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Original Research

Fibroblastic FAP promotes intrahepatic cholangiocarcinoma growth via MDSCs recruitment

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Abstract

Desmoplasia is a hallmark of intrahepatic cholangiocarcinoma (ICC), which constitutes a barrier to infiltration of lymphocyte, but not myeloid cells. Given that dense desmoplastic stroma has been reported to be a barrier to infiltration of lymphocyte, but not myeloid cells. We here investigated whether fibroblastic FAP influenced ICC progression via nonT cellrelated immune mechanisms. We demonstrated fibroblastic FAP expression was critical for STAT3 activation and CCL2 production, and ICCCAFs were the primary source of CCL2 in human ICC microenvironment by using ICCFbs from six ICC patients. Fibroblastic knockdown of FAP significantly impaired the ability of ICCCAFs to promote ICC growth, MDSCs infiltration and angiogenesis, which was restored by adding exogenous CCL2. Furthermore, interestingly, the tumorpromoting effect of fibroblastic FAP is dependent on MDSCs via secretion of CCL2, as depletion of $Gr1^+$ cells reversed the restoring effects of exogenous CCL2 on tumor growth and angiogenesis. In vitro migration assay confirmed that exogenous CCL2 could rescue the impaired ability of ICCFbs to attract $Gr1^+$ cells caused by fibroblastic FAP knockdown. In contrast, fibroblastic FAP knockdown had no effect on ICC cell proliferation and apoptotic resistance. Depletion MDSCs by antiGr1 monoclonal antibody in subcutaneous transplanted tumor model abrogated tumor promotion by ICCCAFs suggested that the protumor function of Fibroblastic FAP relied on MDSCs. Mechanical, flow cytometry and chamber migration assay were conducted to find Fibroblastic FAP was required by the ability of ICCCAFs to promote MDSC migration directly. Moreover, fibroblastic FAP knockdown had no effect on cell proliferation and apoptotic resistance. Here, we revealed the Tcell independent mechanisms underlying the ICCpromoting effect of fibroblastic FAP by attracting MDSCs via CCL2, which was mainly attributed to the ability of FAP to attract MDSCs and suggests that specific targeting fibroblastic FAP may represent a promising therapeutic strategy against ICC.

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Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common type of primary liver cancer with a very poor prognosis [1]. The incidence and mortality rates of ICC are recently increasing worldwide, however, the

Abbreviations: CAFs, cancerassociated fibroblasts, EMT, epithelial–mesenchymal transition, FAP, fibroblast activation protein, MDSCs, myeloid derived suppression cells, Fbs, fibroblasts, ICC, intrahepatic cholangiocarcinoma, MVD, microvessel density, TAMs, tumorassociated macrophages

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cellular and molecular pathogenesis of ICC is poorly understood [2,3]. Dense desmoplastic stroma has long been considered to be a hallmark histological feature of ICC, although ICC is heterogeneous in many aspects such as cellular and molecular phenotypes and genomic differences [4–6]. The desmoplastic stroma of ICC is characterized by the enrichment of activated myofibroblasts that express αSMA, which is also a common marker for cancerassociated fibroblasts (CAFs) [4,5]. Clinical studies suggest that desmoplastic stroma is associated with ICC prognosis [2,7]. For example, abundance in the fibrous stroma and the higher levels of αSMA in ICC patients with resected tumor were reported to negatively correlate with the clinic prognosis of ICC [7]. Moreover, increasing evidence demonstrates that fibroblasts contribute to the progression of ICC by influencing tumor cell biology [8–11].

αSMA$^+$CAFs have been considered to be heterogeneous with phenotypically distinct subpopulations, for example, ICC patients with CAFs expressing a mucinlike transmembrane glycoprotein have increased lymph node metastasis and worse clinic outcome [12,13]. Fibroblast activation protein (FAP), a serine protease, is selectively expressed on CAFs in many human solid tumor [14,15]. Accumulating data demonstrate that targeting FAP genetically, or with vaccines or pharmacological agents including inhibitor and CART cells [16–18], as well as targeting FAPexpressing cells [19,20], could impair tumor progression in several mouse models via different mechanisms. Our previous study using tumor model of immunocompetent mouse demonstrated that FAP is responsible for the inflammatory phenotype of CAFs by activating STAT3CCL2 signaling, which mediates tumor recruitment of myeloidderived suppressive cells (MDSCs), thereby antagonizing antitumor T cell immunity, leading to tumor growth [21]. Importantly, we demonstrated the adverse predictive role of stromal FAP levels in human ICC by immunohistochemical staining of ICC tissue microarrays, suggesting that FAP$^+$CAFs may contribute to ICC growth [21].

Infiltration of immature myeloid cells is one of the hallmarks of cancer [22]. Tumorassociated macrophages (TAMs) and MDSCs are two major myeloid cell types found in solid tumors [22,23]. CD163$^+$ TAMs with a M2 phenotype have been implicated in ICC progression, predicting a poor clinical outcome [24]. In addition to their immunosuppressive function, TAMs and MDSC aggravate tumor progression via many other mechanisms including the promotion of angiogenesis and epithelialmesenchymal transition (EMT) [25–27]. Thus, the understanding of their functions of myeloid cells in addition to their immunosuppressive function would be helpful to develop a more specific anticancer strategy targeting the patients with weak immune system.

In the present study, we aimed to examine whether and how FAP expression in ICC patients derived fibroblasts promoted ICC tumor growth in immunodeficient situations by using nude mice. We confirmed the FAP was necessary for inflammatory phenotype of ICCCAFs. On the mechanism, we found that fibroblastic FAP contributed to CAFmediated tumor growth by promoting tumor cell proliferation and angiogenesis, which relay on CCL2MDSCs axis.

Materials and methods

Mice and cell lines

Male nude mice were purchased from the Chinese Academy of Sciences (Shanghai, China). All mice were kept and bred in a specific pathogenfree environment in the animal facility of Shanghai Medical College, Fudan University, and all animal experiments were approved by the Animal Care and Use Committee at Fudan University, Shanghai, China. ICC cell lines of QBC939, HCCC9810, and RBE were obtained from Liver Cancer Institute, Zhongshan Hospital, Fudan University [28]. All cell lines were tested to exclude mycoplasma contamination before experiments.

Human samples

Freshly resected tumor, noncancerous liver tissues and blood samples from ICC patients were obtained from Liver Cancer Institute, Zhongshan Hospital between July 2014 and March 2016. The study protocol was approved by the Institute Review Board of Zhongshan Hospital, Fudan University.

The isolation of human fibroblasts

Fibroblasts were isolated as previously described [21]. Briefly, tumor or noncancerous liver tissues resected from ICC patients were digested in 1 mg/ml collagenase IV (Sigma) at 37 C for 1 h. Fibroblasts were enriched by antihuman Fibroblast Microbeads (#130050601, Miltenyi Biotec) and cultured in complete DMEM.

Lentivirus vector preparation and fibroblast transduction

The sequences for siRNA targeting *fap* (*fap1,* GCTTCAAATTACGGCTTAT, *fap2,* GCTCTCTGGTGGTCTC CTA, *fap3,* GGTGGATTCTTTGTTTCAA) were designed and synthesized by GenePharma (GenePharma). Western blotting data showed that siRNA *fap3* sequence was most efficient in inhibiting FAP expression. Thus, siRNA *fap3* was used for FAP knockdown. Shorthairpin RNA (shRNA) specifically targeting human FAP was constructed in lentiviral vector pSIH1H1copGFP vector (System Biosciences). Shorthairpin RNA (shRNA) specifically targeting scramble siRNA (GGTGCATTCTATGTATCAA) was constructed in lentiviral vector pSIH1H1copGFP vector as the control vector. Lentivirus was generated and transduced vectors into ICCCAFs according to the manufacturer's protocol to generate Control CAF or FAP^{kd}CAF.

