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Targeting MFGE8 secreted by cancer-associated fibroblasts blocks angiogenesis and metastasis in esophageal squamous cell carcinoma

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Cancer-associated fibroblasts (CAFs) play vital roles in establishing a suitable tumor microenvironment. In this study, RNA sequencing data revealed that CAFs could promote cell proliferation, angiogenesis, and ECM reconstitution by binding to integrin families and activating PI3K/AKT pathways in esophageal squamous cell carcinoma (ESCC). The secretions of CAFs play an important role in regulating these biological activities. Among these secretions, we found that MFGE8 is specifically secreted by CAFs in ESCC. Additionally, the secreted MFGE8 protein is essential in CAF-regulated vascularization, tumor proliferation, drug resistance, and metastasis. By binding to Integrin αVβ3/αVβ5 receptors, MFGE8 promotes tumor progression by activating both the PI3K/AKT and ERK/AKT pathways. Interestingly, the biological function of MFGE8 secreted by CAFs fully demonstrated the major role of CAFs in ESCC and its mode of mechanism, showing that MFGE8 could be a driver factor of CAFs in remodeling the tumor environment. In vivo treatment targeting CAFs-secreting MFGE8 or its receptor produced significant inhibitory effects on ESCC growth and metastasis, which provides an approach for the treatment of ESCC.

CAFs | angiogenesis | metastasis | neutralizing antibody therapy

Esophageal squamous cell carcinoma (ESCC) is a leading cause of cancer death globally and is highly prevalent in China, Northeastern Iran, Southeastern United States, and Southern Africa (1). Despite significant advances in the diagnosis and treatment of ESCC in recent years, the 5-y survival rate for ESCC patients is still less than 20% (2). The lack of effective treatments for tumor metastasis, recurrence, and chemoresistance is the main reason for cancer recurrence and poor clinical prognosis of ESCC (3, 4). Therefore, advanced diagnostic approaches and effective treatment strategies are urgently needed to achieve better outcomes.

CAFs play critical roles in tumor metabolism, angiogenesis, immune escape, and metastasis (5, 6), primarily through extrinsic signals provided by secreted factors, exosomes, and metabolites that promote tumor growth and progression (7–9). Despite being a promising therapeutic target, CAF-targeted treatment strategies have mostly failed and may even accelerate tumor growth, resulting in poor outcomes (10). CAF characteristics and interactions may change dynamically in various cancers, making treatment difficult. While single-cell RNA sequencing has improved our understanding of CAFs, further studies are necessary to fully characterize their effects on cancer progression and develop more effective treatments.

Milk fat globule-EGF factor 8 (MFGE8) is a lactadherin preproprotein (11) involved in recognizing and engulfing apoptotic cells, wound healing, ECM remodeling, and inflammation resolution (12). It has also been found to promote tumor growth, angiogenesis, and cancer stemness in various cancers. (13, 14) Despite this, its role in ESCC has not been fully understood. Our study focused on CAF-secreted MFGE8 in ESCC and found that it specifically promotes ESCC invasion through AKT/STAT3 signaling and enhances angiogenesis via the ERK/AKT pathway by binding integrins αVβ3/αVβ5. This suggests that inhibition of CAF-secreted MFGE8 could be a promising therapeutic approach for ESCC treatment.

Results

RNA-Seq Reveals That CAFs-Secreted Protein Plays An Important Role in ESCC Development. The secretion of specific regulatory factors by CAFs plays a crucial role in driving cancer progression. In our prior investigation, we presented evidence showing that fibrocytes derived from FGFR2+ hematopoietic stem cells can be induced by ESCC

Significance

The secretions of Cancer-associated fibroblasts (CAFs) play an important role in regulating biological activities, but the role and mechanism of CAFs-secreted MFGE8 in esophageal squamous cell carcinoma (ESCC) pathogenesis are still unknown. The secreted MFGE8 protein is essential in CAF-regulated vascularization, tumor proliferation, drug resistance, and metastasis. By binding to Integrin αVβ3/αVβ5 receptors, MFGE8 promotes tumor progression by activating both the PI3K/AKT and ERK/AKT pathways. Targeting CAFs-secreting MFGE8 or its receptors produced significant inhibitory effects on ESCC growth and metastasis. These findings provide insight into previously underrecognized roles of the MFGE8 in cancer development. Targeting MFGE8 or its receptors might be a therapeutic strategy for ESCC patients.

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cells, recruited into tumor xenografts, and then differentiated into functional cancer-associated fibroblasts (CAFs) (15). To gain a better understanding of the role of CAFs in ESCC, RNA-seq was performed to compare gene expression profiles between CAFs and their progenitor cells, circulating fibrocytes. CAFs and their paired circulating fibrocytes were isolated from nine ESCC patients. The samples (total RNA) were divided into two clusters: Cluster 1 included five CAFs (T1) and paired fibrocytes (N1) samples, while cluster 2 included four CAFs (T2) and paired fibrocytes (N2) samples. In the combination of the two sample pools, a total of 944 and 528 genes were found to be up-regulated or down-regulated, respectively (logFC≥ |4|, $P \le 0.05$) (Fig. 1*A*). GO enrichment analysis showed that most of the up-regulated genes in CAFs were mainly involved in cell adhesion, cell metastasis, cell proliferation, and angiogenesis (*SI Appendix,* Fig. S1*A*). Integrin binding emerged as a crucial regulator of CAF function within the tumor microenvironment (TME). Here, the upregulation of MFGE8 also showed a GO enrichment in integrin binding (*SI Appendix,* Fig. S1*A*). Using pathway analysis based on the KEGG database, we identified up-regulated genes involved in signaling pathways, including cancer and PI3K-AKT signaling pathways (*SI Appendix,* Fig. S1*A*). These findings suggest that CAFs play an important role in ESCC development and progression. Notably, cellular component analysis revealed that most of the CAF-mediated effects occurred in the extracellular region (*SI Appendix,* Fig. S1*A*), which indicates that CAFs may be involved in cell–cell contact via secreted proteins within the TME.

