Supplementary Information

Supplementary experimental procedures

Clinical specimens

All colorectal cancer and corresponding adjacent normal tissues were obtained under informed consent from patients undergoing surgical resection at West China Hospital or Sichuan Provincial People's Hospital (Chengdu, China). Samples were immediately frozen in liquid nitrogen or used for isolation of TAFs. Ethics approval was obtained from the Institutional Ethics Committee of Sichuan University. Tumor differentiation was characterized according to WHO classification, and the surgical pathologic stage was analyzed according to the TNM classification system of the International Union against Cancer.

Co-culture of TAFs and cancer cells

Colorectal tumor-associated fibroblasts were isolated from primary colorectal tumors from five individual patients (TAFs), or liver metastatic foci from five individual patients (TAF-Ms). Normal colonic fibroblasts were isolated from non-cancerous colonic tissues from five patients undergoing segmental colonic resection for injuries. Tissue samples were minced and enzymatically dissociated in serum-free DMEM medium at 37 °C for 16 h. Fibroblasts in the supernatant were pelleted by centrifugation at 100 g for 10 min followed by two washes with DMEM medium. The cells were re-suspended and cultured with DMEM medium containing 10% fetal calf serum (Hyclone, Logan, UT), penicillin (10^7 U/L) and streptomycin (10 mg/L) at 37 °C in a humidified chamber containing 5% CO_2. TAFs between passages 3-8 were used in the experiments. A non-contact co-culture system was used to observe the TAF-cancer cell communication. Colorectal cancer cells were

serum-starved overnight before experiment. TAFs and cancer cells were co-cultured separately using a Transwell filter (polycarbonate membrane insert, 0.45-mm pore; Corning Inc.).

Reagents and plasmids

The following primary antibodies were used: rabbit-anti-E-cadherin (Abcam), rabbit-anti-Snail (Abcam), mouse-anti-Vimentin (Santa Cruz, Abcam), mouse-anti-FGFR1 (Abcam), rabbit-anti-FGFR2 (Abcam), goat-anti-FGF19 (Abcam), rabbit-anti-FGFR3 (Abcam), rabbit-anti-Histone H3 (Abcam), rabbit-anti-FGFR4 (Cell Signaling), rabbit-anti-p-Erk-Thr202/Tyr204 (Cell Signaling), rabbit-anti-FRS2 (Abcam), rabbit-anti-p-FRS2-Tyr436 (Abcam), rabbit-anti-Erk (Cell Signaling), rabbit-anti-β-catenin (Millipore), mouse-anti-CD44 (Cell Signaling), rabbit-anti-CCL2 (Abcam), rabbit-anti-p-β-catenin-Y142 (Abcam), rabbit-anti-CD133 (Cell Signaling) and mouse-anti-p-Tyrosine (Cell Signaling).

SU5402 was purchased from Sigma and used as indicated dose.

Active FGF19 recombinant protein was purchased from Abcam and used as indicated doses.

Plasmid encoding full-length human FGFR4 was purchased from Gene Copoeia (Guangzhou, China). FGFR4-DN and FGFR4 promoter constructs were prepared as described previously (1-3).

Immunoblot

Cells were lysed with RIPA buffer (50 mM Tris base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF). The protein sample was separated on 12% or 15% SDS-PAGE, and transferred to PVDF

membranes (Amersham Biosciences). After blocking at 37 °C for 2 h, the blots were probed by the primary antibodies at 4 °C overnight. After washing three times with TBS containing 0.1 % Tween 20, the blots were incubated with HRP-conjugated secondary antibody (diluted 1:10,000; Santa Cruz Biotechnology) 2 h at room temperature. Finally, the blots were visualized by enhanced chemiluminescence (Amersham Biosciences).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS for 30 min followed by two washes with ice-cold PBS. The cells were then probed with primary antibodies at 4 °C overnight. After washing again twice, the cells were treated with TRITC or FITC-conjugated secondary antibodies (diluted 1:100, Santa Cruz) for 1 h at room temperature. The slides were visualized using a Zeiss Imager. Z1 fluorescence microscope equipped with an AxioCamMRc5 digital CCD camera (Carl Zeiss Microimaging, Jena, Germany).

Immunohistochemistry

The slides were stained using the Envision System horseradish peroxidase method (DakoCytomation Inc., Carpinteria, CA) according to the manufacturer's instructions. To estimate the score of each slide, at least eight individual fields were chosen, and 100 cancer or stroma cells were counted for each field. Cells with membrane and/or cytoplasmic FGFR4 immunoreactivity were considered positive. The score for each slide was measured as the cross product of the value of immunostaining intensity (A) and the value of proportion of staining-positive cells (B), as described previously (4). Immunostaining intensity was divided into five

grades: 0, negative; 1, weak; 2, moderate; 3, strong; 4, very strong. The proportion of staining-positive cells was divided into five grades: 0, <5%; 1, 6 –25%; 2, 26 –50%; 3, 51–75%; 4, >75%. The results were defined as: 0-4, low; 5-16, high. Results were assessed and confirmed by two independent experienced pathologists. To determine the correlation of the expression of FGFR4, p-β-catenin (Y142) and CCL2, consecutive sections used and the corresponding similar regions used to score the immunostaining intensity.