Coimmunoprecipitation and Western blotting

For coimmunoprecipitation (CoIP), fap gene (NM_007986.2) was cloned into pCMVTag2Bflag vector, and *Plaur* gene (NM_011113.3) into PCDNA3.1HA vector. Primary antibodies against uPAR (R&D Systems), FAP (Abgent), HA (Santa Cruz Biotechnology) were used. The rabbit normal IgG antibodies (Santa Cruz Biotechnology) were added as a control. AntiFLAG M2 Affinity Gel (Sigma) was used for immunoprecipitation. For Western blotting, Protein concentration was measured by using BCA protein assay kit (Pierce). 20 g protein samples were used and transferred onto polyvinylidene fluoride (PVDF) transfer membrane (Millipore). The membrane was blocked with 5% blotting grade milk powder in TBST (50 mM TrisHCl, 0.15 M NaCl, 0.1% Tween20, pH: 7.4). The Western blotting was performed as previously described [21]. The antibodies were shown in Supporting Table S1.

Gr-1$^+$ cells depletion

Gr1$^+$ Cells were depleted by 100 g antiGr1 monoclonal antibody (αGr1, clone RB68C5) (Bioxcell) via intravenous injection 24 hr before tumor cells challenge for every 3 days.

RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA was isolated by TRIzol (Invitrogen) and reversetranscribed into cDNA by PrimeScript RT Master Mix (TaKaRa). qRTPCR was performed by Applied Biosystems 7500 using Power SYBR Green Master

kit (TaKaRa). The relative expression of target gene was calculated using the $2C_{(t)}$ method. Fold induction of target gene expression were calculated by normalization to control group. The primer sequences of all genes for PCR are shown in Supporting Table S3.

Enzyme-linked immunosorbent assay (ELISA)

ICCCAFs or FAP^{kd}ICCCAFs were cultured for 24 h, and the supernatants were collected. CCL2 concentrations were measured using a human CCL2 ELISA Kit (R&D Systems).

Subcutaneous transplanted tumor model

Fibroblasts derived from ICC tissues (ICCCAFs) or FAP^{kd}ICCCAFs (in which FAP was knockdown) ($2X10^5$ each) were subcutaneously coinjected with QBC939 cells ($1\ \ 10^6$) into nude mice. The tumor volume was calculated by the following formula: V = /6X (larger diameter) (smaller diameter)^2. CCL2 protein (5 g/mouse, Peprotech) or PBS as control were administered around the site of the tumor every other day starting from day 7.

Immunohistochemistry (IHC)

IHC staining of tumor tissue sections was performed using the avidinbiotinperoxidase complex method. Antibodies against CD31 (1:200, sc1506, Santa Cruz) or Ki67 (1:100, AF7689, R&D Systems) were used. The proliferation index was quantified according to the mean numbers of ki67$^+$ cells in five high power fields (HPF) [17]. For the evaluation of microvessel density (MVD), brownstained endothelial cell or endothelial cell cluster that clearly separated from tumor cells or stromal cells were counted as one microvessel [29].

Chemotaxis assay

Blood samples from ICC patients were obtained from Liver Cancer Institute, Zhongshan Hospital. CD14$^+$ cells were sorted by CD14$^+$ beads (Miltenyi Biotec). Ten thousand sorted CD14$^+$ cells were added to the upper chambers of Transwell (5 m, 24well format; Corning, USA). The supernatants from various fibroblasts were added to the lower chambers with or without CCL2 (100 ng/ml). Cells migrated to the lower chambers were counted by CyAn (Beckman Coulter) after 4 h incubation.

Flow cytometry

The tumor tissue was cut into pieces and digested by collagenase IV (1 mg/ml) for 1 hour at 37 C. Resuspended cells were blocked with Fc antibody first and then stained with antibody. Ly6G$^+$CD11b$^+$F4/80 cells were considered as PMNMDSCs. Ly6C$^+$CD11b$^+$F4/80 cells were considered as MoMDSCs. F4/80$^+$CD11b$^+$ cells were considered as tumorassociated macrophages. Sorted CD14$^+$ myeloid cells from peripheral blood of ICC patients were further confirmed by staining with antibodies as CD33$^+$CD14$^+$CD11b$^+$ cells. The following antibodies were shown in Supporting Table S2. Samples were acquired by FACS Cyan instrument and analyzed with Summit (Beckman Coulter).

Statistical analysis

The comparisons between two groups were performed by twotailed Student's *t*tests. Multiplegroup comparisons were performed by twoway ANOVA followed by a Bonferroni correction to compare each group. The 2 test or Fishers exact test was used for qualitative variables comparison. Statistical analyses were performed by Graph Prism V5.2. $p < 0.05$ was considered statistically significant.

Results

Fibroblasts derived from tumor tissues of ICC patients express FAP, which promotes STAT3 activation

We first attempted to confirm the FAP expression in fibroblasts derived from ICC tissues (ICCCAFs). Fibroblasts isolated from paired noncancerous liver tissues were used as control (CtrlFbs). ICCCAFs from six ICC patients each expressed FAP, while CtrlFbs expressed very little, if any, FAP by western blotting (Figure 1A). Immunofluorescence staining revealed extensive expression of αSMA in ICCCAFs, and only a subset of αSMA$^+$CAFs expressed FAP (Figure 1B), indicating that FAP$^+$CAFs may represent a phenotypically distinct subpopulation. Although FAP has a short intracellular domain, our previous study suggested that it interacts with uPAR in mouse hepatocellular cancer (HCC) CAFs [21]. Firstly, we examined that ICCCAFs expressed uPAR. Moreover, CoIP results showed that FAP interacts with uPAR in ICCCAFs, which suggesting that FAP may induce the intercellular signal in ICCCAFs through uPAR (Figure 1C). STAT3 was an important transcription factor for inducing inflammatory signals in CAFs [30]. To investigate whether FAP effected on the activation of STAT3 in CAFs, we specifically knocked down *FAP* in ICCCAFs by using FAP specific shRNA adenovirus (FAP^{kd}CAF). ICCCAFs transfected with vector contained scramble shRNA as control CAFs (CAF). The efficiency of *FAP* specific siRNA was confirmed by Western blotting (Figure 1D). Compared with CtrlFbs, ICCCAFs had marked increases in pSTAT3 protein levels, which was significantly reduced by knockdown of FAP (Figure 1A and E). Collectively, these data demonstrate that fibroblasts isolated from ICC patients express FAP, which is important for the activation of fibroblastic STAT3 inflammatory signaling.

