ml) for 2, 6, 12, 24, and 48 h. Cell proliferation was assessed using a CCK-8 assay kit. As shown in Fig. 7A, in response to CCN1 treatment, ESCC cells grew steadily while the number of EAC cells was getting lower and lower with time. After 2-day of CCN1 treatment, more than half of the EAC cells were gone. However, the addition of the active Furin peptide kept the EAC cells growing almost like the ESCC cells for the first 24 h, and then the cells started to die again (Fig. 7B), likely due to the different length of active life between recombinant CCN1 and Furin, as CCN1 is known capable to stay active for > 2 days[19,20]. To confirm our speculation, we administered additional recombinant Furin at 24 h. The number of EAC cells at 48 h was restored by 20% compared to before, indicating the loss of Furin activity played a role in EAC cell viability (Fig. 7C). Treatments with either recombinant APRIL or BAFF (Fig. 7D and E), on the other hand, created similar effects on both ESCC and EAC cells, i.e., cell numbers increased in the first 24 h but started to decline then after due to the exhaustion of the ligand activity (Fig. 7F and G).

Discussion

CCN1 has been one of our primary research targets for the past decade, mainly focusing on its role in the digestive system[2]. To date, we have found that CCN1 expresses at minimal levels in the epithelial lining of the
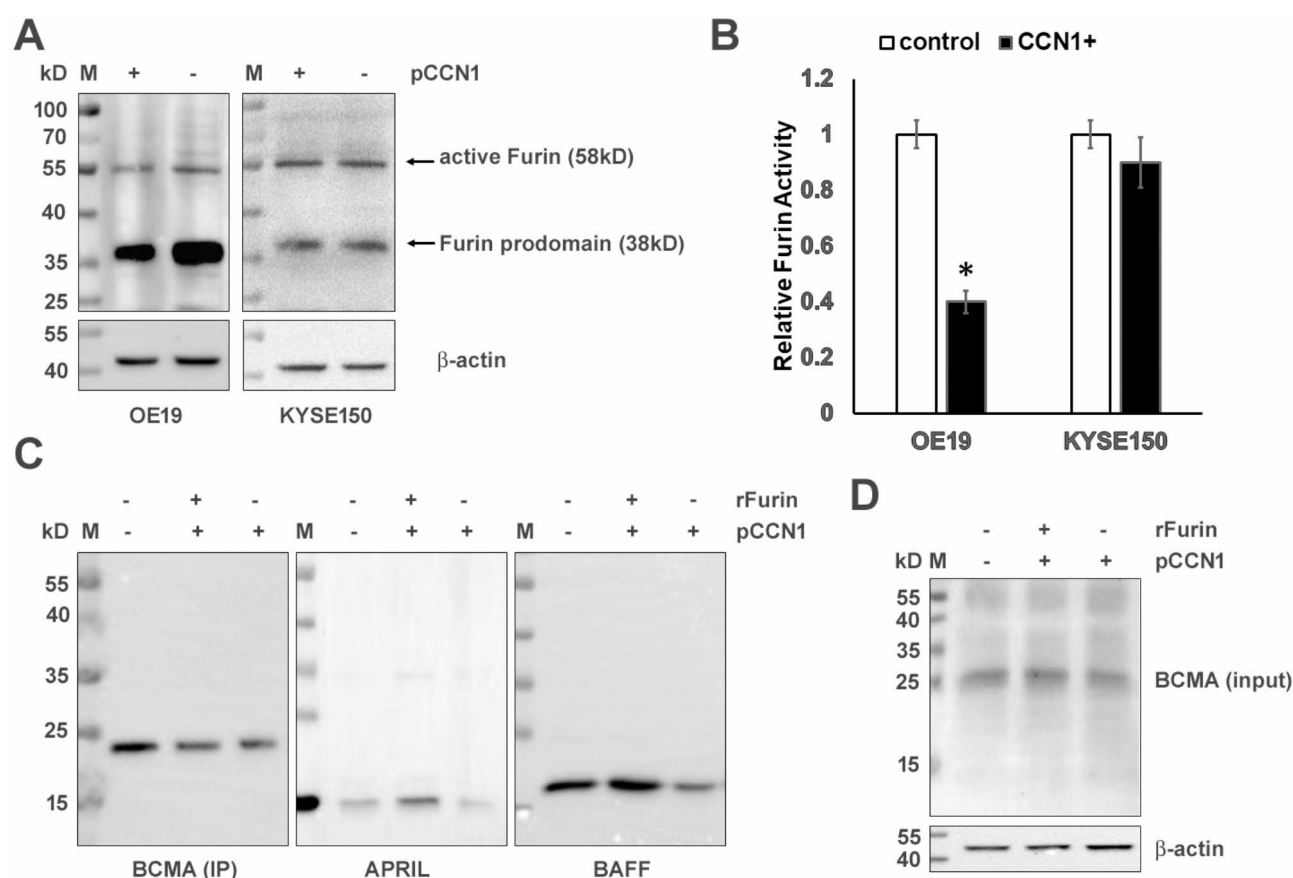