BrdU labeling assay

BrdU labeling assay was performed in 96 well plates. BrdU was purchased from Roche Applied Science (Indianapolis, IN). After treatment, BrdU was added to a final concentration of 10 mM, and the cells were incubated for another 12 h. BrdU signal was measured by using 5-Bromo-2´-deoxy-uridine Labeling and Detection Kit III (Roche).

2-DE and MS/MS analysis

2-DE was performed as previously reported with minor modifications (5, 6). Briefly, the cell samples were dissolved in lysis buffer (7M urea, 2Mthiourea, 4% CHAPS, 100 mM DTT, 0.2% pH3–10 ampholyte, Bio-Rad, USA) containing protease inhibitor (Sigma). Protein samples was loaded into an IPG strip (17 cm, pH3–10NL, Bio-Rad), separated by isoelectric focusing (first dimension) and SDS-PAGE (second dimension). Quantitation and comparison of each gel spot was determined using PDQuest software 7.1 (Bio-Rad). In-gel digestion of proteins was carried out using mass spectrometry grade trypsin (Trypsin Gold, Promega, V5280). MS analysis was performed on a Q-TOF mass spectrometer (Micromass, Manchester,

UK) fitted with an ESI source (Waters). The MS/MS data were processed with MassLynx V 4.1 software (Micromass) which converts MS/MS data into PKL files. The PKL files were analyzed using the online MASCOT search engine (http://www.matrixscience.com).

Wound healing, cell migration and invasion assays

For the scratch wound healing assay, wounds were created in confluent cells by scraping the cells with a sterile pipette tip. The cell culture was then washed with medium (five times) to remove free-floating cells and debris. The number of the cells migrated into the scraped region was documented over 48 h.

Transwell 24-well chambers (Corning) were used for *in vitro* cell migration and invasion assay. For cell migration assay, 2.5×10^4 cells were seeded in the upper well of a transwell chamber. For invasion assays, Matrigel (1:3, BD, USA) was added to the transwell chambers, and cells were seeded after incubation at 37 °C for 4 h. Cells on the upper side of the filter were removed after 24 h for the migration assay or 48 h for the invasion assay. The filter membrane was stained with crystal violet, and the number of the cells that remained adherent to the underside of the membrane were counted using an inverted microscope (Zeiss Axiovert).

Top/Fop flash assay

Colorectal cancer cells were transfected with Top-flash plasmid (Upstate) plus pRL-CMV plasmid (Promega) or Fop-flash plasmid (Upstate) plus pRL-CMV plasmid. The activity of Top-flash and Fop-flash was measured using a Dual Luciferase Kit (Promega) and normalized by the activity of pRL-CMV. The transcriptional activity of TCF/LEF was calculated as a ratio of normalized Top-flash

and Fop-flash.

RNA interference

SiRNA targeting FGFR3 and Ets-1 were purchased from Santa Cruz. Other siRNAs used in this study were synthesized using the following sequences: FGFR1, 5'-CCU GGA GCA UCA UAA UGG A-3'; FGFR2, 5'-TTA GTT GAG GAT ACC ACA TTA-3'; FGFR4, 5'-GGC AUG CAG UAU CUG GAG U-3'; β-catenin, 5'-AGC UGA UAU UGA UGG ACA G-3'; CCR2: 5'-GCT GCA AAT GAG TGG GTC TTT-3'; Negative control oligonucleotide (NC):5'-UUC UCC GAA CGU GUC ACG U-3'.

***In vivo* metastasis assays**

An experimental mouse liver metastasis model was generated as described previously (7). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University. Briefly, mice were anesthetized, and then a total of 5×10^6 cells were injected into the spleens of the nu/nu nude mice. To determine survival time, mice were inspected daily and the date of death for each mouse has been documented. To examine the liver metastasis of tumor cells, mice were killed 8 weeks after injection. The liver tissues were analyzed by H&E staining, and liver metastasis was determined by counting the liver metastatic nodules.

FGF19 sensitivity assay

Cancer cells were co-cultured with TAFs for indicated times. Cells were washed with fresh serum-free DMEM medium (three times), and then treated with 200 ng/ml

FGF19 and 1 ng/ml heparin for 15 min. Phosphorylation of FRS2 was examined by immunoblot and used as a marker for cell sensitivity to FGF19.

Data analysis and statistics

Unpaired *t*-test or Pearson's correlation test was used to compare quantitative variables; Patients' survival curve was plotted by the Kaplan-Meier method, and the log-rank test was used to determine the significant difference among groups; the Cox regression model was used to perform multivariate analysis. Linear regressions were tested by using the Spearman rank correlation. $P < 0.05$ was considered statistically significant.

References

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Supplementary Figure legends

Figure S1

Co-culture with TAFs induces a stem cell-like phenotype of colorectal cancer cells

(A) NAFs, TAFs and TAF-Ms were isolated. Expression of Vimentin and pan-Cytokeratin in these fibroblasts were examined by immunocytochemistry. Scale bar, 25 μm.