CAF-Derived MFG-E8 Is A Potential Meditator of ESCC Progression. Among the up-regulated secreted factors, MFGE8 exhibited the highest expression levels in the two CAF sample pools compared to circulating fibrocytes (Fig. 1*B*). To further investigate the expression

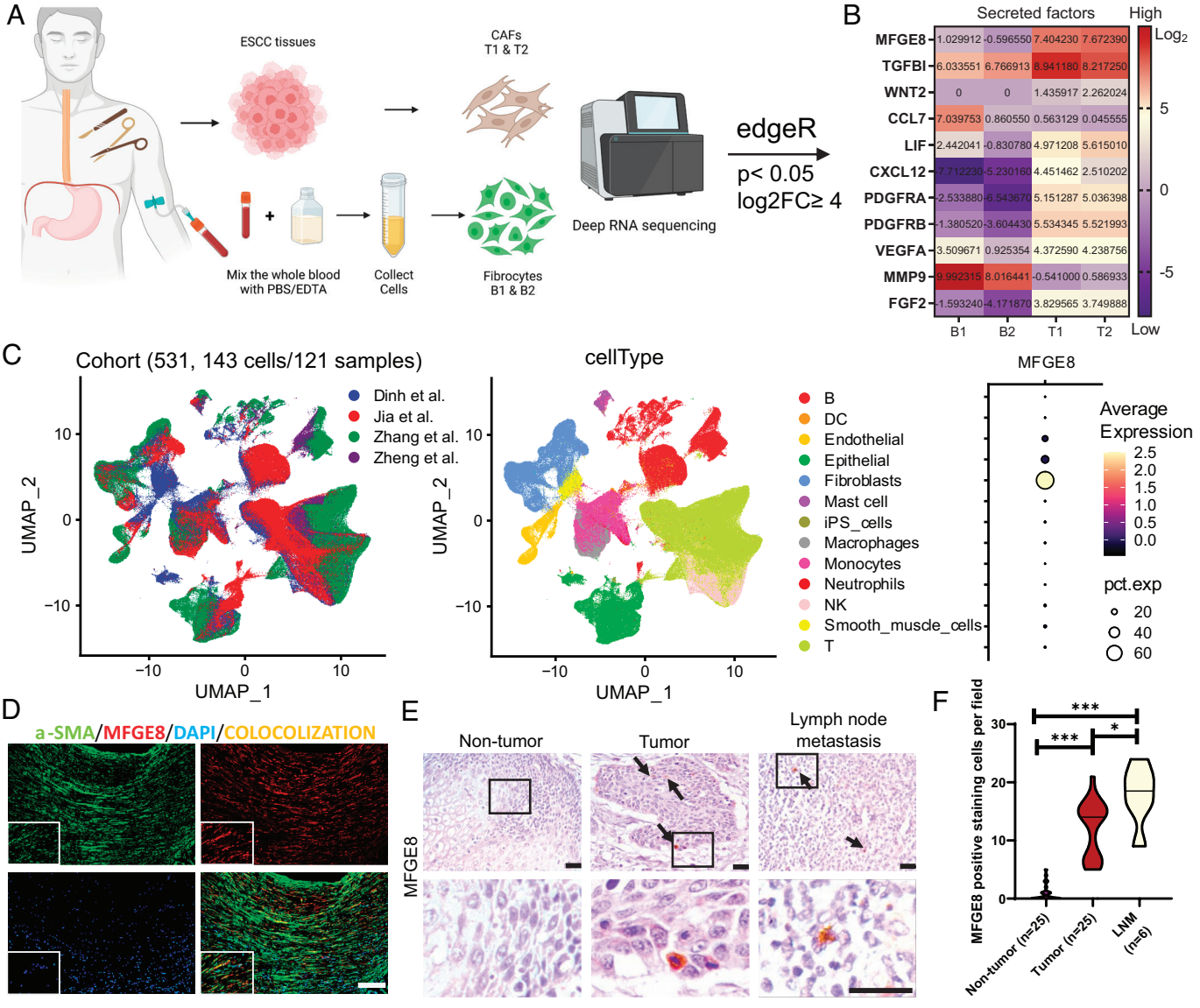

Fig. 1. MFGE8 is specifically overexpressed in CAFs but not in ESCC tumor cells, circulating fibrocytes, and other cells. (*A*) CAFs and their paired circulating fibrocytes were isolated from nine ESCC patients. A total of 944 and 528 genes were found to be up-regulated or down-regulated in the combined set of two CAF sample pools. The figure was created with BioRender.com. (*B*) MFGE8 is the top secreted protein in the two CAFs sample pools compared with circulating fibrocytes (*Right*). Compared with other functional CAF-secreted proteins, MFGE8 showed extremely significant differences between the CAFs group and the circulating fibroblast group (*Left*). (*C*) Single-cell sequencing data reveals high expression of MFGE8 in cancer-associated fibroblasts (CAFs) compared to tumor cells, endothelial cells, and immune cells. (*D*) Representative IF staining of protein MFGE8 and α-sma in ESCC specimens. (Scale bar, 100 μm.) (*E*) Representative IHC staining of protein MFGE8 in nontumor, tumor, and lymph node metastasis ESCC specimens. MGFE8+ cells were pointed by arrows. (Scale bar, 100 μm.) (*F*) Scoring statistics of MFGE8 expression in ESCC nontumor, tumor, and lymph node metastases. Statistical significances: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

of MFGE8, we analyzed single-cell sequencing data from ESCC patients (16–19). After quality filtering and doublet removal, 531,143 cells underwent principal component analysis and UMAP algorithm in Seurat software, revealing distinct cell clusters. These clusters were assigned to major cell types using established marker genes, including B cells, epithelial cells, fibroblast cells, myeloid cells, and endothelial cells. We found that CAFs had higher expression levels of MFGE8 compared to tumor and immune cells (Fig. 1*C*). Additionally, we evaluated the expression levels of MFGE8 in a subcutaneous model of ESCC in mice. Our results demonstrated significantly higher expression of MFGE8 in CAFs as compared to other cell types in the model (*SI Appendix,* Fig. S1*B*). Moreover, real-time PCR and ELISA results also found that MFGE8 was up-regulated in CAFs compared with normal fibroblasts, circulating fibrocytes, normal epithelial cells, and tumor cells (*SI Appendix,* Fig. S1 *C* and *D*). Immunofluorescence (IF) results confirmed that MFGE8 was mainly expressed in α-SMA-positive CAFs (Fig. 1*D*). Immunohistology (IHC) results also showed that MFGE8 was expressed in CAFs in tumor and metastatic lymph node tissues (Fig. 1*E*). Interestingly, IHC results revealed that the frequency of $MFGE8^{+}$ CAFs increased with ESCC progression (Fig. 1*F*). Moreover, correlation analysis of the TCGA ESCC dataset showed that high expression of MFGE8 was significantly associated with poor overall survival ($P < 0.05$), poor disease-free survival ($P < 0.05$), and tumor staging (*SI Appendix,* Fig. S1*E*). Using the single-cell sequencing data, we conducted a gene ontology (GO) analysis by comparing MFGE8+ CAFs with MFGE8- CAFs. The analysis revealed that MFGE8 primarily functions in cell adhesion and angiogenesis (*SI Appendix,* Fig. S2*A*). We then performed gene set enrichment analysis (GSEA) on the TCGA database using samples from 25 patients (1/4) with high MFGE8 expression and 25 patients with low MFGE8 expression, with a significance threshold of P <0.05 and q < 0.25.

The analysis also indicated that high expression of MFGE8 was associated with increased cell motility and angiogenesis processes in ESCC (*SI Appendix,* Fig. S2 *B* and *C*). Taken together, these results suggest that MFGE8 is highly expressed in CAFs specifically, and high expression of MFGE8 is significantly associated with poor clinical outcomes.