Fig. 6. The effect of CCN1 expression on Furin activity. (**A**) Western blot analyses show active Furin (58 kD) and the cleaved prodomain (38 kD) in response to CCN1 transfection (pCCN1) in OE19 and KYSE150. (**B**) Quantitative analysis of active Furin based on at least 3 replicates. (**C**) Immunoprecipitation (IP) of BCMA from the cell extracts of OE19 and Western blot analyses for its association with APRIL and BAFF with or without the addition of recombinant active Furin (rFurin). (**D**) Western blot analyses of BCMA input. *Statistical significance. M – molecular weight ladder.
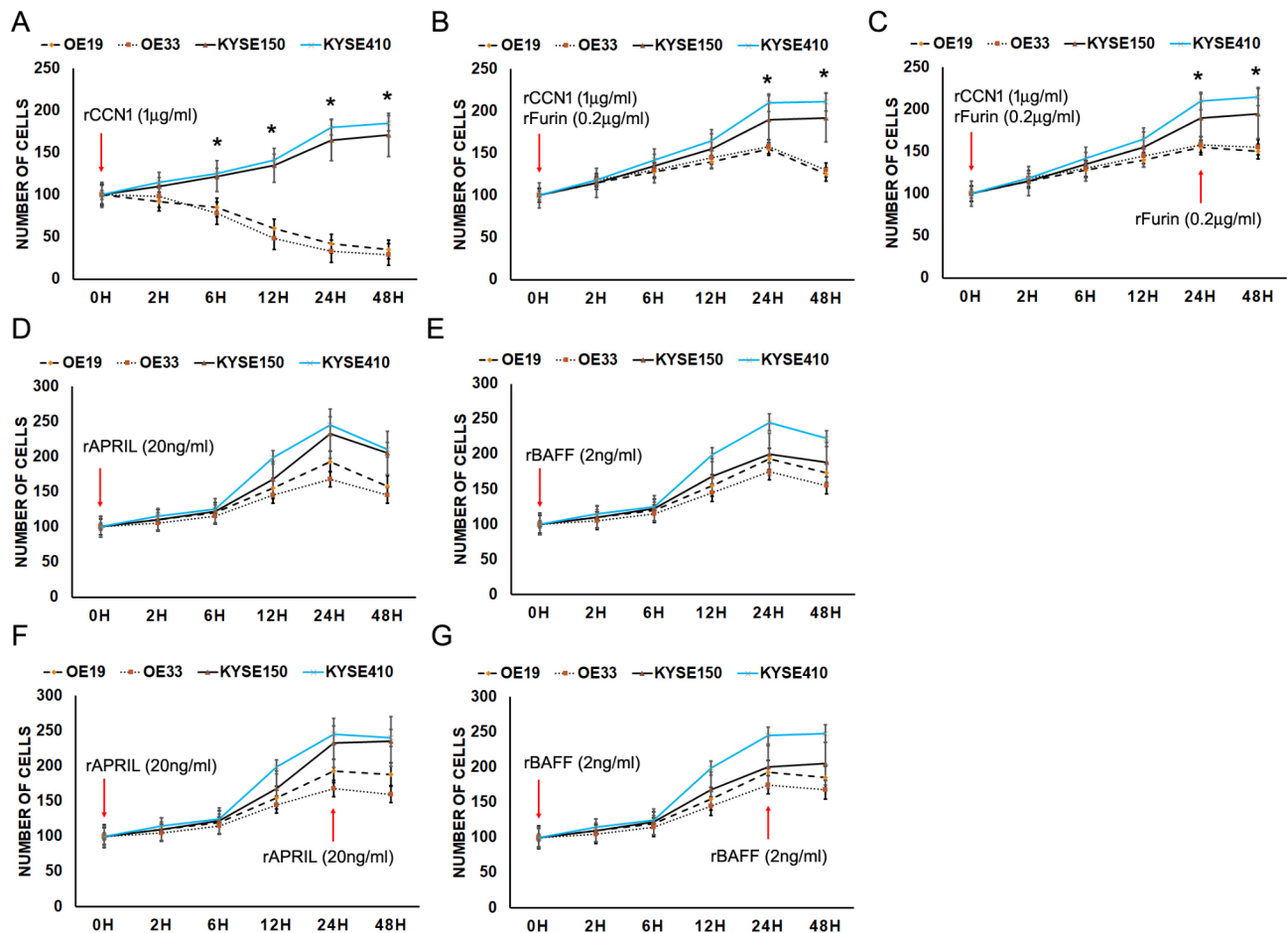
Fig. 7. Cell proliferation assays using CCK-8 kit in response to treatment with either recombinant CCN1 (rCCN1), recombinant APRIL (rAPRIL), recombinant BAFF (rBAFF), or recombinant CCN1 (rCCN1) plus recombinant active Furin (rFurin). (**A**) Quantitative analysis of the relative cell numbers of OE19, OE33, KYSE150, and KYSE410 after incubation with recombinant CCN1 for up to 48 h based on at least 3 replicates. The initial number of cells was set to 100 arbitrarily. (**B**) Quantitative analysis of the relative cell numbers of OE19, OE33, KYSE150, and KYSE410 after incubation with recombinant CCN1 plus recombinant active Furin for up to 48 h based on at least 3 replicates. The initial number of cells was set to 100 arbitrarily. (**C**) Quantitative analysis of the relative cell numbers of OE19, OE33, KYSE150, and KYSE410 after incubation with recombinant CCN1 plus recombinant active Furin for up to 24 h, and then Furin was replenished as indicated by the arrow. Based on at least 3 replicates. The initial number of cells was set to 100 arbitrarily. (**D**) Quantitative analysis of the relative cell numbers of OE19, OE33, KYSE150, and KYSE410 after incubation with recombinant APRIL for up to 48 h based on at least 3 replicates. The initial number of cells was set to 100 arbitrarily. (**E**) Quantitative analysis of the relative cell numbers of OE19, OE33, KYSE150, and KYSE410 after incubation with recombinant BAFF for up to 48 h based on at least 3 replicates. The initial number of cells was set to 100 arbitrarily. (**F**) Quantitative analysis of the relative cell numbers of OE19, OE33, KYSE150, and KYSE410 after incubation with recombinant APRIL for up to 24 h, and then APRIL was replenished as indicated by the arrow. Based on at least 3 replicates. The initial number of cells was set to 100 arbitrarily. (**G**) Quantitative analysis of the relative cell numbers of OE19, OE33, KYSE150, and KYSE410 after incubation with recombinant BAFF for up to 24 h, and then BAFF was replenished as indicated by the arrow. Based on at least 3 replicates. The initial number of cells was set to 100 arbitrarily. *Statistical significance.