Knockdown of FAP in ICC-CAFs inhibits tumor growth in a xenograft mouse model of human ICC

To further investigate whether FAP contributed to the ability of ICCCAFs to promote ICC growth in vivo, we established a xenograft model of human ICC by subcutaneously injecting QBC939 ICC cells alone or with ICCCAFs or FAP^{kd}ICCCAFs in the flanks of nude mice. Coinjection with ICCCAFs significantly promoted the volume and the weight of tumors, which was markedly reduced by specifically knockdown of FAP in ICCCAFs (Figure 2A and B). To evaluate the ICC cell proliferation rate, tumor sections were stained with antiKi67 antibodies. There were significantly less Ki67positive cells in tumors coinjected with FAP^{kd}-ICCCAFs than those with CAFs (Figure 2C), indicating that fibroblastic FAP is required for promoting tumor cell proliferation in vivo. We therefore analyzed MVD by staining CD31 and gene expression of several proangiogenic factors in tumors coinjected with CAFs or FAP^{kd}CAFs. FAP knockdown caused an obvious decrease in CD31positive staining, suggesting a less MVD in tumors (Figure 2C). Consistently, significantly decreased gene expression of angiogenic factors was detected in tumors coinjected with FAP^{kd}ICCCAFs (Figure 2D). Take together, FAP knockdown in ICCCAFs still could suppress tumor growth in nude mice that are deficient in T cells prompted us to propose that FAP may promote tumor growth via nonT cellrelated mechanism.

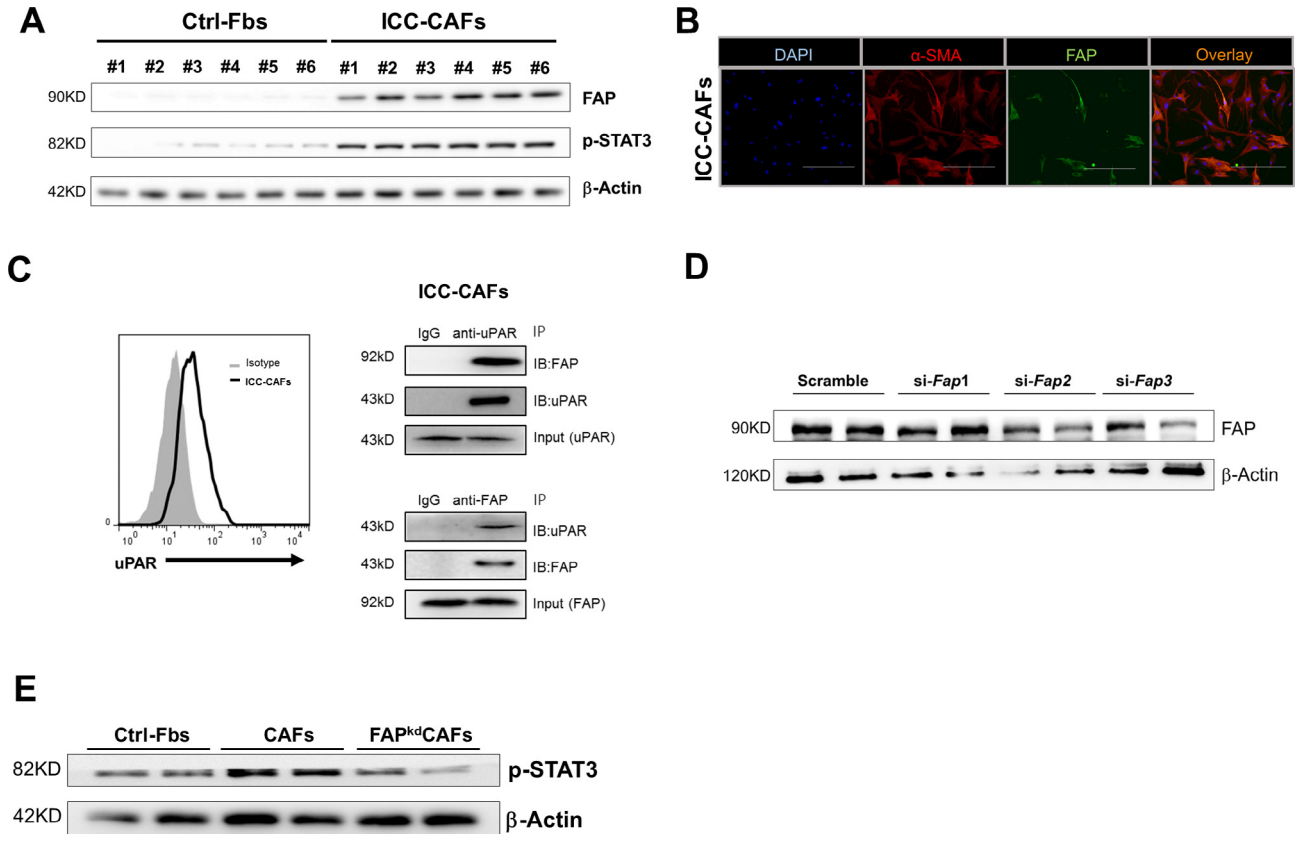

Figure 1. Fibroblasts derived from tumor tissues of ICC patients express FAP, which promotes STAT3 activation. (A) Western blots showing the protein levels of FAP and phosphorylated STAT3 (p-STAT3) in fibroblasts derived from ICC tissues (CAFs) and paired non-cancerous liver tissues (Ctrl-Fbs). (B) Representative images of immunofluorescence staining of cultured CAFs with α-SMA (red) and FAP (green) antibodies and counterstained with DAPI (blue) (400X magnification). The scale bars represent 200 M. (C) Representative flow cytometry analysis of uPAR expression and CoIP assays to analyze the interaction between FAP and uPAR in ICC tissue-derived fibroblasts. Normal rabbit IgG antibodies were served as a negative control. (D) Western blots showing the efficiency of FAP knockdown. (E) Western blots showing the protein levels of p-STAT3. Results are representative of at least three independent experiments.

Fibroblastic FAP does not directly promote ICC cells proliferation and apoptosis

Accumulating data showed that CAFs play a crucial role in accelerating the progression of ICC via the direct effect on the biology of tumor cells [5]. Our in vivo study suggested that fibroblastic FAP induced tumor cells proliferation. We then asked whether FAP contributed to the effect of ICCCAFs on the biology of ICC cells including proliferation and apoptosis directly. To this end, we incubated three ICC cell lines, QBC939, HCCC9810 and RBE, with supernatants from ICCCAFs or FAP^{kd}-ICCCAFs. Incubation with supernatants from ICCCAFs or FAP^{kd}-ICCCAFs did not affect the proliferation of all three ICC cell lines as determined by CCK8 and BrdU assay (Figure 3A and B). In contrast, incubation with supernatants from ICCCAFs significantly decreased the percentages of Annexin V^+ apoptotic cells in the culture of three ICC cell lines, which, however, was not affected by FAP knockdown (Figure 3C). These data suggest that ICCCAFs have no effect on the proliferation of ICC cells, but could promote apoptosis resistance of ICC cells in a FAPindependent manner.

