FGFR4 promotes stroma-induced epithelial to mesenchymal transition in colorectal cancer

Rui Liu1*, Jingyi Li2*, Ke Xie3*, Tao Zhang2*, Yunlong Lei1, Yi Chen4, Lu Zhang1, Kai Huang1, Kui Wang1, Hong Wu4, Min Wu5, Edouard C. Nice6, Canhua Huang1§, Yuquan Wei1

1. The State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu, 610041, P. R. China.
2. The School of Biomedical Sciences, Chengdu Medical College, Chengdu, 610083, People’s Republic of China.
3. Department of Oncology, Sichuan Provincial People’s Hospital, Chengdu, 610072, P. R. China.
4. Department of Hepatobiliary Pancreatic Surgery, West China Hospital, Sichuan University, Chengdu, 610041, People’s Republic of China.
5. Department of Biochemistry and Molecular Biology, University of North Dakota, Grand Forks, North Dakota 58203-9037.
6. Monash University, Department of Biochemistry and Molecular Biology, Clayton, Victoria 3800, Australia.

*These authors contributed equally to this work.

Running title: FGFR4 promotes stroma-induced EMT in CRC

§ Corresponding FGFR4 author:
Dr. Canhua Huang
The State Key Laboratory of Biotherapy
Chengdu, Sichuan University, People’s Republic of China
Tel: + 86-13258370346
Fax: + 86-28-85164060
Email: hcanhua@hotmail.com

Conflicts of interest
We declare that we have no conflicts of interest.

Funding
This work was supported by grants from the National 973 Basic Research Program of China (2013CB911300, 2011CB910703, 2012CB518900), the National Science and Technology Major Project (2011ZX09302-001-01, 2012ZX09501001-003), and Chinese NSFC (81225015, 81072022, 81172173).

Word count
4970 words
Abstract

Tumor cells evolve by interacting with the local microenvironment; however, the tumor-stroma interactions that govern tumor metastasis are poorly understood. In this study, proteomic analyses reveal that co-culture with tumor-associated fibroblasts (TAFs) induces significant overexpression of FGFR4, but not other FGFRs, in colorectal cancer cell lines. Mechanistic study shows that FGFR4 plays crucial roles in TAF-induced epithelial to mesenchymal transition (EMT) in colorectal cancer cell lines. Accumulated FGFR4 in cell membrane phosphorylates β-catenin, leading to translocation of β-catenin into nucleus. Further, TAF-derived CCL2 and its downstream transcription factor, Ets-1, are prerequisite for TAF-induced FGFR4 up-regulation. Further, FGFR4-associated pathways are shown to be preferentially activated in colorectal tumor samples, and direct tumor metastasis in mouse metastasis model. Our study demonstrates a pivotal role of FGFR4 in tumor-stroma interactions during colorectal cancer metastasis, and suggests novel therapeutic opportunities for the treatment of colorectal cancer.

Key words: FGFR4 / Colorectal cancer / Metastasis / Tumor-associated fibroblast / Wnt signaling pathway / CCL2
Introduction

Colorectal cancer (CRC) is one of the most frequent causes of cancer-related death worldwide (1). Although significant advances have been made in the treatment of metastatic colorectal cancers, in particular the introduction of novel chemotherapies and targeted agents, approximately 60% of patients receiving curative resection will undergo local recurrence or distant metastases, and 85% of patients will relapse within the first 2.5 years after surgery (2). Thus, a thorough understanding of the molecular mechanisms of colorectal cancer metastasis is urgently required to facilitate early diagnosis of individuals with a high risk of metastasis.

Fibroblast growth factor (FGF) ligands bind and activate cell surface FGF receptors (FGFR) to mediate a broad spectrum of biological processes in both embryonic and adult organisms (3). FGFs exert biological effects as potent growth factors for primary epithelial cells, which makes FGF signaling susceptible to hijacking by cancer cells. Accumulating evidence has linked carcinogenesis in a range of tissue types with the dysregulation of FGF signaling, including control of cancer cell proliferation and motility, and support of tumor angiogenesis (4, 5).