gastrointestinal tract under normal circumstances, but goes up in case of injury or hazardous insults, e.g., ulcers, acid reflux, etc., stays high until the condition returns to normal, and then drops to the low levels again. During this process, CCN1 defends the epithelium against detrimental substances and restores tissue homeostasis by regulating cell proliferation, cell migration, and other extracellular components, e.g., TNFSF cytokines.

EAC mostly originated from gastroesophageal reflux disease (GERD). GERD causes esophagitis initially, and if the refluxate contains bile, it may lead to esophageal metaplasia, also known as Barrett's esophagus, a precancerous condition to EAC. During the process of GERD to EAC, the esophageal CCN1 expression goes up extremely high at the beginning (esophagitis) and declines gradually if the GERD episodes continue, but remains still higher in the metaplastic esophageal epithelium compared to its normal counterpart. Once EAC appears, however, CCN1 essentially withdraws from the malignant mucosa[4]. In other words, EAC must eliminate CCN1

to grow. Compared to EAC, ESCC seems more evolved. Instead of eliminating CCN1, ESCC utilizes CCN1 to support its tumor growth and metastasis.

In the battle against hazardous insults, CCN1 constantly interacts with the TNFSF cytokines in the extracellular matrix. As demonstrated in our previous study[21], acid/bile exposure stimulates esophageal epithelial cells to express multiple TNFSF/TNFRSF molecules, including CD40L/CD40 (TNFSF5/TNFRSF5), LTA (TNFSF1), LTBR (TNFRSF3), RANK (TNFRSF11A), and thereby activating non-canonical NFκB. Then NFκB transcribes CDX2, which makes esophageal cells express intestinal genes, turning the squamous epithelial cells into columnar phenotype. Overexpression of CCN1 suppresses these genes thus preventing the metaplastic transformation.