CCL2 mediates ICC-promoting effect of fibroblastic FAP

As inflammatory CAFs in tumor microenvironment was important for tumor cell proliferation and angiogenesis, we examined the inflammatory associated genes regulated by STAT3 in ICCCAFs. Compared with CtrlFbs, ICCCAFs had marked increases in gene expression of STAT3 regulating inflammatory cytokines, particularly CCL2 (Figure 4A). Consistently, FAP knockdown significantly reduced the secretion of CCL2 production by ICCCAFs (Figure 4B). Moreover, we found that CAFs were the major source for CCL2 in ICC microenvironment, as evidenced by much higher levels of CCL2 gene expression in CAFs derived from 7 patients than those in tumor tissues from the same patients (Figure 4C). To determine whether CCL2 mediated the tumorpromoting effect of FAP in ICCCAFs, exogenous CCL2 was injected into the tumor coinjected with FAP^{kd}ICCCAFs. Addition of CCL2 could restore the reduction in tumor growth accompanied by increased tumor cell proliferation and angiogenesis (Figure 2A–D). These results together suggest that ICCpromoting effect of fibroblastic FAP is mediated by CCL2. In addition to their immunosuppressive functions, MDSCs are reported to promote tumor growth and metastasis by promoting angiogenesis [25,26], and MDSCs are reported to be the important source for angiogenic factors in the tumor microenvironment [26,31]. Flow cytometry results showed significantly increased frequencies of two subsets of MDSCs, PMNMDSCs and MMDSCs, as well as macrophages, were observed in tumors coinjected with ICCCAFs which was significantly reduced by specifically knockdown of fibroblastic FAP (Figure 4D). We then investigated how FAP expression in ICCCAFs influenced MDSCs. We first examined the chemotaxis of sorted myeloid cells from peripheral blood of ICC patients toward supernatants from ICCCAFs or FAP^{kd}ICCCAFs

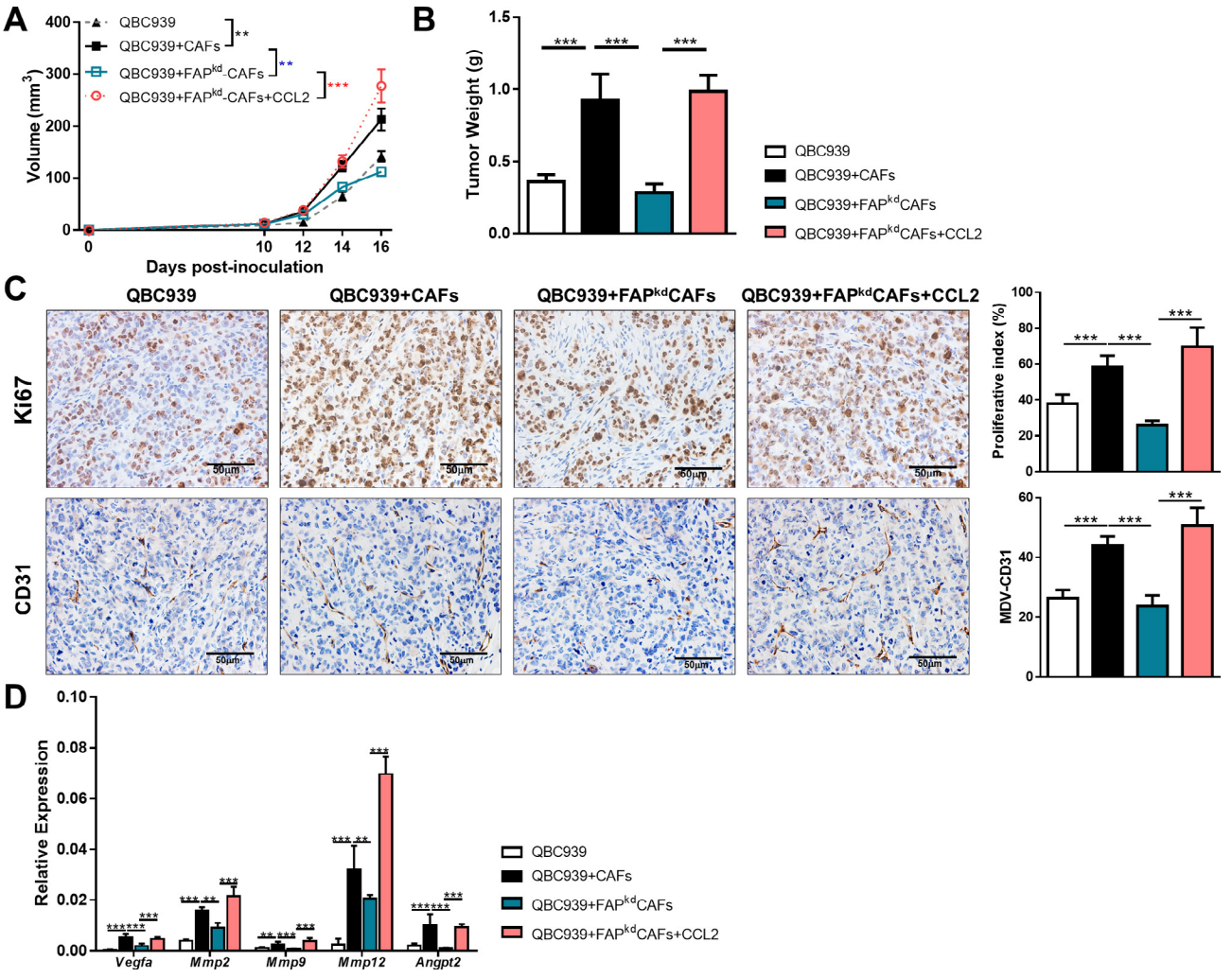

Figure 2. Knockdown of FAP in ICC-CAFs inhibits tumor growth in a xenograft mouse model of human ICC. QBC939 cells were injected alone or co-injected with various fibroblasts subcutaneously into nude mice with or without CCL2, respectively. (A) Tumor growth was measured at the indicated time points. (B) Tumor weight (C) representative photomicrographs of IHC-staining of Ki67 and CD31 of tumor sections and proliferation index calculated by surface quantification of Ki67 positive and CD31 positive cells. Original magnification, 200X; scale bar, 50 m. (D) qRT-PCR analysis of the expression of proangiogenic in tumor tissues. Data are represented as mean SEM, n = 5–7. Results are representative of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

by transwell assay. FAP knockdown significantly impaired the ability of ICCCAFs to attract myeloid cells, which was rescued by addition of CCL2 (Figure 4E). In contrast, compared with supernatants from ICCCAFs, those from FAP^{kd}ICCCAFs had a significantly impaired ability to enhance angiogenic gene expression, which could not be restored by addition of CCL2 (Figure 4F). These results together suggest that FAP is required for the ability of ICCCAFs to mediate the migration in a CCL2dependent way, and angiogenic function in a CCL2independent way.

ICC-promoting effect of fibroblastic FAP is dependent on Gr-1^{+} MDSCs

Given that exogenous CCL2 restored infiltrating MDSCs in tumors coinjected with FAP^{kd}ICCCAFs, we then investigated whether ICCpromoting effect of fibroblastic FAP was dependent on Gr1^{+} MDSCs via CCL2. To this end, we depleted Gr1^{+} MDSCs by i.v. injecting αGr1 neutralizing antibodies into nude mice which were s.c coinjected QBC939 ICC cells with FAP^{kd}ICCCAFs followed by CCL2 treatment. The depletion deficiency was confirmed by flow cytometric analysis of tumor tissues showing no detectable Gr1^{+} MDSCs (Figure 5A). Depletion of Gr1^{+} MDSCs abrogated the restoring effects of CCL2 in tumors coinjected with FAP^{kd}ICCCAFs, as evidenced by marked reduction in tumor volume and weight of tumors (Figure 5B and C). These findings were further supported by IHC staining showing significant less Ki67 positive cells observed in tumor sections after Gr1^{+} MDSCs Depleted (Figure 5D and E). Consistently, the number of CD31^{+} cells and the expression of angiogenesis associated genes also significantly decreased in MDSCs Depleted group (Figure 5E and F). These data together demonstrate that tumorinfiltrating Gr1^{+} MDSCs are required for the ability of fibroblastic FAP to promote ICC growth.