(B) RKO cells were co-cultured with NAFs, TAFs or TAF-Ms for 48 h. Expression of CD133 and CD44 was examined by immunoblot.

(C) Proliferation rate of RKO cells were examined by BrdU labeling assay.

Figure S2

Co-culture with TAFs induces an aggressive phenotype of colorectal cancer cells

(A) RKO cells were co-cultured with TAFs for 48 h, and cell migration was examined by wound healing assay. Scale bar, 400 μm.

(B) Cell migration was examined by transwell assay. Scale bar, 100 μm.

(C) Cell invasion was examined by Matrigel assay. Scale bar, 100 μm.

(D) Representative phase-contrast images of cell morphology of RKO cells. Scale bar, 40 μm.

(E) Expression of Snail, E-cadherin and Vimentin was examined by immunoblot.

Figure S3

Proteomic analysis of RKO cells co-cultured with TAFs

(A) Representative 2-DE gel images of RKO cells solo-cultured or co-cultured with TAFs for 48 h. Total protein were loaded on pH 3–10 nonlinear immobilized pH gradient strips, then separated by 12% SDS-PAGE, and stained by CBB staining. The protein spots referring to FGFR4 and their surrounding areas were boxed.

(B) Enlarged view of the protein spots referring to FGFR4. Arbitrary expression values of the protein spots were quantified using PDQuest software.

All data were representative of at least three independent experiments. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$.

Figure S4

Up-regulation of FGFR4 is required for TAF-induced aggressive phenotype of colorectal cancer cells

(A) HCT116, SW480 and LoVo cells were co-cultured with TAFs or NAFs for 48 h. Expression of FGFR4 was examined by immunoblot.

(B) HCT116 cells were co-cultured with TAFs or NAFs for 24 h and 48 h. Phosphorylation of FRS2 (Y436) was examined by immunoblot. Phosphorylation of FGFR4 was examined by immunoprecipitation using FGFR antibody followed by immunoblot using an antibody specific for p-Tyrosine.

(C) HCT116 cells were co-cultured with TAFs for indicated time. Cells were washed with fresh serum-free medium, and then treated with 200 ng/ml FGF19 and 1 ng/ml heparin for 15 min. Phosphorylation of FRS2 (Y436) was examined by immunoblot.

(D) HCT116 cells were co-cultured with TAFs for 48 h. Expression of FGFR1 and FGFR2 were examined by immunoblot.

(E) HCT116 cells were transfected with siFGFR1, siFGFR2, siFGFR3, siFGFR4 or siNC, respectively, and co-cultured with TAFs for 48 h. Phosphorylation of FRS2 (Y436) was examined by immunoblot.

(F) Statistical analyses for Fig. 2E.

(G) Statistical analyses for Fig. 2F.

(H) HCT116 cells were transfected with mock vector, FGFR4-DN, siNC or siFGFR4, respectively, and then co-cultured with TAFs for 48 h. Cell migration was examined by transwell assay.

(I) Cell invasion was examined by Matrigel invasion assay.

All data were representative of at least three independent experiments. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$.

Figure S5

FGFR4 cooperates with Wnt signaling and CCL2/CCR2 axis to mediate TAF-induced EMT

(A) SW480 cells were co-cultured with TAFs or NAFs for 48 h. TCF/LEF transcription activity was examined by Top/Fop flash. Expression of Cyclin D1 was examined by immunoblot.

(B) SW480 cells were transfected with siNC or siβ-catenin, respectively, and then

co-cultured with TAFs for 48 h. Expression of E-cadherin and Vimentin was examined by immunoblot.

(C) SW480 cells were transfected with mock vector, FGFR4-DN, siNC or siFGFR4, respectively, and then co-cultured with TAFs for 48 h. TCF/LEF transcription activity was examined by Top/Fop flash. Expression of Cyclin D1 was examined by immunoblot.

(D) SW480 cells were transfected with mock vector or a plasmid coding full-length of FGFR4, and then treated with or without 10 μM SU5402. Phosphorylation of β-catenin (Y142) was examined by immunoblot by using whole cell lysate. The level of nuclear β-catenin was examined by immunoblot by using nucleus extracts. Histone H3 was used as internal control. H3, Histone H3.

(E) SW480 cells were transfected with mock vector, FGFR4-DN, siNC or siFGFR4, respectively, and co-cultured with TAFs for 48 h. Phosphorylation of β-catenin (Y142) was examined by immunoblot.

(F) SW480 cells were transfected with siNC or siEts-1, and then co-cultured with TAFs for 48 h in presence or absence of neutralizing antibodies against CCL2 (40 μg/ml). Expression of FGFR4 was determined by immunoblot.

(G) Normal RKO cells or RKO cells that were stably transfected with shFGFR4, shNC, shβ-catenin or shCCR2 were injected into the spleens of nude mice with or without TAFs. Liver metastatic nodules were analyzed by H&E staining. The border between normal liver tissues and metastatic nodules were indicated by dotted line. Scale bar, 30 μm.

All data were representative of at least three independent experiments. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$.