In the case of EAC, the expression of CCN1 stimulates the cancer cells to express TRAIL (TNFSF10) and DR5 (TNFRSF10B), thereby resulting in apoptosis[3]. Acid/bile exposure can also stimulate EAC cells to overexpress TRAIL and DR5 but does not result in apoptosis, because under such conditions EAC cells overexpress TRADD (TNFR1 associated death domain protein) to misguide the death signal in the direction of NFκB activation instead of caspase activation, promoting cell survival rather than cell death[22]. Also in EAC cells, CCN1 upregulates TNFR1 (TNFRSF1A) expression but does not promote TNF (TNFSF2) signaling because CCN1 activates ADAM17-mediated TNFR1 cleavage. Consequently, more soluble TNFR1 fragments are produced to neutralize TNF signals[23]. Here in this study, we found a similar situation with APRIL and BAFF. CCN1 upregulated APRIL and BAFF in both ESCC and EAC but did not promote their signaling in the latter because CCN1 suppressed Furin-mediated APRIL/BAFF cleavage. As a result, the soluble APRIL and BAFF were insufficient to bind BCMA activating NFκB-mediated cell proliferation. In this way, the presence of CCN1 allows TRAIL/DR5 to mediate tumor cell apoptosis without interference from APRIL and BAFF.

APRIL and BAFF are the essential cytokines supporting B cell maturation. Although three receptors, namely, BAFFR, TACI, and BAMA, can mediate APRIL/BAFF signaling, they are expressed in B cells sequentially in the order of BAFFR-TACI-BAMA. Mature B cells mainly express BCMA to receive signals from APRIL and BAFF. Here in esophageal cancer cells, we found BCMA also to be the predominant receptor for APRIL/BAFF signaling, reflecting the maturity of these tumor cells. Compared to ESCC, which utilizes the APRIL/BAFF system craftily, EAC requires the elimination of CCN1 as a precondition to have the APRIL/BAFF system functioning properly, as the presence of CCN1 not only interrupts APRIL/BAFF production but also lowers the BCMA availability. Two molecular mechanisms are known to regulate BCMA protein levels. One is BCMA shedding by γ-secretase, converting BCMA from a 20kD functional membrane-bound receptor to a 6kD soluble decoy receptor, thereby blocking APRIL/BAFF signaling. The second way to lower BCMA protein expression is the transcription of the antisense RNA of BCMA, which has been documented in several B cell lines[24]. In this study, we did not see BCMA cleavage but we saw CCN1-induced BCMA mRNA elevation, which means that the reduction of BCMA protein in the presence of CCN1 is likely caused by transcription from the antisense strand of the BCMA gene. Further study is needed.

Materials and methods
Cell culture, transfection, and treatment reagents
Human EAC cell lines OE19 and OE33 (Sigma-Aldrich, St Louis, USA), and ESCC cell lines KYSE150 and KYSE410 (BeNa Culture Collection, Beijing, China) were cultured in RPMI medium (Thermal Fisher Scientific, Waltham, USA) plus 10% fetal bovine serum (Sigma-Aldrich). For cell treatment, an equal number of cells were plated in 6-well plates and cultured to 80% confluency. They were then synchronized in the basal medium for 6 h before experiments. Recombinant human CCN1 protein (NM_001554) was purchased from Origene (Rockville, USA). Recombinant human APRIL (O75888), BAFF (Q9Y275.1), and Furin (P09958) were purchased from R&D Systems (Minneapolis, USA). Plasmid pcDNA3.1 with or without a 3-flag tagged CCN1 open reading frame was obtained from Origene for cell transfection with lipofectamine LTX-plus (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. After transfection, the cells were selected using Neomycin (Invitrogen), and the transfection efficiency was monitored via Western blot analysis.

Real-time RT-PCR
Total RNA was extracted from the cells using Trizol reagent (Invitrogen) and purified using an RNeasy kit (QIAGEN, Valencia, USA). These RNA extracts were then used as templates to synthesize cDNA probes by reverse transcription using a GEArray kit (SABiosciences, Frederick, USA). Reverse transcription was done following the procedure: 25 °C/10 min – 55 °C/30 min – 85 °C/5 min – 4 °C/∞. A 96-well cDNA array containing all the members of TNF/TNFR families and associated molecules (Origene) was used to monitor gene expression by real-time PCR following the two-step program using the SYBR Green master mix (SABiosciences). Data were generated from 4 independent experiments and analyzed according to the $\Delta\Delta C_t$ method. Briefly, ΔC_t was calculated by subtracting the C_t value of GAPDH from the C_t value for each gene; and then $\Delta\Delta C_t$ was calculated by subtracting the ΔC_t of the control from the ΔC_t of the treatment, and finally, the fold change was calculated using the formula: Fold Change $= 2^{(-\Delta\Delta Ct)}$.