Discussion

Despite accumulating evidence demonstrating that fibroblasts play a crucial role in ICC development and FAP is expressed in CAFs from many tumor types, the direct evidence that FAP is expressed in fibroblasts derived from ICC is still lacking and how fibroblastic FAP contributes

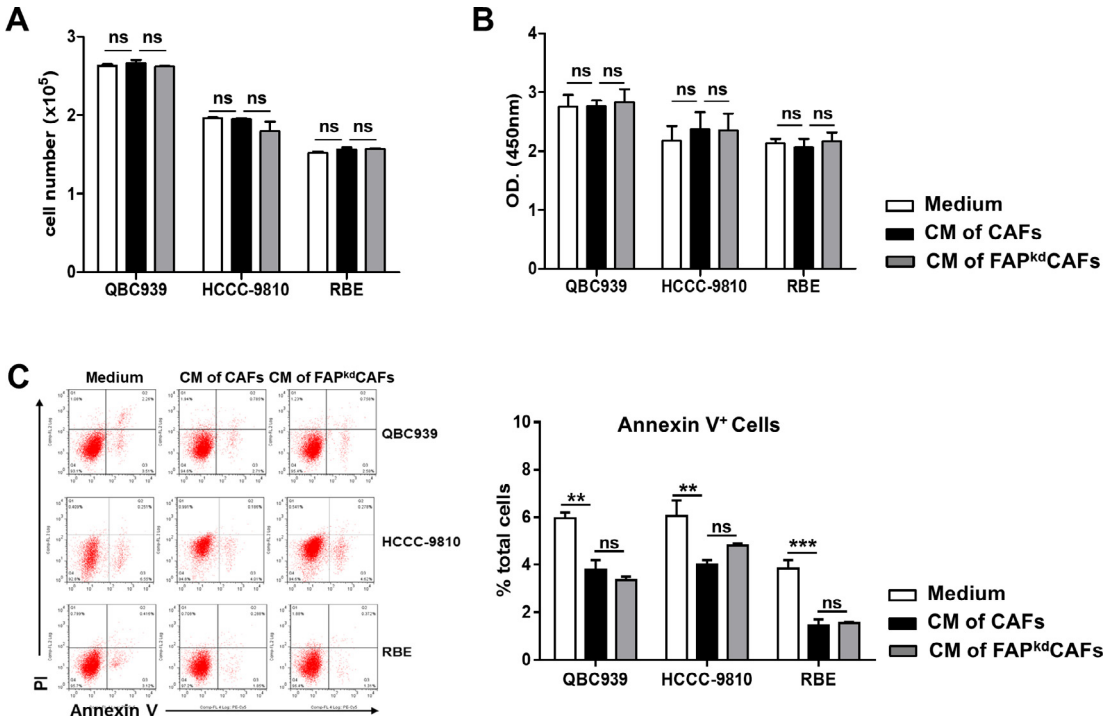

Figure 3. Fibroblastic FAP does not directly promote ICC cells proliferation and apoptosis. ICC cell line QBC939, HCCC9810 and RBE were treated with supernatants of various fibroblasts. (A and B) CCK8 and BrdU analysis of tumor cell number and proliferation. (C) Representative flow cytometry data of AnnexinV/PI double staining and averaged percentages of Annexin V^+ cells. Data represented as mean SEM, n = 5–7. Results are representative of at least three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

to ICCpromoting effect of fibroblasts remains unknown. First, this study provides the direct evidence that FAP was expressed in fibroblasts isolated from 6 individual surgical samples of ICC patients by western blotting and immunofluorescence staining. Moreover, we here demonstrate that fibroblasts derived from tumors of ICC patients express FAP have higher STAT3 activation and CCL2 production, which was greatly reduced by FAP knockdown. Furthermore, fibroblastic FAP is required for tumor growth and MDSC infiltration in a CCL2dependent manner by using a xenograft mouse model of human ICC. Moreover, in vitro studies reveal that fibroblastic FAP is critical for ICCCAFs to mediate migration of MDSCs via CCL2.

Lots of studies have suggested that there were various fibroblast subsets in the tumor microenvironment and inflammatory diseases, and only part fibroblast subsets contribute to inflammation, tumor progression and cancer therapy resistance [21,32–34]. A recent study found that it was FAP^+ fibroblasts contributing to inflammation in arthritis by using singlecell RNA sequencing [32]. Our previous study showed that FAP^+CAFs were a subset of inflammatory CAFs in HCC [21]. Although CAFs isolated from ICC patients were αSMA positive fibroblasts, we found that FAP only expressed on a subset of αSMA^+CAFs In the ICC tumor microenvironment. Moreover, our data demonstrated that FAP knockdown in ICCCAFs has lower STAT3 activation and inflammatory cytokines expression, suggesting that FAP was a functional marker of activated CAFs, more than a byproduct marker. MDSCs are a crucial immune component in the tumor microenvironment, facilitating all stages of tumor development via various mechanisms [22,35]. Despite accumulating data about MDSCs in tumorbearing mouse model [21,35], the information of MDSCs is relatively limited in human cancers. FAP was previously demonstrated to induce immunosuppression in the tumor microenvironment by antagonizing antitumor T cell immunity [19–21]. Considering limited infiltration of T cells, but not myeloid cells, in cancer with dense desmoplastic stroma [36,37], we speculated that fibroblastic FAP may also facilitate ICC development via nonT celldependent mechanisms. Using a tumor model by coinjecting fibroblasts and tumor cells into immunodeficient mice, we demonstrated that ICCCAFs promoted QBC939 ICC cell growth in vivo accompanied by increases in tumor infiltration of MDSCs and macrophages, and gene expression of angiogenic factors. Importantly, FAP knockdown completely impaired the tumorpromoting effect of ICCCAFs, which was reversed by adding exogenous CCL2. Altogether, these data emphasize a nonT celldependent mechanism underlying ICC growth inhibition by targeting fibroblastic FAP. Otherwise, the previous study showed that FAP expressed on the surface of tumorassociated macrophage in human breast cancer [38]. However, we found that the TAM of ICC did not express FAP (data not shown). Therefore, whether FAP expressed on TAM may rely on its tumor microenvironment and tumor types.

We further clarified the possible nonT celldependent mechanisms by which fibroblastic FAP promoted ICC growth. FAP can promote tumor growth via its enzyme activity or nonenzyme activity to shape the protumorigenic functions of CAFs [39,40]. Ours and other studies have demonstrated that FAP regulated the expression of CCL2. Moreover, FAP did not cleave CCL2 spite FAP cleavage sites being present in CCL2 [39]. Taking together, these results suggested that protumorigenic functions of FAP in ICC via CCL2 were dependent on its introcellular activation of STAT3 signal in CAFs. Moreover, we demonstrated that fibroblastic FAP mediated tumor recruitment of MDSCs in CCL2dependent manner. In addition to mediating immunosuppression, MDSCs could promote tumor progression by enhancing angiogenesis in a paracrine pathway [26]. We demonstrated that ICCCAFs enhanced the ability of MDSCs to express various angiogenic factors, particularly MMP9 that was reported to be primarily produced by MDSCs [26], in a FAPdependent manner, however, considering that ICC is a hypovascular cancer [41], the enhancement of tumor angiogenesis may not be the major attribution to the ICCpromoting effect of