MFG-E8 Secreted by CAFs Promotes Angiogenesis and Metastasis in ESCC. Single-cell transcriptome GO analysis and GSEA of the TCGA patient pool suggest that MFGE8 plays a crucial role in promoting angiogenesis and metastasis in ESCC. Therefore, we posit that the primary function of MFGE8 secreted by CAFs in the ESCC microenvironment is to modulate tumor progression via the regulation of angiogenesis and metastasis. To test the effect of MFGE8 on angiogenesis, human umbilical vein endothelial cells (HUVECs) were treated with MFGE8 recombinant protein or PBS as a negative control. Functional assays showed that MFGE8 treatment could significantly enhance the cell proliferation (*SI Appendix,* Fig. S3*A*), cell migration (*SI Appendix,* Fig. S3*B*), and tube formation ability (*SI Appendix,* Fig. S3*C*) compared to PBS-treated HUVECs. Additionally, we performed functional assays using CAFs supernatant and an anti-MFGE8 neutralizing antibody as a control. Briefly, CAFs were isolated from a patient with ESCC as previously described (20). HUVECs were treated with CAFs-conditioned medium either alone or with an anti-MFGE8 neutralizing antibody. The functional assays indicated that the CAFs-conditioned medium significantly enhanced the migratory (*SI Appendix,* Fig. S3*D*) and tube formation (*SI Appendix,* Fig. S3*E*) ability of HUVECs compared to the Anti-MFGE8-blocked HUVECs. These results further support the notion that MFGE8 plays a critical role in promoting angiogenesis in ESCC. Meanwhile, western blotting results showed that the MFGE8 recombinant protein promoted the phosphorylation of ERK1/2 and AKT (*SI Appendix,* Fig. S3*F*), thereby activating the ERK/AKT pathway in HUVECs. Importantly, IF with MFGE8 and CD31 antibodies showed a positive correlation between vascular density and MFGE8 expression in ESCC clinical samples (*SI Appendix,* Fig. S3 *G* and *H*). These results indicate that MFGE8 secreted by CAFs could promote angiogenesis through the ERK/AKT pathway.

To determine the influence of MFGE8 on the metastasis of ESCC, we performed a comparative analysis of the migration and invasion abilities of ESCC cell lines (K180 and K410) treated with MFGE8 recombinant protein, PBS, CAFs-conditioned medium, and CAFs-conditioned medium supplemented with anti-MFGE8. The results of migration and invasion assay showed that MFGE8 could significantly enhance the migration and invasion abilities of ESCC cell lines (*SI Appendix,* Fig. S3 *I* and *J*). The western blotting results revealed that MFGE8 could promote epithelial-to-mesenchymal transition (EMT) by up-regulating mesenchymal markers (Fibronectin and N-cadherin) and down-regulating epithelial markers (E-cadherin and β-cadherin) (*SI Appendix,* Fig. S3*K*). Since MFGE8 has been reported to promote cell proliferation and drug resistance, these effects were studied in K180 and K410 cells treated with MFGE8 recombinant protein (500 ng/mL), PBS, CAFs-conditioned medium, and CAFs-conditioned medium supplemented with anti-MFGE8 by the XTT assay and flow cytometry. The results showed that MFGE8 could promote cell proliferation (*SI Appendix,* Fig. S3*L*) and increase drug resistance (*SI Appendix,* Fig. S3*M*) in ESCC cell lines. Collectively, these data suggest that MFGE8 secreted by CAFs enhances the cell motility via the EMT pathway and inhibits the apoptotic trigger of cancer cells in ESCC.

MFGE8 Binds to Integrin αVβ3/αVβ5 Receptors to Activate Downstream Pathways. To gain a deeper understanding of the molecular mechanism underlying MFGE8-mediated ESCC progression, we conducted KEGG pathway enrichment analysis on 478 coexpressed genes from the TCGA database (Spearman coefficient >0.5, P <0.05). The results showed that the activation of "ECM-receptor interaction," "Focal adhesion," and "PI3K-AKT signaling pathway" in ESCCs was significantly related to high MFGE8 expression (*SI Appendix,* Fig. S4*A*). Moreover, we performed GSEA (P <0.05, q < 0.25) in patients with high (Top 25%) and low (Bottom 25%) expression of *MFGE8* in the TCGA database. GSEA results also found that high expression of MFGE8 was associated with the established gene sets "PI3K-AKT signaling pathway" (*SI Appendix,* Fig. S4*B*). Then, we analyzed the public single-cell sequencing data of ESCC from four different studies (16–19). We analyzed the average expression of MFGE8 in CAFs, cancer cells, and other cells for each sample and then stratified samples into three subgroups [CAF-MFGE8-High (Top 25%), CAF-MFGE8-Low (Bottom 25%), and others] (Fig. 2*A*). As the results shown, the MFGE8 of ESCC TME is mainly originated from CAFs and the expression of MFGE8 is positively correlated with the percentage of cells expressed in each sample (R = 0.72, P = 7.7e-15; Fig. 2 *A* and *B*), indicating that these data are reasonable for GSEA analysis of cancer cells in CAF-MFGE8-High vs CAF-MFGE8-Low. The GSEA analysis results show that the PI3K/AKT signaling pathway is significantly up-regulated in cancer cells of CAF-MFGE8-High samples (NES = 1.532, P = 0.002; Fig. 2*C*). This finding is consistent with the GSEA analysis result of TCGA database using bulk RNA sequencing data. Western blot analysis revealed that treatment with MFGE8 recombinant protein resulted in increased phosphorylation of AKT and STAT3,