The tumor microenvironment provides the necessary signals which not only support growth and survival of the primary tumor, but also facilitate its metastatic dissemination to distant organs (6). Tumor-associated fibroblasts (TAFs) have recently been linked to a number of pro-metastatic capabilities, including induction of epithelial to mesenchymal transition (EMT) and remodeling of the extracellular matrix (ECM) (7). Although TAFs are considered to be among the key determinants in the metastatic progression of cancer (8), mechanisms underlying the regulatory effect of TAFs on cancer cells are still largely unknown. In this study, we have identified a pivotal role of FGFR4 in the TAF-mediated signaling network, which
confers an aggressive metastatic phenotype on colorectal cancer cells.

Material and Methods

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. To observe the TAF-cancer cell communication, we used a non-contact co-culture system, as described previously (9).

Cell lines

Human colorectal cancer cell line RKO, HCT116, LoVo and SW480 were purchased from and tested by American Type Culture Collection (ATCC, Rockville, MD). All the cell lines were used within 6 months after receipt or resuscitation. All the cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) containing 10% fetal calf serum (Hyclone, Logan, UT), penicillin (10^7U/L) and streptomycin (10 mg/L) at 37 °C in a humidified chamber containing 5% CO₂.

Further details of experimental procedures are described in Supplementary Information.

Results

FGFR4 is up-regulated in response to TAF-derived signals
In a pilot study, we analyzed the regulatory role of TAFs on colorectal cancer cells. Organ-specific fibroblasts were isolated from primary colorectal cancer tissues (TAFs), liver metastatic foci (TAF-Ms) and non-cancerous colonic tissues (NAFs). Purity of these fibroblasts was confirmed by expression of Vimentin and loss of pan-Cytokeratin (Fig. S1A). As expected, co-culture with TAFs or TAF-Ms, but not NAFs, induced a stem cell-like phenotype (Fig. S1B-C), enhanced both migratory and invasive capacity (Fig. S2A-C), and triggered significant EMT in colorectal cancer cells (Fig. S2D-E). No apparent differences were found in the ability of TAFs and TAF-Ms to activate colorectal cancer cells.

To explore the molecular mechanisms underlying TAF-induced migration and invasion of colorectal cancer cells, a comparative proteomic analysis of RKO cells solo-cultured or co-cultured with TAFs, was performed (Fig. S3A). A total of 41 proteins were identified with altered expression (Table S1). Notably, bioinformatics analysis of these changed proteins using Batch Query software, a web-based tool, showed a dramatic alteration in the FGF pathway ($P = 0.0103$) (Fig. 1A). Further, using STRING software, a significant group of regulated proteins were also found to be associated with FGFR4 (Fig. 1B), which was shown to be elevated over 8 fold in RKO cells co-cultured with TAFs compared to those solo-cultured cells (Fig. S3B). Overexpression of FGFR4 was further validated in a series of colorectal cancer cell lines of epithelium origin by immunoblot (Fig. 1C and Fig. S4A) and/or immunocytofluorescence analysis (Fig. 1D). Since RKO and HCT116 cells showed most significant alteration in FGFR4 expression, both two cell lines were chosen for further validation. TAF-induced up-regulation of FGFR4 was accompanied by increased phosphorylation of FGFR4 (Fig. 1C and Fig. S4B), enhanced phosphorylation of FRS2, a direct substrate of FGFR4, and Erk, the major
downstream effector of FGFR4 (Fig. 1C and Fig. S4B) (10), and elevated cell sensitivity to FGF19, which uniquely binds to, and signals through FGFR4 (Fig. 1E and Fig. S4C) (11). However, compared to TAFs, NAFs were unable to induce activation of either FGFR4 or its downstream signaling pathways in colorectal cancer cell lines (Fig. 1C-E and Fig. S4A-C). Aware of previous reports that both FRS2 and Erk can be activated by either FGFR1, FGFR2 or FGFR3 (5), we further examined whether expression of these FGFRs was altered in response to TAFs. A slight increase in FGFR1 and a moderate decrease in FGFR2 were found in both RKO and HCT116 cells after 48 h co-culture (Fig. 2A and Fig. S4D). In contrast, expression of FGFR3 was constantly low in RKO (Fig. 2A), and almost undetectable in HCT116 cells.

FGFR4 mediates a TAF-induced aggressive phenotype in colorectal cancer cells

To evaluate the functional role of individual FGFRs in mediating TAF-derived signals, specific siRNAs were introduced to repress the expression of each FGFR, respectively. Intracellular downstream cascades of FGFRs were estimated by measuring the phosphorylation status of FRS2 and Erk (12). Only a slight decrease in phosphorylation of both Erk and FRS2 was found when the cells were treated with either siFGFR1, siFGFR2 or siFGFR3, compared with the siNC control. However, knockdown of FGFR4 substantially reduced TAF-induced phosphorylation of Erk or FRS2 (Fig. 2B and Fig. S4E), suggesting that FGFR4, but not FGFR1, FGFR2 or FGFR3, was a potential surface sensor for mediating TAF-derived signals.