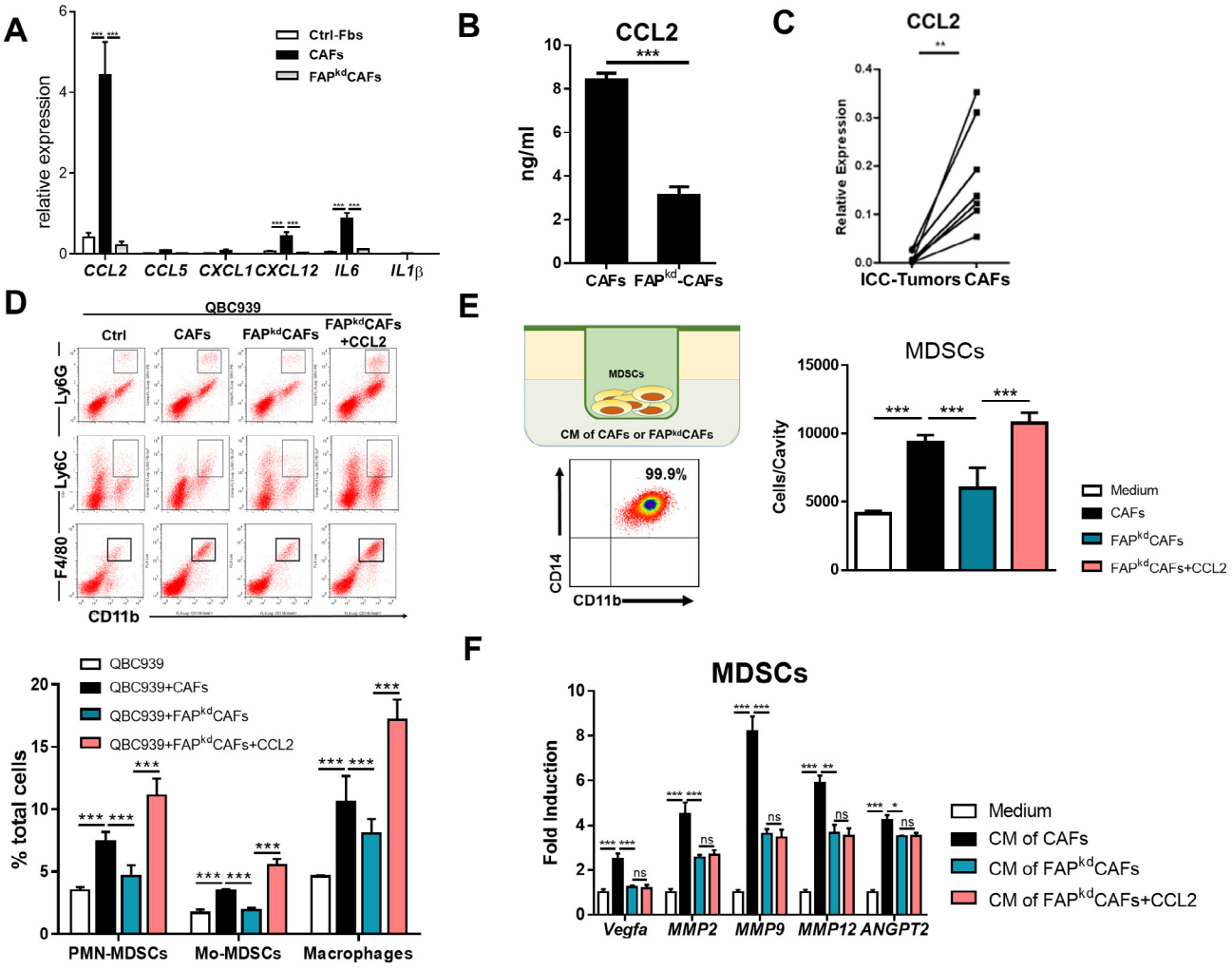

Figure 4. CCL2 mediates ICC-promoting effect of fibroblastic FAP. (A) qRT-PCR analysis of STAT3 regulating inflammatory cytokines particularly CCL2 expression of Ctrl-Fbs, CAFs or FAP^{kd}CAFs. n = 3. (B) ELISA analysis of CCL2 concentrations in the culture of CAFs treated with negative control or fap siRNA. n = 3. (C) qRT-PCR analysis of CCL2 concentrations in the culture of ICC tumor tissues or CAFs. n = 7. (D) Representative flow cytometry analysis of PMN-MDSCs (Ly6G$^+$CD11b$^+$F4/80), Mo-MDSCs (Ly6C$^+$CD11b$^+$F4/80) and tumor-associated macrophages (F4/80$^+$CD11b$^+$). (E) Representative flow cytometry analysis of sorted myeloid cells from peripheral blood of ICC patients (CD33$^+$CD14$^+$CD11b$^+$). Transwell assays of MDSC chemotaxis toward supernatants of various fibroblasts with or without CCL2 protein. (F) qRT-PCR analysis of gene expression of proangiogenic factor in MDSCs treated by supernatants of various fibroblasts with or without CCL2. Values are mean SEM of three replicate wells from a representative of three experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

fibroblastic FAP. CCL2 production regulated the vicious cycle between tumor cells and macrophages that promotes the progression of tumors [42]. Fibroblastderived CCL2 was recently shown to promote breast cancer progression by regulating NOTCH activation and macrophage infiltration [43,44]. Consistent with this study, we showed that fibroblasts were the major source of CCL2 in human ICC microenvironment. However, coculture with supernatants from ICCCAFs failed to enhance cell proliferation. Thus, FAP and CCL2dependent increases in ICC cell proliferation in vivo could not result from the direct effect of ICCCAFs on ICC cells. Given that myeloid cells including MDSCs and macrophages are implicated in promoting cancer growth [25,26,35], it is likely that CCL2 derived from FAP$^+$ ICCCAFs mediates migration of MDSCs to tumor sites, where they support cancer cell proliferation.

Most studies focused on the crosstalk between fibroblasts and cholangiocarcinoma cells, demonstrating that fibroblasts directly promote cancer cell apoptotic resistance, migration and/or EMT by secreting a variety of mediators [8–11,13]. We demonstrated that ICCCAFs directly reduced the frequencies of apoptotic cells in the cultures of three ICC cell lines, but not dependent on FAP. However, the previous study showed that overexpression FAP in LX2, a liver fibroblast cell line, did not increase tumor cells apoptosis directly, but can enhance staurosporine streptomycesinduced tumor cells apoptosis [45]. As the CAFs play an important role in the chemotherapy resistance, therefore, we did not exclude that FAP may play a synergetic effect on apoptosis resistance under some chemotherapeutic treatment.

Conclusion

In summary, our study provides the first evidence that FAP is expressed by ICCderived fibroblasts, which is the main source of CCL2 in the ICC microenvironment. We further demonstrate that fibroblastic FAP promotes ICC growth indirectly by attracting MDSCs to tumor sites via CCL2 specific targeting fibroblastic FAP may represent a more effective and safer strategy against ICC.