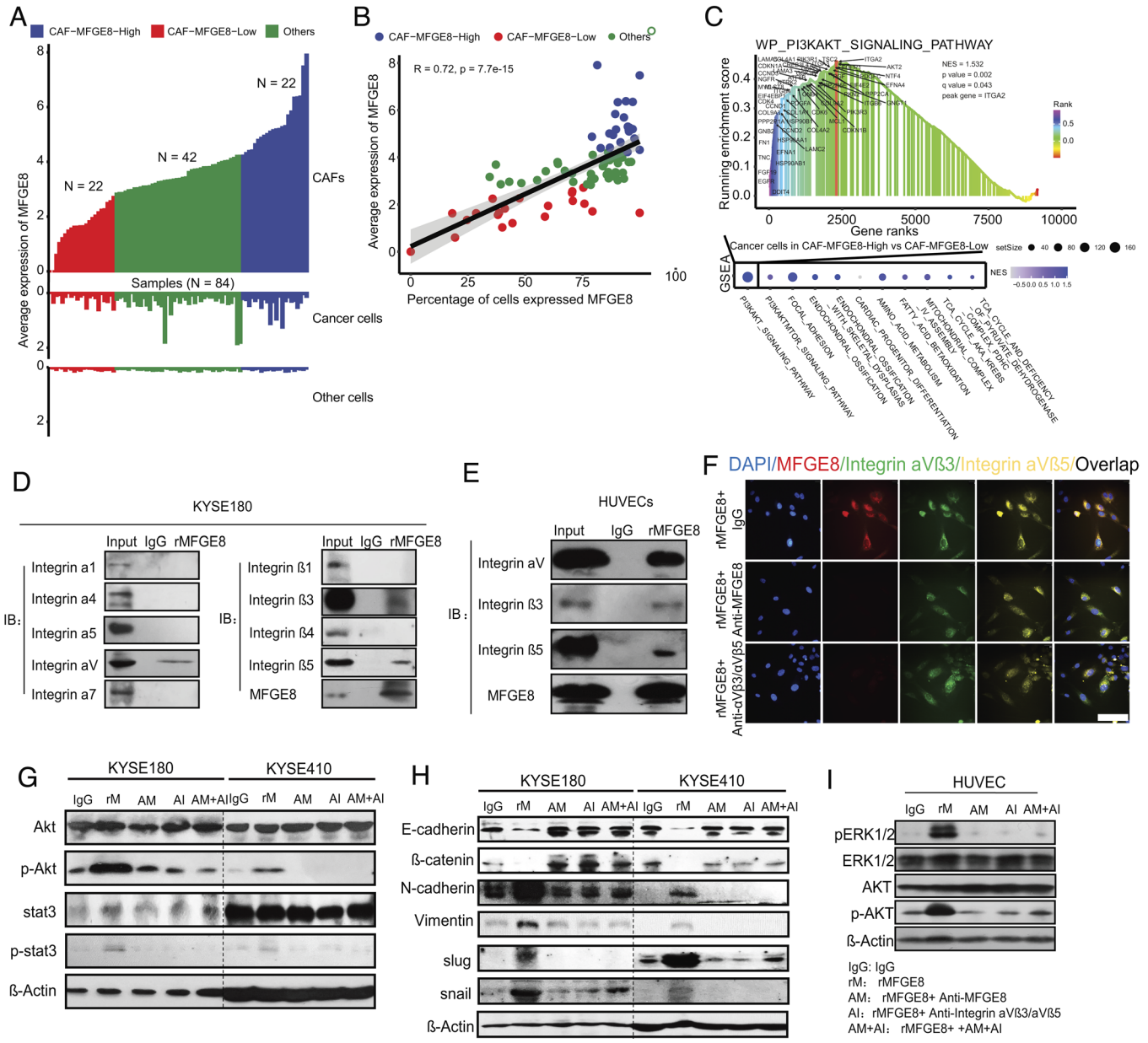

Fig. 2. In ESCC cell lines and HUVECs, MFGE8 binds to Integrin αVβ3/αVβ5 receptors, activating downstream pathways. (*A*) Average expression of MFGE8 in cancer cells in 84 single-cell sequencing data of ESCC. (*B*) The expression of MFGE8 is positively correlated with the percentage of cells expressed in each sample. (*C*) GSEA analysis showed that high expression of MFGE8 was significantly related to the "PI3K/AKT pathway" in single-cell analysis. (*D*) Cell lysates prepared from K180 cell line were subjected to immunoprecipitation (IP) with MFGE8 antibody or control immunoglobulin G (IgG) and then immunoblotted with antibody from Integrin families. rMFGE8: recombinant MFGE8. (*E*) Cell lysates prepared from K180 cell line were subjected to immunoprecipitation (IP) with MFGE8 antibody or control immunoglobulin G (IgG) and then immunoblotted with Integrin αV, Integrin β3, Integrin β5 antibody. rMFGE8: recombinant MFGE8. (*F*) Representative IF staining of MFGE8, Integrin αVβ3, and Integrin αVβ5 in HUVECs cells treated with recombinant protein MFGE8, recombinant protein MFGE8 + anti-MFGE8, or recombinant protein MFGE8 +anti- Integrin αVβ3/αVβ5. Nuclei were stained with DAPI. (Scale bar, 200 μm.) (*G*) Western blotting of AKT, p-AKT, STAT3, and p-STAT3 in K180 and K410 ESCC cell lines treated with PBS, recombinant protein MFGE8, anti-MFGE8, or anti-Integrin αVβ3/αVβ5. β-Actin was used as a loading control. (*H*) Western blotting of E-cadherin, β-cadherin, N-cadherin, vimentin, slug, and snail in K180 and K410 ESCC cell lines treated with PBS, recombinant protein MFGE8, anti-MFGE8, or anti- Integrin αVβ3/αVβ5. β-actin was used as a loading control. (*I*) Western blotting of ERK1/2, p-ERK1/2, AKT, and p-AKT in HUVECs in HUVECs treated with PBS, recombinant protein MFGE8, anti-MFGE8, or anti- Integrin αVβ3/αVβ5. β-actin was used as a loading control. Statistical significances: **P* < 0.05; ***P* <0.01; ****P* < 0.001.

indicating activation of the PI3K/AKT/STAT3 pathway in ESCC cell lines (*SI Appendix*, Fig. S4*C*). Based on the ability of secreted MFGE8 to activate intracellular AKT/STAT3 phosphorylation, we next investigated the receptors on the cell membrane that bind with MFGE8. GSEA analysis showed that upregulation of MFGE8 was highly correlated with integrin cell surface interactions (*SI Appendix*, Fig. S4*D*). CO-Immunoprecipitation (CO-IP) verified the binding between MFGE8 and integrin αV, integrin β3, and integrin β5 (Fig. 2*D*). Binding of MFGE8 to integrin αV, integrin β3, and integrin β5 was also validated in HUVECs by CO-IP (Fig. 2*E*). Moreover, neutralizing antibody against MFGE8 or integrin αVβ3/αVβ5 could effectively block the binding of MFGE8 recombinant protein to integrin αVβ3/αVβ5 in the IF result of HUVECs (Fig. 2*F*). Western blot confirmed that the activation of the PI3K/AKT pathway induced by MFGE8 could be blocked by neutralizing antibodies against MFGE8 or integrin αVβ3/αVβ5 (Fig. 2*G*). As expected, this neutralizing antibody blockade effectively suppressed the MFGE8-induced EMT in ESCC cell lines (Fig. 2*H*). In

HUVEC cells, the ERK/AKT pathway activated by MFGE8 could be also inhibited by neutralizing antibodies against to MFGE8 or Integrin αVβ3/αVβ5 (Fig. 2*I*).

Targeting of MFGE8 by Neutralizing Antibodies Blocks Its Carcinogenesis Effects. Next, we investigated the therapeutic potential of neutralizing antibodies against MFGE8 or its receptors in ESCC cell lines and HUVECs. Cell proliferation, invasion, drug resistance, and angiogenesis-related functional assays were performed using cells treated with MFGE8 recombinant protein alone or containing excess neutralizing antibodies for 3 d (Fig. 3*A*). Intriguingly, MFGE8 or Integrin αVβ3/αVβ5 neutralizing antibodies could effectively block the promotion effects of MFGE8 recombinant protein on cell proliferation (Fig. 3*B*), chemoresistance (Fig. 3*C*), and cell migration and invasion (Fig. 3*D*). In addition, the neutralizing antibodies also inhibited MFGE8 induced EMT, including upregulation of E-cadherin and downregulation of vimentin and snail (Fig. 3*E*). Likewise, MFGE8 or Integrin αVβ3/αVβ5 blockade completely abolished the angiogenesis-promoting effect of MFGE8 in HUVECs (Fig. 3 *F*–*H*), suggesting that MFGE8-mediated angiogenesis mainly depends on the interaction between MFGE8 and integrin. As part of our inquiry, we scrutinized the involvement of MFGE8 in the functional medium of CAFs. To this end, a series of experimental assays were conducted within the conditional media of CAFs, wherein neutralizing antibodies targeting MFGE8 or its receptors were utilized. The objective of these assays was to evaluate the effects of MFGE8 blockade on a range of cellular processes, including apoptosis, transwell migration, and HUVECs tube formation. According to the results, the inhibition of MFGE8 or its receptor was found to effectively counteract the MFGE8-mediated promotion of drug resistance (*SI Appendix,* Fig. S5*A*), metastasis (*SI Appendix,* Fig. S5*B*), and angiogenic potential (*SI Appendix,* Fig. S5*C*). The data suggest that anti-MFGE8 or Integrin has strong therapeutic potential by inhibiting MFGE8-promoting cell proliferation, metastasis, and angiogenesis.