To determine the role of FGFR4 in the TAF-induced aggressive phenotype of colorectal cancer cells, the migratory and invasive capabilities of colorectal cancer cells were compared in the presence or absence of FGFR4. Repression of FGFR4, by either siFGFR4 or a dominant negative form of FGFR4 (FGFR4-DN), resulted in a
remarkable decrease in motility of colorectal cancer cells co-cultured with TAFs, accompanied with reversion to a more compact epithelium-like morphology (Fig. 2C-E and Fig. S4F-I). Correspondingly, loss of FGFR4 increased expression of the epithelial marker E-cadherin and reduced the levels of mesenchymal markers vimentin and Snail in TAF-treated cells (Fig. 2F-G). Together, these results indicated that FGFR4 is required for the TAF-induced aggressive phenotype of colorectal cancer cells.

It was of particular interest to examine the contribution of FGF signaling in the TAFs themselves. As shown in Fig. 2H, treatment with SU5402, a pan FGFR inhibitor, markedly reduced the phosphorylation of both Erk and FRS2. Further, siRNA-mediated knockdown of either FGFR1, FGFR2, FGFR3 or FGFR4 substantially decreased the phosphorylated forms of both Erk and FRS2 (Fig. 2I), suggesting that each FGFR was involved in FGF signal transduction in TAFs.

**FGFR4 mediates TAF-induced activation of Wnt signaling pathway via β-catenin Y142 phosphorylation**

Cross-talk between FGF and Wnt signaling pathways is involved in a range of biological processes, including carcinogenesis (5). To explore the potential signaling cascades downstream of FGFR4, of our particular interest, Wnt signaling was examined. As results, TCF/LEF transcription activity and expression of Cyclin D1, a Wnt target gene, were both enhanced in either RKO or SW480 cells co-cultured with TAFs, compared to those cells solo-cultured or co-cultured with NAFs (Fig. 3A and Fig. S5A). Further, repression of Wnt signaling by siRNA targeting β-catenin markedly abrogated TAF-induced EMT in these two cell lines (Fig. 3B and Fig. S5B), suggesting Wnt signaling is required for TAF-induced metastasis of colorectal cancer.
Next, we investigated whether FGFR4 plays a role in TAF-induced activation of Wnt signaling. Inhibition of FGFR4 by either specific siRNA or FGFR4-DN markedly repressed TCF/LEF transcription activity and expression of Cyclin D1 in both RKO and SW480 cells co-cultured with TAFs (Fig. 3C and Fig. S5C).

Further, we set out to explore the molecular basis of the regulatory effect of FGFR4 on Wnt signaling. It is known that disruption of the APC/Axin/GSK-3β complex stabilizes β-catenin, which is a crucial step in the activation of Wnt signaling (13). However, the observation that FGFR4 repression could inhibit TAF-induced activation of Wnt signaling in SW480 cells, which harbors a truncated APC (14), suggested other mechanisms likely involved in. It has been reported that some receptor tyrosine kinases, including EGFR, are capable of directly phosphorylating β-catenin at Y142, leading to β-catenin translocation from membrane to nucleus (15). Indeed, co-immunoprecipitation detected an FGFR4-β-catenin complex in SW480 cells, and such complex was accumulated when the cells were co-cultured with TAFs (Fig. 3D-E). Further, overexpression of FGFR4 resulted in significant β-catenin phosphorylation at Y142 and accumulation of nuclear β-catenin in both RKO and SW480 cells, which could be reversed by treatment with SU5402 (Fig. 3F and Fig. S5D) (16). Furthermore, co-culture with TAFs markedly enhanced β-catenin Y142 phosphorylation, which could be largely abolished upon FGFR4 suppression (Fig. 3G and Fig. S5E). These results suggested that FGFR4 mediated TAF-induced activation of Wnt signaling pathway via β-catenin Y142 phosphorylation.