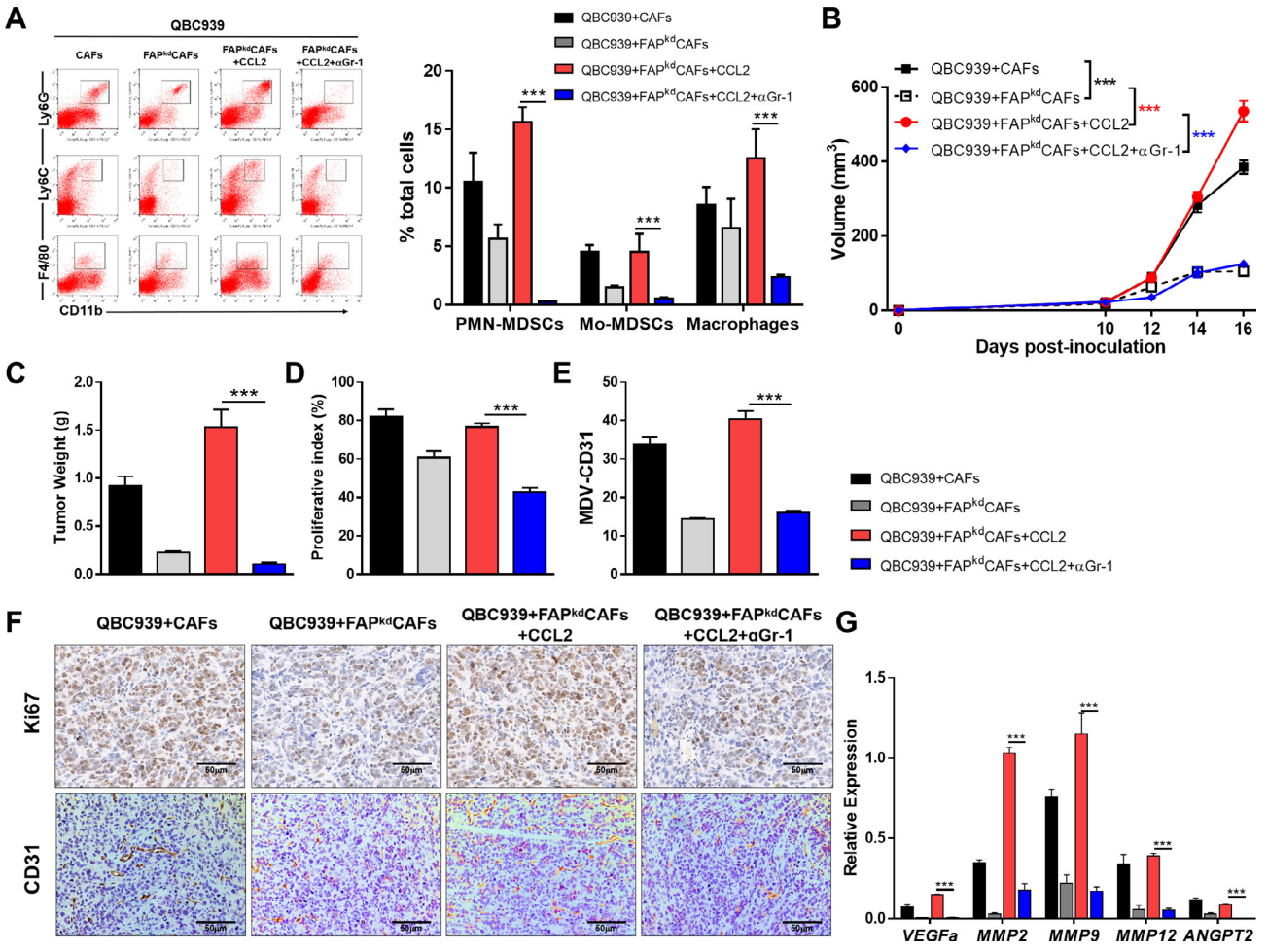

Figure 5. ICC-promoting effect of fibroblastic FAP is dependent on Gr-1 + MDSCs. QBC939 cells were injected alone or co-injected with various fibroblasts subcutaneously into nude mice with or without CCL2 and Gr-1 antibody, respectively. (A) Representative flow cytometry data and averaged percentages of PMN-MDSCs, M-MDSCs, and macrophages. (B) Tumor growth was measured at the indicated time points. (C) Tumor weight. (D–F). Representative photomicrographs of IHC-staining of Ki67 and CD31 (F) of tumor sections and proliferation index calculated by surface quantification of Ki67 positive (D) and CD31 positive cells (E). Original magnification, 200; scale bar, 50 m. (G) qRT-PCR analysis of the expression of proangiogenic in tumor tissues. Data are represented as mean SEM, n = 5–7. Results are representative of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Ethic approval and consent to participate

The current study was approved by Ethical Committee of Zhongshan Hospital and the Institutional Animal Care and Use Committee of Fudan University.

Consent for publication

All authors reached an agreement to publish the study in this journal.

Availability of data and materials

Not applicable.

Authors contributions

R.H. and Y.L. designed the study, Y.L., B.L., X.G. performed the experiments and analyzed data, Y.C., Q.C., W.Y. and Y.S. performed the experiments, W.L., M.T. and Y.S. provided the clinical samples, R.H. and Y.L. wrote the manuscript, and all authors have read the manuscript critically.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.09.004.