Antibodies against MFGE8 and Its Receptors Inhibits Tumor Growth In Vivo. To evaluate the potential of an MFGE8$^+$ CAF-targeting strategy to blocking the tumor growth in ESCC, anti-MFGE8 and anti-Integrin αVβ3/αVβ5 neutralizing antibodies were applied alone or in combination with cisplatin to explore their antitumor effects in the xenograft model. Subcutaneous tumor formation was induced by ESCC cell line KYSE180 in BABL/C mice, and a formed tumor was uniformly cut into 1 mm^3 size and subcutaneously implanted into different mice. Mice received drug treatment 4 d after implantation (Fig. 4*A*). Tested mice were divided into four groups, which were treated with IgG, cisplatin, anti-MFGE8 antibody, and anti-MFGE8 antibody plus cisplatin, respectively. The results showed that cisplatin or MFGE8 antibody alone significantly reduced tumor volume and mass compared to IgG controls, while the combination of cisplatin and MFGE8 antibody further reduced tumor volume and mass (Fig. 4*B*). We next tested four treatments in mice in the same manner: IgG, cisplatin, Anti-Integrin αVβ3/αVβ5, and cisplatin combined with Anti-Integrin αVβ3/αVβ5. The results showed that cisplatin or Anti-Integrin αVβ3/αVβ5 alone had a significant therapeutic effect in tumor-burdened mice compared to the IgG group, while the combination of cisplatin and Anti-Integrin αVβ3/αVβ5 shrank the tumor volume and mass more significantly (Fig. 4*C*).

Next, we investigated the combined treatment of Anti-MFGE8 and Anti-Integrin. It was found that the tumors grew faster with larger mass in mice given MFGE8 intraperitoneally compared to the IgG group. The results of Anti-MFGE8 or Anti-Integrin monotherapy remained consistent with those shown in Fig. 4 *B* and *C*. However, there were no significant differences in tumor size and mass between the combination treatment group and the single-drug groups (Fig. 4*D*). To investigate the therapeutic effect of the same treatment regimen in combination with cisplatin on ESCC, we expanded the dose in mice and performed the same treatment experiments on KYSE180 and KYSE410 subcutaneously tumor-forming mice. The results showed that the recombinant protein MGFE8 was resistant to cisplatin treatment. Consistent with previous studies, the combination of Anti-MFGE8 or Anti-Integrin with cisplatin was more effective in slowing tumor growth. In addition, the combination of the three treatment regimens achieved the best efficacy compared to the other groups (Fig. 4 *E* and *F*).

Decreasing in tumor volume coincides with reduction in PCNA, CD31, and staining levels. Consistent with these biological effects, we observed inhibition of phosphorylation levels of AKT and STAT3 in the monotherapy or drug combination groups (Fig. 4*G*). Notably, anti-MFGE8 and anti-Integrin αVβ3/αVβ5 treatments showed a high degree of safety in mouse tumor models, as anti-MFGE8 and anti-Integrin prefer to bind to tumor tissue rather than normal tissue such as the heart, liver, stomach, spleen, lung, and kidney (*SI Appendix,* Fig. S6 *A* and *B*). Furthermore, there are no significant histologic changes observed in the above normal vital organs upon treatment with neutralizing antibodies (*SI Appendix,* Fig. S6*C*). During the experiment, the animals receiving the combination treatment did not show any signs of toxicity (infection, diarrhea, damage to vital organs, or loss of body weight) (*SI Appendix,* Fig. S7).

To further confirm the role of CAFs-secreted MFGE8 in promoting tumor growth, we conducted a coinjection study of ESCC cells (K180 and K410) and CAFs in mice with neutralizing antibody treatment to monitor tumor growth (*SI Appendix,* Fig. S8*A*). The results showed that anti-MFGE8 and anti-Integrin effectively suppressed tumor growth in mice coinjected with both K180 and CAFs, and the combined treatment showed no significant difference in tumor size and mass compared to the groups treated with only anti-MFGE8 or anti-Integrin (*SI Appendix,* Fig. S8 *B*–*D*). The results of treatment experiments in mice coinjected with K410 and CAFs were consistent with those of K180 and CAFs (*SI Appendix,* Fig. S8 *E*–*G*). Collectively, CAFs-secreted MFGE8 was found to significantly promote tumor growth in vivo, while inhibition of MFGE8 and its receptor resulted in suppression of this growth-promoting effect. Notably, a combination of cisplatin and neutralizing antibodies exhibited the most potent efficacy in vivo.

Blocking MFGE8 and Its Receptor Substantially Suppresses Tumor Metastasis in ESCC. We have demonstrated the inhibitory effects of neutralizing MFGE8 or Integrin αVβ3/αVβ5 on ESCC migration triggered by MFGE8 in vitro. The tail vein injection lung metastasis mouse model (Fig. 5*A*) and hock injection lymph node metastasis mouse model (Fig. 5*B*) were used to investigate the in vivo effects of blocking MFGE8 or Integrin αVβ3/αVβ5 on MFGE8-induced ESCC metastasis. In the lung metastasis mouse model, MFGE8 recombinant protein–induced lung metastasis could be effectively inhibited by anti-MFGE8 and anti-Integrin αVβ3/αVβ5 monotherapy or combination therapy. However, there was no significant difference between the monotherapy and combination therapy groups (Fig. 5*C*). Similar results were obtained in the lymph node metastasis model (Fig. 5*D* and *SI Appendix,* Fig. S9), suggesting that targeting MFGE8 secreted by CAFs or its tumor cell surface receptor could effectively inhibit tumor metastasis in vivo. In particular, during the treatment of tumor metastasis with neutralizing antibodies targeting MFGE8 and integrin αVβ3/αVβ5, the behavior and body weight of mice