Protein isolation, immunoprecipitation, and western blot analysis
After indicated cell treatment, the cellular protein was extracted using a modified RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, and 1% NP-40. The supernatant was collected after centrifugation at 10,000 g for 10 min.

For immunoprecipitation (IP), 100 µg of each cellular protein extract was adjusted to 1 µg/ml concentration and incubated overnight at 4 °C with protein A-agarose beads (Invitrogen) coated with 1 µg of specific antibody against APRIL (sc-374674), BAFF (sc-271809), or BCMA (sc-390147). The beads were then washed twice in the lysis buffer and boiled in the SDS sample buffer for 5 min to release the targeted protein and its associates.

For Western blot analysis, equal amounts of protein samples (30–50 µg each) or IP products were separated in a mini-gel at 100 V and transferred to a nitrocellulose membrane at 390 mA for 90 min. The membrane was first blocked with 5% nonfat milk for an hour and then incubated with a primary antibody for 2 h on a shaker. After a 3 × 10 min wash with TBST buffer, the blot was incubated with an HRP-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology) on a shaker for an hour. The signal was developed in ECL solution (Amersham Pharmacia, Netherlands) and the image was captured and quantified using Gel Doc XR (Bio-Rad, Hercules, USA). Protein expression of interest was compared among the experimental conditions within the same blot against β-actin. The data were collected from multiple independent experiments and assessed for statistical significance. The following primary antibodies were used in this study: CCN1 (TA349858) and BCMA (TA382649) from Origene, APRIL (sc-374674), BAFF (sc-271809), TACI (sc-365253), BAFFR (sc-365409), Furin (sc-133142), and β-actin (sc69879) from Santa Cruz Biotechnology.

Cell proliferation assay

Cell Counting Kit 8 (CCK-8, Sigma-Aldrich) was used to quantify cell viability after the indicated treatment following the manufacturer's protocol. Briefly, an equal number of cells was distributed in a 96-well plate at 100 µl/well. The plate was incubated in the CO_2 incubator for 24 h and then switched to the serum-free medium for 6 h. The cells were treated with one of the following formulas: (1) recombinant CCN1 (1 µg/ml); (2) recombinant APRIL (20ng/ml); (3) recombinant BAFF (2ng/ml); (4) recombinant CCN1 (1 µg/ml) plus recombinant active Furin (0.2 µg/ml), for 2, 6, 12, 24, and 48 h. Before termination of the experiment,10 µl of the CCK-8 reagent was added to each well and the plate was put back in the CO_2 incubator for another 4 h. Cell proliferation was measured using a plate reader (Fisher Scientific, USA) at 450 nm wavelength. The control reading was arbitrarily set to 100, and the rest of the readings were multiplied by 100. To determine whether protein decay plays a role in the treatments, the recombinant proteins above were replenished after 24 h.

Immunofluorescence cell staining

For immunofluorescence, after serum starvation and indicated treatment, cells were fixed for 10 min in 4% paraformaldehyde, followed by incubation with the antibody against APRIL (sc-374674, Santa Cruz Biotechnology) or BAFF (sc-271809, Santa Cruz Biotechnology) or BCMA (TA382649, Origene) for 2 h, and then washed in PBS and incubated with a FITC- (ab6785) or Texas Red-conjugated (ab6787) anti-mouse or FITC-conjugated anti-rabbit (ab6717) secondary antibody (Abcam, USA) for an hour. Nuclei were counter-stained with Propidium Iodide (Invitrogen).

Statistical analysis

All numerical data were expressed as mean ± standard deviation and analyzed by single classification one-way ANOVA. In brief, the sums of squares among groups and within groups were calculated using the formula SS_{among} = sample size/2 × (mean of the treatment + mean of the control)^2, and SS_{within} = Σ (treatment reading – treatment mean)^2 + Σ (control reading – control mean)^2, respectively. The means of squares among groups and within groups were calculated using the formula MS_{among} = SS_{among}/df_{among} and MS_{within} = SS_{within}/df_{within} respectively. Here df_{among} = number of groups – 1 and df_{within} = number of groups × (sample size – 1), standing for the degree of freedom among groups and within-group respectively. The ratio of MS_{among}/MS_{within} was the F_s value for the experiment. By referring to the F table, if F_s > F value in the table at $P < 0.05$, the effect of the treatment was considered significant from control.

Data availability

Data are available upon reasonable request from the corresponding author Dr. Chai.

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Author contributions

L.X. and Z.J. conducted most of the experimental work and prepared the manuscript drifts. R.X. and J.W. participated in some of the experiments. T.D. provided some research funds and administrated the project. X.M. provided additional funds and other resources. J.C. conceptualized the study, curated the data, and finalized the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to J.W., J.C. or X.M.

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