**TAF-derived CCL2 is required for TAF-induced FGFR4 up-regulation**

To explore the mechanisms responsible for TAF-induced FGFR4 up-regulation,
bioinformatics analysis was employed to highlight the key regulatory factors based on the proteomic profiling data. Intriguingly, a cluster of altered proteins were found to be associated with the CCL2/CCR2 axis (Fig. 4A). CCL2 is highly expressed in TAFs (17), and its downstream transcription factor, Ets-1, can initiate FGFR4 expression by direct binding to FGFR4 promoter (18, 19). Therefore, we investigated whether CCL2 was required for TAF-induced FGFR4 up-regulation. As shown in Fig. 4B, TAFs showed a markedly elevated expression of CCL2, compared to NAFs. Notably, co-culture with colorectal cancer cells further enhanced the CCL2 production in TAFs. Further, addition of CCL2 in the culture induced a dramatic up-regulation of FGFR4 in colorectal cancer cell lines (Fig. 4C). Inhibition of CCL2 signaling via either a neutralizing antibody against CCL2 or siRNA targeting CCR2, the receptor for CCL2, markedly attenuated TAF-induced FGFR4 expression and its promoter activity (Fig. 4D and Fig. S5F), as well as the subsequent phosphorylation of β-catenin Y142 (Fig. 4E). We also investigated whether Ets-1 was involved in FGFR4 up-regulation. As shown, siRNA-mediated suppression of Ets-1 dramatically abolished TAF-induced FGFR4 overexpression (Fig. 4F and Fig. S5F). These results suggested that TAF-derived CCL2 was required for TAF-induced FGFR4 up-regulation in an Ets-1-dependent manner.

**TAF-derived FGF19 is required for TAF-induced FGFR4/Wnt activation**

It has been reported that FGF19 is a specific FGFR4 activator which has been implicated in colorectal cancer development (5). Therefore, we next examined the expression of FGF19 in TAFs and NAFs. As shown in Fig. 4G, expression of FGF19 was enhanced in TAFs compared to NAFs, revealed by both immunoblot and ELISA. Further, inhibition of FGF19 by a neutralizing antibody decreased TAF-induced
phosphorylation of both FGFR4 and β-catenin (Fig. 4H), suggesting that FGF19 played a role in TAF-induced FGFR4/Wnt activation.

**FGFR4 governs colorectal cancer cell metastasis in vivo**

To investigate whether FGFR4 accelerated colorectal cancer metastasis in vivo, we generated a mouse liver metastatic model by injecting mixed TAFs and colorectal cancer cells in spleen (20). Histopathological analysis confirmed that the liver metastatic nodules were composed of cancer cells, but not fibroblasts (Fig. S5G). As shown, inhibition of FGFR4-associated pathways markedly decreased the number of liver metastatic nodules (TAFs + RKO-shNC vs. TAFs + RKO-shFGFR4, \( P = 0.0002 \); TAFs + RKO-shNC vs. TAFs + RKO + shβ-catenin, \( P < 0.0001 \); TAFs + RKO-shNC VS. TAFs + RKO-shCCR2, \( P = 0.001 \); n=8. Fig. 5A-B) and prolonged the survival time of tumor-bearing mice (TAFs + RKO-shNC vs. TAFs + RKO-shFGFR4, \( P = 0.0018 \); TAFs + RKO-shNC vs. TAFs + RKO + shβ-catenin, \( P = 0.0024 \); TAFs + RKO-shNC VS. TAFs + RKO-shCCR2, \( P = 0.0062 \); n=8. Fig. 5C). These studies suggested that FGFR4 could function as a crucial signaling node in tumor-stroma interactions by promoting a TAF-induced CCL2/FGFR4/Wnt signaling pathway.

**FGFR4 and its associated pathways are preferentially activated in colorectal cancer**

To verify whether our findings with cancer cell lines and animal models were of clinical relevance, we evaluated the correlation between FGFR4 expression and clinico-pathological parameters in clinical samples. Clinico-pathological information for the clinical samples is summarized in Table S2. FGFR4 immunoreactivity was more intense in cancer tissues compared to non-cancerous adjacent tissues (\( P < \)
Further, a high level of FGFR4 expression was more likely to be associated with lymph node metastasis (Figure. 6B) and poor outcome (Figure. 6C). We also analyzed the correlation between the distribution of fibroblasts and the expression of FGFR4. As shown in Figure. 6D, the tumor cells adjoining the stromal fibroblasts displayed strong FGFR4 immunoreactivity, while those tumor cells residing in the central part of tumor region expressed a relatively low level of FGFR4, which suggested the occurrence of a paracrine effect of TAFs on FGFR4 expression. Furthermore, immunostaining using consecutive sections revealed positive correlations between the expression of FGFR4, CCL2 and phosphorylated β-catenin (Y142) (Figure. 6E-F). These data suggested that FGFR4 and its associated pathways were preferably activated in colorectal cancer, and were strongly related to a high risk of tumor metastasis and poor patient outcome.