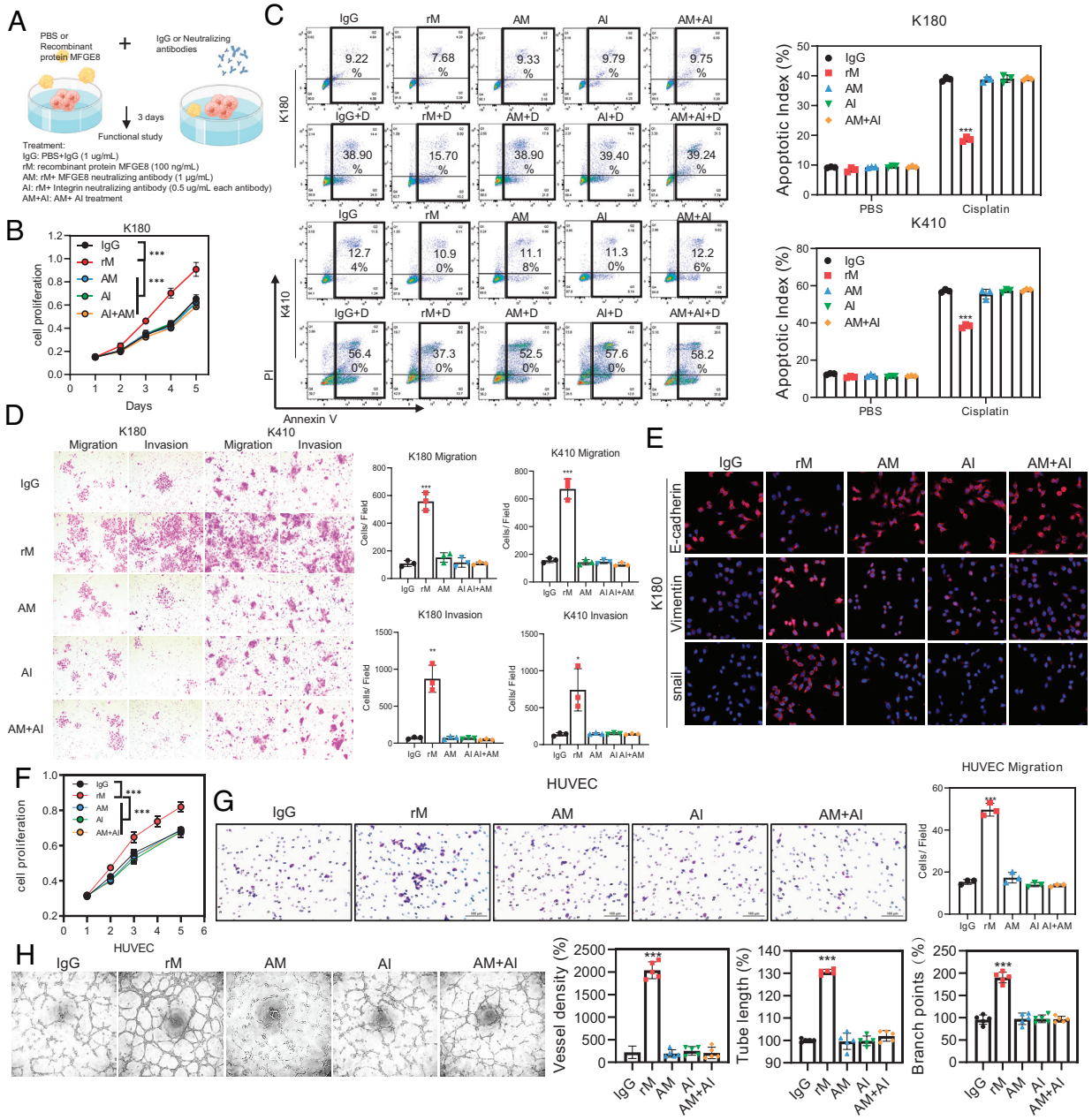

Fig. 3. Functional effects of anti-MFGE8 or Anti-Integrin αVβ3/αVβ5 receptors and combination therapy in ESCC cell lines or HUVECs. (*A*) Schematic of the drug intervention protocol for anti-MFGE8 and/or anti-αVβ3/αVβ5 in ESCC cell lines or HUVECs. The figure was created with BioRender.com. (*B*) The XTT assay showed that the proproliferative ability of recombinant protein MFGE8 (500 ng/mL) was blocked by anti-MFGE8 or Anti-Integrin αVβ3/αVβ5 in K180 and K410 ESCC cell lines. (*C*) Representative flow cytometry plots (*Left*) and summaries (*Right*) of a percentage of apoptotic cells in K180 and K410 ESCC cell lines treated with PBS, recombinant protein MFGE8, anti-MFGE8, or anti-Integrin αVβ3/αVβ5. (*D*) Representative images of migration and invasion assays (*Upper*) and their summaries (*Lower*) in K180 and K410 ESCC cell lines treated with PBS, recombinant protein MFGE8, anti-MFGE8, or anti-Integrin αVβ3/αVβ5. (*E*) Representative IF staining of protein E-cadherin, vimentin, and snail in K180 and K410 ESCC cell lines treated with PBS, recombinant protein MFGE8, anti-MFGE8, or anti-Integrin αVβ3/αVβ5. (*F*) The XTT assay showed that the proproliferative ability of recombinant protein MFGE8 was blocked by anti-MFGE8 or Anti-Integrin αVβ3/αVβ5 in HUVECs. (*G*) Representative images of migration assays (*Left*) and their summaries (*Right*) showed that the prometastatic ability of recombinant protein MFGE8 was blocked by anti-MFGE8 or Anti-Integrin αVβ3/αVβ5 in HUVECs. (*H*) Representative images of tube formation (*Left*) and the statistical result (*Right*) indicated that the proangiogenesis ability of recombinant protein MFGE8 was blocked by anti-MFGE8 or Anti-Integrin αVβ3/αVβ5 in HUVECs. Statistical significances: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

were not abnormal, indicating that MFGE8 or Integrin αVβ3/αVβ5 neutralizing antibody treatment provides a safe and effective strategy for ESCC treatment (*SI Appendix,* Fig. S10).

Discussion

A growing number of evidence suggests that cancer is a wound that never heals (21). Similar to normal wound healing, cancer development and progression require the participation of many cell types. Host cells, including fibroblasts, endothelial cells, and immune cells, are mobilized to cancer microenvironments in order to modulate a range of critical processes, including cancer cell survival, proliferation, invasion, metastasis, angiogenesis, cancer stem cell maintenance, and, ultimately, therapeutic response (22). CAFs, stromal cells, are one of the most important members of the TME, with different origins and multiple phenotypes (23). Activated CAFs can promote tumor development, angiogenesis, invasion, and metastasis, ECM remodeling, and chemoresistance