References

1. Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol* 2014;**60**(6):1268–89.
2. Fujita T. Analyzing risk factors for intrahepatic cholangiocarcinoma. *Hepatology* 2013;**58**(5):1862–3.
3. Razumilava N, Gores GJ. Cholangiocarcinoma. *Lancet* 2014;**383**(9935):2168–79.
4. Coulouarn C, Clement B. Stellate cells and the development of liver cancer: therapeutic potential of targeting the stroma. *J Hepatol* 2014;**60**(6):1306–9.
5. Sirica AE. The role of cancer-associated myofibroblasts in intrahepatic cholangiocarcinoma. *Nat Rev Gastroenterol Hepatol* 2011;**9**(1):44–54.
6. Sirica AE, Gores GJ. Desmoplastic stroma and cholangiocarcinoma: clinical implications and therapeutic targeting. *Hepatology* 2014;**59**(6):2397–402.
7. Chuaysri C, Thuwajit P, Paupairoj A, Chau-In S, Suthiphongchai T, Thuwajit C. Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma. *Oncol Rep* 2009;**21**(4):957–69.
8. Fingas CD, Bronk SF, Werneburg NW, Mott JL, Guicciardi ME, Cazanave JC, Mertens JC, Sirica AE, Gores GJ. Myofibroblast-derived PDGF-BB promotes Hedgehog survival signaling in cholangiocarcinoma cells. *Hepatology* 2011;**54**(6):2076–88.
9. Ohira S, Sasaki M, Harada K, Sato Y, Zen Y, Isse K, Kozaka K, Ishikawa A, Oda K, Nimura Y, etal. Possible regulation of migration of intrahepatic cholangiocarcinoma cells by interaction of CXCR4 expressed in carcinoma cells with tumor necrosis factor-alpha and stromal-derived factor-1 released in stroma. *Am J Pathol* 2006;**168**(4):1155–68.
10. Claperon A, Mergey M, Aoudjehane L, Ho-Bouldoires TH, Wendum D, Prignon A, Merabtene F, Firrincieli D, Desbois-Mouthon C, Scatton O, etal. Hepatic myofibroblasts promote the progression of human cholangiocarcinoma through activation of epidermal growth factor receptor. *Hepatology* 2013;**58**(6):2001–11.
11. Claperon A, Mergey M, Nguyen Ho-Bouldoires TH, Vignjevic D, Wendum Y, Chretien Y, Merabtene F, Frazao A, Paradis V, Housset C, etal. EGF/EGFR axis contributes to the progression of cholangiocarcinoma through the induction of an epithelial-mesenchymal transition. *J Hepatol* 2014;**61**(2):325–32.
12. Aishima S, Nishihara Y, Iguchi T, Taguchi K, Taketomi A, Maehara Y, Tsuneyoshi M. Lymphatic spread is related to VEGF-C expression and D2–40-positive myofibroblasts in intrahepatic cholangiocarcinoma. *Mod Pathol* 2008;**21**(3):256–64.
13. Fiori ME, Di Franco S, Villanova L, Bianca P, Stassi G, De Maria R. Cancer-associated fibroblasts as abettors of tumor progression at the crossroads of EMT and therapy resistance. *Mol Cancer* 2019;**18**(1):70.
14. Scanlan MJ, Raj BK, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, Healey JH, Old LJ, Rettig WJ. Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. *Proc Natl Acad Sci U S A* 1994;**91**(12):5657–61.
15. Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J Biol Chem* 1999;**274**(51):36505–12.
16. Lo A, Wang LCS, Scholler J, Monslow J, Avery D, Newick K, O'Brien S, Evans RA, Bajor DJ, Clendenin C, etal. Tumor-promoting desmoplasia is disrupted by depleting FAP-expressing stromal cells. *Cancer Res* 2015;**75**(14):2800–10.
17. Santos AM, Jung J, Aziz N, Kissil JL, Pure E. Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. *J Clin Invest* 2009;**119**(12):3613–25.
18. Lee J, Fassnacht M, Nair S, Boczkowski D, Gilboa E. Tumor immunotherapy targeting fibroblast activation protein, a product expressed in tumor-associated fibroblasts. *Cancer Res* 2005;**65**(23):11156–63.
19. Kraman M, Bambrough PJ, Arnold JN, Roberts EW, Magiera L, Jones JO, Gopinathan A, Tuveson DA, Fearon DT. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science* 2010;**330**(6005):827–30.
20. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, Connell CM, Roberts EW, Zhao Q, Caballero OL, etal. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc Natl Acad Sci U S A* 2013;**110**(50):20212–7.
21. Yang X, Lin Y, Shi Y, Li B, Liu W, Yin W, Dang Y, Chu Y, Fan J, He R. FAP promotes immunosuppression by cancer-associated fibroblasts in the tumor microenvironment via STAT3-CCL2 signaling. *Cancer Res* 2016;**76**(14):4124–35.
22. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 2012;**12**(4):253–68.
23. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;**9**(3):162–74.
24. Hasita H, Komohara Y, Okabe H, Masuda T, Ohnishi K, Lei XF, Beppu T, Baba H, Takeya M. Significance of alternatively activated macrophages in patients with intrahepatic cholangiocarcinoma. *Cancer Sci* 2010;**101**(8):1913–9.
25. Wan S, Zhao E, Kryczek I, Vatan L, Sadovskaya A, Ludema G, Simeone DM, Zou W, Welling TH. Tumor-associated macrophages produce interleukin 6 and signal via STAT3 to promote expansion of human hepatocellular carcinoma stem cells. *Gastroenterology* 2014;**147**(6):1393–404.
26. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 2008;**8**(8):618–31.
27. Yeung OW, Lo CM, Ling CC, Qi X, Geng W, Li CX, Ng KT, Forbes SJ, Guan XY, Poon RT, etal. Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. *J Hepatol* 2015;**62**(3):607–16.
28. Zhang C, Bai DS, Huang XY, Shi GM, Ke AW, Yang LX, Yang XR, Zhou J, Fan J. Prognostic significance of Capn4 overexpression in intrahepatic cholangiocarcinoma. *PLoS One* 2013;**8**(1) e54619.
29. Zhang JP, Yan J, Xu J, Pang XH, Chen MS, Li L, Wu C, Li SP, Zheng L. Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. *J Hepatol* 2009;**50**(5):980–9.
30. Karakasheva TA, Lin EW, Tang Q, Qiao E, Waldron TJ, Soni M, Klein-Szanto AJ, Sahu V, Basu D, Ohashi S, etal. IL-6 mediates cross-talk between tumor cells and activated fibroblasts in the tumor microenvironment. *Cancer Res* 2018;**78**(17):4957–70.
31. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000;**103**(3):481–90.
32. Croft AP, Campos J, Jansen K, Turner JD, Marshall J, Attar M, Savary L, Wehmeyer C, Naylor AJ, Kemble S, etal. Distinct fibroblast subsets drive inflammation and damage in arthritis. *Nature* 2019;**570**(7760):246–51.
33. Huelsken J, Hanahan D. A subset of cancer-associated fibroblasts determines therapy resistance. *Cell* 2018;**172**(4):643–4.
34. Su S, Chen J, Yao H, Liu J, Yu S, Lao L, Wang M, Luo M, Xing Y, Chen F et al. CD10(+)GPR77(+) cancer-associated fibroblasts promote cancer formation and chemoresistance by sustaining cancer stemness. Cell 2018, 172(4):841–856 e816..
35. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 2009;**182**(8):4499–506.
36. Ene-Obong A, Clear AJ, Watt J, Wang J, Fatah R, Riches JC, Marshall JF, Chin-Aleong J, Chelala C, Gribben JG, etal. Activated pancreatic stellate cells sequester CD8+ T cells to reduce their infiltration of the juxtatumoral compartment of pancreatic ductal adenocarcinoma. *Gastroenterology* 2013;**145**(5):1121–32.
37. Watt J, Kocher HM. The desmoplastic stroma of pancreatic cancer is a barrier to immune cell infiltration. *Oncoimmunology* 2013;**2**(12) e26788.
38. Tchou J, Zhang PJ, Bi Y, Satija C, Marjumdar R, Stephen TL, Lo A, Chen H, Mies C, June CH, etal. Fibroblast activation protein expression by stromal cells and tumor-associated macrophages in human breast cancer. *Hum Pathol* 2013;**44**(11):2549–57.
39. Zhang HE, Hamson EJ, Koczorowska MM, Tholen S, Chowdhury S, Bailey AJ, Lay AJ, Twigg SM, Lee Q, Roediger B, etal. Identification of novel natural substrates of fibroblast activation protein-alpha by differential degradomics and proteomics. *Mol Cell Proteomics* 2019;**18**(1):65–85.

40. Koczorowska MM, Tholen S, Bucher F, Lutz L, Kizhakkedathu JN, De Wever UF, Wellner UF, Biniossek ML, Stahl A, Lassmann S, etal. Fibroblast activation protein-alpha, a stromal cell surface protease, shapes key features of cancer associated fibroblasts through proteome and degradome alterations. *Mol Oncol* 2016;**10**(1):40–58.
41. Kawahara N, Ono M, Taguchi K, Okamoto M, Shimada M, Takenaka K, Hayashi K, Mosher DF, Sugimachi K, Tsuneyoshi M, etal. Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma. *Hepatology* 1998;**28**(6):1512–7.
42. Yoshimura T. The chemokine MCP-1 (CCL2) in the host interaction with cancer: a foe or ally?. *Cell Mol Immunol* 2018;**15**(4):335–45.
43. Tsuyada A, Chow A, Wu J, Somlo G, Chu P, Loera S, Luu T, Li AX, Wu X, Ye W, etal. CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. *Cancer Res* 2012;**72**(11):2768–79.
44. Fujimoto H, Sangai T, Ishii G, Ikehara A, Nagashima T, Miyazaki M, Ochiai A. Stromal MCP-1 in mammary tumors induces tumor-associated macrophage infiltration and contributes to tumor progression. *Int J Cancer* 2009;**125**(6):1276–84.
45. Wang XM, Yu DM, McCaughan GW, Gorrell MD. Fibroblast activation protein increases apoptosis, cell adhesion, and migration by the LX-2 human stellate cell line. *Hepatology* 2005;**42**(4):935–45.