Discussion

Multiple interactions between tumor cells and TAFs govern tumor progression, promoting the escape of tumor cells from immune-surveillance or hormonal control, and achieving the invasive phenotype required for translocation to a distant organ (4). However, the key molecular regulator and signaling pathways involved in the crosstalk between tumor cells and TAFs remain largely unknown. In this study, we demonstrate that FGFR4 is a potential regulator of this process. FGFR4, but not other FGFRs, was markedly up-regulated in colorectal cancer cells co-cultured with TAFs. Inhibition of FGFR4 attenuated TAF-induced intracellular signaling cascades, including phosphorylation of Erk and FRS2. Further, in both in vitro and in vivo models, TAF-induced migration and invasion of colorectal cancer cells could be reversed upon suppression of FGFR4. These data suggest that exposure to TAFs
prime colorectal cancer cells with a FGFR4-rich surface, which initiates intracellular signaling leading to an aggressive phenotype.

We found that the Wnt signaling pathway was required for the TAF-induced EMT in colorectal cancer cells. More importantly, we found in the SW480 cell line, which possessed a defective APC, that FGFR4 mediated TAF-induced activation of Wnt signaling pathway via β-catenin Y142 phosphorylation. Gene mutations of the APC tumor suppressor, which result in disruption of APC/Axin/GSK-3β complex, are among the most frequent oncogenic molecular abnormalities observed in colorectal cancer (21). Therefore, the present data might suggest that TAF-induced and FGFR4-mediated metastasis of colorectal cancer cells is, at least partially, APC/Axin/GSK-3β complex-independent.

CCL2/CCR2 chemokine signaling has been previously demonstrated to be involved in cancer metastasis (22). TAFs are an important reservoir of CCL2, which is released into the tumor microenvironment (17). Our data show that expression of CCL2 was elevated in TAFs compared to NAFs. Further, treating colorectal cancer cells with CCL2 triggered FGFR4 expression. In contrast, inhibition of the CCL2/CCR2 axis attenuated TAF-induced FGFR4 up-regulation and subsequent activation of Wnt signaling, as well as liver metastasis of colorectal cancer cells in mouse model. In addition, Ets-1, a downstream transcription factor of CCL2, also seemed to be involved in this process: siRNA-mediated knockdown of Ets-1 markedly suppressed TAF-induced FGFR4 up-regulation. These results suggest that the CCL2 signaling pathway plays crucial roles in early steps of TAF-induced colorectal cancer metastasis by mediating FGFR4 up-regulation.

Analysis of FGFR4 expression in colorectal tumor tissues suggested that the mechanistic results obtained from in vitro and in vivo experiments were of clinical
relevance. We demonstrate that expression of FGFR4 directly correlated with lymph node metastasis and metastasis-free survival rate. Furthermore, expression of FGFR4 was preferentially expressed in the tumor cells neighbouring the fibroblasts, and was positively correlated with expression of CCL2 or phosphorylated β-catenin (Y142), respectively. These data suggest FGFR4 and its associated pathways as potential biomarkers that could be used to predict the risk of metastasis and guide further diagnostic and therapeutic decisions.

Efforts have been made to understand the inter-communication between tumor and stroma cells, but few rational signaling pathways have been identified thus far. The present data suggest that FGFR4 is a key regulator in TAF-induced colorectal cancer metastasis (Fig. 7). Briefly, TAF-derived CCL2 induces expression of FGFR4 in an Ets-1-dependent manner. Overexpression of FGFR4 phosphorylates membranous β-catenin at Y142, which results in its translocation into the nucleus. Increased nuclear β-catenin initiates expression of Snail and subsequently represses expression of E-cadherin, leading to induction of EMT in colorectal cancer cells.