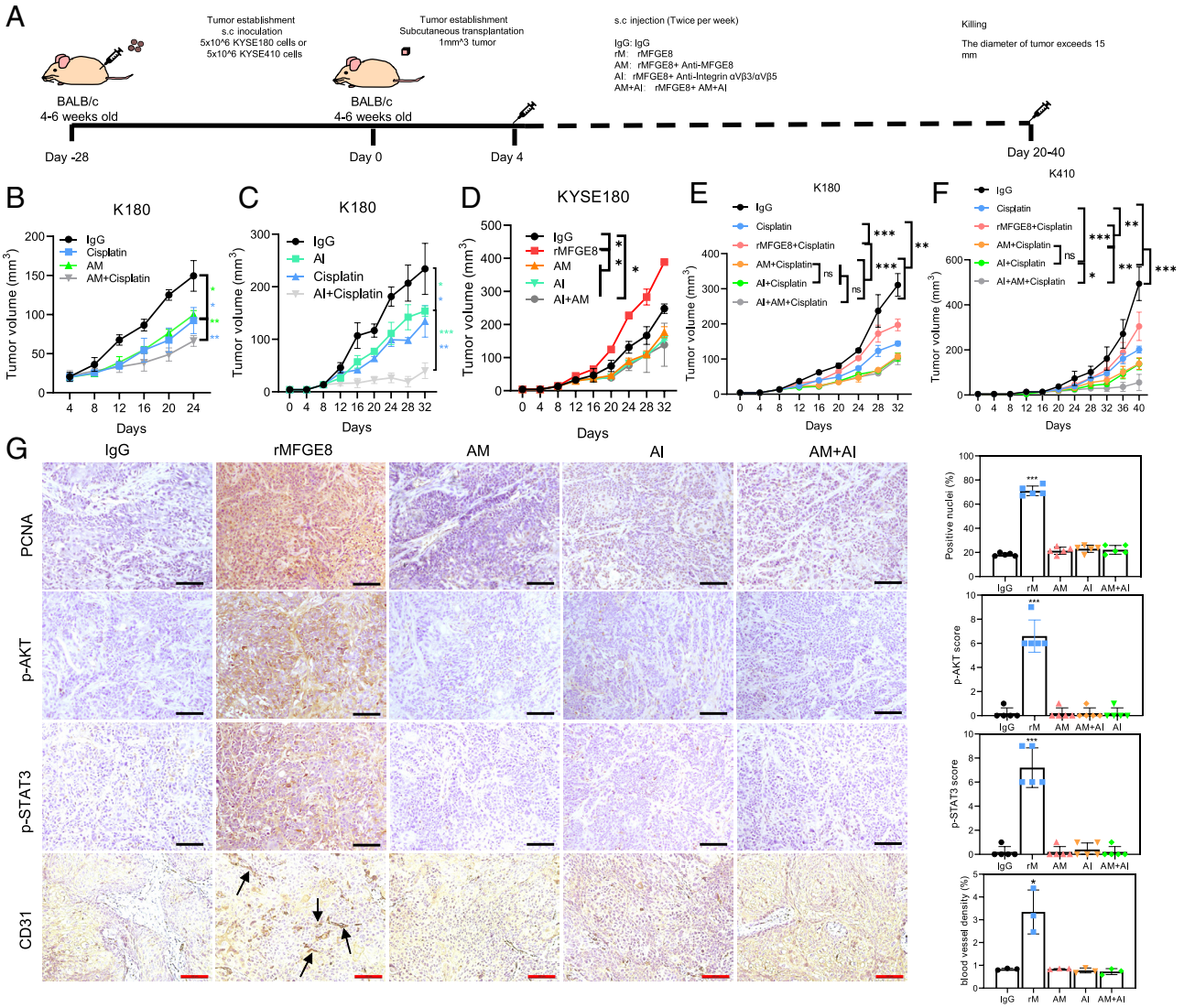

Fig. 4. Targeting MFGE8 and its receptor is effective in reducing the size of ESCC tumors. (*A*) Schematic of the drug intervention protocol for anti-MFGE8 (intraperitoneally) and/or anti-αVβ3/αVβ5 (intraperitoneally) in nude mice subcutaneously implanted with 1 mm3 tumors from K410 or K180 formed tumor. rMFGE8: recombinant MFGE8. (*B–F*) Mice burdened with K180 or K410 formed tumor were treated with IgG, cisplatin, recombinant protein MFGE8 (rMFGE8), anti-MFGE8 antibody (AM), or anti-Integrin αVβ3/αVβ5 antibody (AI). The tumor volumes were monitored for 20-40 days. rMFGE8: recombinant MFGE8. (*G*) Representative IHC staining of protein PCNA, p-AKT, p-STAT3, and CD31 in K180 formed tumor treated with PBS, recombinant protein MFGE8, anti-MFGE8, or anti- Integrin αVβ3/αVβ5. Statistics of protein expression are shown in the *Right*. Statistical significances: **P* < 0.05; ***P* <0.01; ****P* < 0.001.

through activating a variety of signaling (24). The interaction of CAFs and other cells in TME plays a critical role in tumor growth. CAFs interact with those cells by secreting cytokines, growth factors, chemokine, exosome, and other effector chemicals, consequently, generating an aggressive TME that helps tumor grow and invasion.

In the present study, our findings suggest that CAFs exert a pivotal influence on ESCC development by activating the PI3K/AKT pathway and facilitating cell metastasis, proliferation, angiogenesis, and ECM remodeling via their secretome. RNA-seq analysis identified differentially expressed genes related to cell–cell contact, including cell adhesion, angiogenesis, extracellular matrix regulation, and receptor binding activity. The study also highlighted MFGE8 as a potential pivotal gene in oncogenic CAFs and confirmed its high expression in CAFs in tumor tissue through various techniques. These findings align with previous reports on the role of CAFs in tumors.

There are a number of biological processes where MFGE8 plays a key and nonredundant role, including apoptotic cell clearance (24), angiogenesis (25), and adaptive immunity (26). In cellular clearance, MFG-E8 specifically targets the phosphatidylserine found on apoptotic cells, which ensures that the macrophages can engulf apoptotic cells. Another important function of MFGE8 is proangiogenesis. Previous studies have demonstrated its role in combining with integrin αVβ3/αVβ5 to activate proliferation of vascular smooth muscle cells via up-regulating phosphorylation of ERK1/2 (27). In melanoma cells, knocking down MFGE8 was found to inhibit AKT and twist signaling, thereby compromising tumor cell survival, EMT, and invasion (13). In addition, MFGE8-deficient melanoma was more sensitive to small molecule inhibitors (28). MFGE8 has also been reported to be associated with the ability to promote tumor invasion and metastasis within triple-negative breast and ovarian cancers (29, 30). So far as we know, the role of MFGE8 in ESCC is rarely studied. In this study, we found that MFGE8 is specifically produced and secreted by CAFs in ESCC, which promotes tumor angiogenesis, tumor proliferation, drug resistance, and metastasis. MFGE8 promotes related protumor progressive functions by

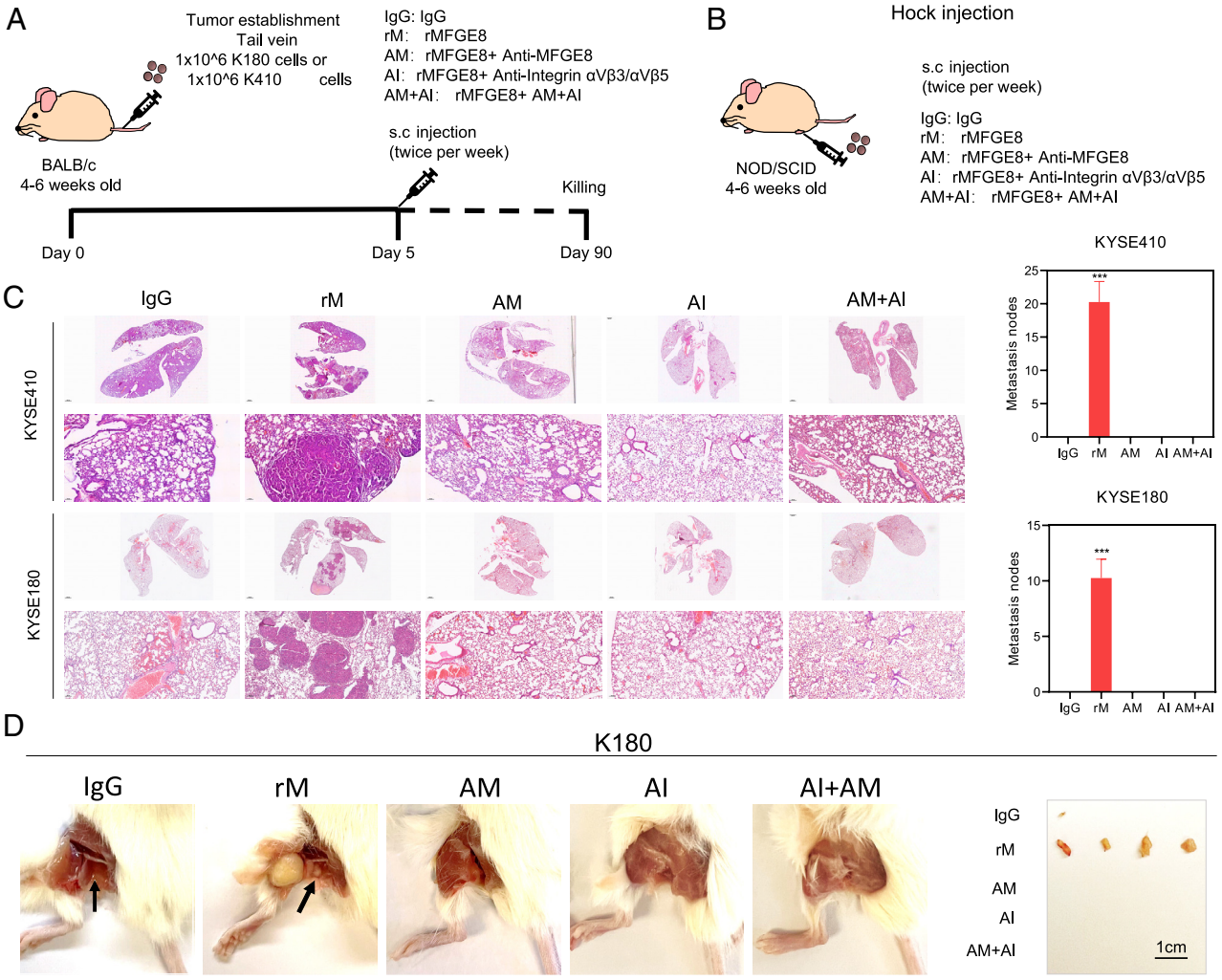

Fig. 5. CAF-secreted MFG-E8 facilitates tumor progression. (*A*) Schematic of the drug intervention protocol for anti-MFGE8, anti-αVβ3/αVβ5, and/or cisplatin in the NOD SCID mice model. (*B*) Schematic of the drug intervention protocol for anti-MFGE8, anti-αVβ3/αVβ5, and/or cisplatin in the NOD SCID mice model. (*C*) Representative HE images (*Left*) of the tail vein mice model treated with PBS, MFGE8 recombinant protein, anti-MFGE8 antibody, or anti-Integrin αVβ3/αVβ5 antibody. Statistics of lung metastasis nodes are shown in the *Right*. (*D*) Mice burden with hock injected tumor and lymph node metastasis are shown in the *Left*, and tumors in each indicated group are shown in the *Right*.

binding to receptors integrin αVβ3/αVβ5 in tumor cells or vascular endothelial cells and activating the PI3K/AKT pathway and ERK/AKT pathway, respectively, which presented a high degree of consistency with both the role and manner of CAFs in regulating the tumor environment.

For the mechanism part, MFGE8 has been mainly reported to bind with integrins to induce the activation of downstream pathways, including the TIGβ5/PI3K/CXCL12 axis (31), AKT signaling (32), twist signaling (13), or ERK signaling (33). Interestingly, our data revealed that the regulation of MFGE8-related functions in both vascular endothelial cells and ESCC cells is mediated by binding with Integrin αVβ3/αVβ5 receptors. Furthermore, the downstream AKT/STAT3 pathway and ERK/AKT signaling were activated by the stimulation of MFGE8 in vascular endothelial cells and tumor cells.

Next, we tested the therapeutic potential by blocking the interaction of MFGE8 and its receptors. This MFGE8 blockade has been performed in several in vitro functional experiments in many diseases other than cancer. For example, MFGE8-blocking antibody reduces phagocytic and macrophage activity (34). In acute pancreatitis, the effect of MFGE8 could be abrogated by cilengitide, a selective integrin αVβ3/αVβ5 inhibitor (35). In the current investigation, the interaction between MFGE8 and integrin αVβ3/αVβ5 was disrupted through the application of neutralizing antibodies, and their impact was assessed via in vitro and in vivo experimentation. Our findings indicate that the employment of MFGE8-neutralizing antibodies effectively impedes MFGE8-mediated processes such as cell proliferation, drug resistance, metastasis, and angiogenesis. In particular, we studied the therapeutic effects of blocking the interaction of MFGE8 and its receptors in three animal models: subcutaneous tumor formation mouse model, lung metastasis mouse model, and lymph node metastasis mouse model. The findings of this study suggest that interrupting the binding between MFGE8 and its receptors can effectively impede the tumorigenesis and metastasis of ESCC. Moreover, the combination of such intervention with chemotherapeutic drugs exhibits a synergistic effect, indicating a promising therapeutic target for the treatment of ESCC.

Materials and Methods

Additional details about the experimental procedures, including descriptions of in vitro functional assays, coimmunoprecipitation, IF staining, and western blotting, are provided in *SI Appendix, Materials and Methods*.

Statement. The study was approved by the ethical committee at the University of Hong Kong-Shenzhen Hospital and the Committee on the Use of Live Animals in Teaching and Research (CULATR) at the University of Hong Kong. Written informed consent was obtained for the original human work that produced the tissue samples. Animal experiments were under the obligation of the CULATR at the University of Hong Kong and Shenzhen TopBiotech Co., Ltd. SPF Laboratory Animal Center.

Data, Materials, and Software Availability. Previously published data were used for this work. The RNA data reported in this paper have been deposited in Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE103111) (36). Public scRNA-seq data were downloaded (16), GSE160269 (17), GSE145370 (18) and PRJNA777911 (19). All other data are included in the article and/or *SI Appendix*.

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