Many other signaling pathways were also found to be altered in colorectal cancer cells exposed to TAFs, including the platelet-derived growth factor (PDGF) signaling pathway, as shown by our proteomic profiling and bioinformatics analysis (Fig. 1A). The FGFRs are phylogenetically closely related to the platelet-derived growth factor receptors (PDGFRs). Both FGF and PDGF signaling pathways were found to be implicated in colorectal cancer development (5, 23). Therefore, further studies will be conducted to determine other potential interactions of these pathways in stroma-induced EMT in colorectal cancer cells.

**Acknowledgments**
This work was supported by grants from the National 973 Basic Research Program of China (2013CB911300, 2011CB910703, 2012CB518900), the National Science and Technology Major Project (2011ZX09302-001-01, 2012ZX09501001-003), and Chinese NSFC (81225015, 81072022, 81172173).

References


Figure legends

Figure 1

Co-culture with TAFs induces FGFR4 up-regulation

(A) The regulated proteins were analyzed by the web-based software Batch Query. The pathways showing significant changes ($P < 0.05$) are shown with the corresponding $P$ value shown at the top of the column. The FGF signaling pathway is highlighted.

(B) The protein-protein interaction network of identified proteins was analyzed using String software. A significant group of regulated proteins were found to be associated with FGFR4.

(C) RKO cells were co-cultured with TAFs or NAFs for 24 h or 48 h. Expression of FGFR4, and phosphorylation of FRS2 (Y436) and Erk (Thr202/Tyr204) were examined by immunoblot. Phosphorylation of FGFR4 was examined by immunoprecipitation using FGFR antibody followed by immunoblot using an antibody specific for p-Tyrosine.

(D) Subcellular location of FGFR4 was examined by immunocytochemistry. Scale bar, 10 μm.

(E) RKO cells were co-cultured with TAFs for indicated time. 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 15min. Phosphorylation of FRS2 (Y436) was examined by immunoblot.

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

Figure 2
Up-regulation of FGFR4 is required for TAF-induced aggressive phenotype of colorectal cancer cells

(A) RKO cells were co-cultured with TAFs for 24 h or 48 h. Expression of FGFR1, FGFR2 and FGFR3 was examined by immunoblot.

(B) RKO cells were transfected with siFGFR1, siFGFR2, siFGFR3, siFGFR4 or siNC, respectively, and then co-cultured with TAFs for 48 h. Phosphorylation of FRS2 (Y436) and Erk (Thr202/Tyr204) were examined by immunoblot.

(C) RKO 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 wound healing assay. Scale bar, 500 μm.

(D) Cell invasion was examined by Matrigel assay. Scale bar, 250 μm.

(E) Representative phase-contrast images of cell morphology of RKO cells. Scale bar, 30 μm.

(F) Expression of Snail, E-cadherin and Vimentin was examined by immunocytochemistry. Scale bar, 20 μm.

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

(H) TAFs were treated with SU5402 (10 μM) for 24 h. Phosphorylation of FRS2 (Y436) and Erk (Thr202/Tyr204) were examined by immunoblot.

(I) TAFs were transfected with siFGFR1, siFGFR2, siFGFR3, siFGFR4 or siNC, respectively. Phosphorylation of FRS2 (Y436) and Erk (Thr202/Tyr204) were examined by immunoblot.

All data were representative of at least three independent experiments.

Figure 3

FGFR4 mediates TAF-induced EMT via activating the Wnt signaling pathway
(A) RKO cells were co-cultured with TAFs or NAFs for 24 h or 48 h. TCF/LEF transcription activity was examined by Top/Fop flash. Expression of Cyclin D1 was examined by immunoblot.

(B) RKO cells were transfected with siβ-catenin or siNC, respectively, and then co-cultured with TAFs for 48 h. Expression of E-cadherin and Vimentin was examined by immunoblot.

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

(D) SW480 cells were co-transfected with β-catenin-Flag and FGFR4-Myc. Interaction between β-catenin and FGFR4 was determined by co-immunoprecipitation.

(E) SW480 cells were co-cultured with TAFs for 48 h. Interaction between endogenous β-catenin and FGFR4 was determined by co-immunoprecipitation.

(F) RKO 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 using whole cell lysate. The level of nuclear β-catenin was examined by immunoblot by using nuclear extracts. Histone H3 was used as internal control. H3, Histone H3.

(G) RKO 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.

All data were representative of at least three independent experiments. ***, P<0.001; **, P<0.01; *, P<0.05.
Figure 4

TAF-derived CCL2 is required for TAF-induced FGFR4 up-regulation

(A) The protein-protein interaction network of the regulated proteins identified by proteomic profiling was analyzed by String software. A significant group of proteins were found to be involved in CCL2-associated pathways.

(B) TAFs were co-cultured with RKO cells for 48 h (co-cultured TAFs). Expression of CCL2 in untreated NAFs, untreated TAFs and co-cultured TAFs were examined by immunoblot. The untreated NAFs, untreated TAFs and co-cultured TAFs were incubated with serum-free medium for 24 h. The CCL2 concentration in the supernatant was determined by ELISA.

(C) RKO and SW480 cells were treated with CCL2 (50 ng/ml) for 12 h. Expression of FGFR4 was examined by immunoblot.

(D) RKO cells were transfected with siCCR2 or siNC, and then co-cultured with TAFs for 48 h in presence or absence of neutralizing antibodies against CCL2 (40 μg/ml). FGFR4 promoter activity was examined by luciferase assay; expression of FGFR4 was determined by immunoblot.

(E) RKO cells were co-cultured with TAFs for 48 h in presence of neutralizing antibodies against CCL2 (40 μg/ml). TCF/LEF transcription activity was examined by Top/Fop flash. Phosphorylation of β-catenin (Y142) was examined by immunoblot.

(F) RKO cells were transfected with siEts-1 or siNC, and then co-cultured with TAFs for 48 h. FGFR4 promoter activity was examined by luciferase assay, and expression of FGFR4 was determined by immunoblot.

(G) Expression of FGF19 in TAFs and NAFs was examined by immunoblot. TAFs or
NAFs were incubated with serum-free medium for 24 h, and the concentration of FGF19 in the supernatant was determined by ELISA.

(R) RKO cells were co-cultured with TAFs for 48 h in presence or absence of neutralizing antibodies against FGF19 (30 μg/ml). Phosphorylation of FGFR4 was examined by immunoprecipitation using FGFR antibody followed by immunoblot using an antibody specific for p-Tyrosine. Phosphorylation of β-catenin (Y142) was examined by immunoblot.

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

**Figure 5**

**FGFR4 governs colorectal cancer cell metastasis in vivo**

(A) Representative images of liver metastasis. Normal RKO cells or RKO cells that were stably transfected with shNC, shFGFR4, shβ-catenin or shCCR2 were injected in the spleens of nude mice with or without TAFs.

(B) Tumor cell metastasis was examined by counting the metastatic nodules in mouse liver.

(C) Mice survival curves were plotted by the Kaplan-Meier method, and the log-rank test was used to determine the significant difference among groups.

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

**Figure 6**

**FGFR4 and its associated pathway is preferably activated in colorectal cancer**

(A) Representative images of FGFR4 immunostaining in colorectal tumor tissues and
adjacent non-cancerous tissues. Scale bar, 500 μm.

(B) Expression of FGFR4 in the primary tumors without (N0) or with (N1/N2) lymph node metastasis was analyzed. Left, overall tumors; middle, stage T1-T2; right stage T3-T4.

(C) Metastasis-free survival curves based on the FGFR4 expression in primary tumors. Left, overall tumors; middle, stage T1-T2; right stage T3-T4.

(D) Representative images of tumor cells neighbouring TAFs. Arrows, the tumor cells neighbouring TAFs with high FGFR4 expression; asterisks, the tumor cells far from TAFs with low FGFR4 expression. Scale bar, 75 μm.

(E) Representative images of concurrent expression of FGFR4 with phosphorylated β-catenin (Y142) or CCL2, respectively, in consecutive sections of colorectal cancer tissues. Scale bar, 75 μm.

(F) Linear regression analyses of immunostaining intensity between FGFR4 and CCL2 or phosphorylated β-catenin (Y142), respectively.

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

**Figure 7**

**Schematic illustrating the potential role of FGFR4 in TAF-induced metastasis in colorectal cancer**

TAF-derived CCL2 up-regulates FGFR4 in bystander colorectal cancer cells in an Ets-1-dependent manner. Subsequently, FGFR4 phosphorylates membranous β-catenin at Y142, resulting in its dissociation with α-catenin and translocation into nucleus. Nuclear β-catenin enhances expression of Snail and represses expression of E-cadherin, leading to induction of EMT in colorectal cancer cells.
Figure 1

A. Graph showing statistical significance of various signaling pathways.

B. Network diagram illustrating interactions among proteins such as FGFR4, GRB2, SEPT2, GFAP, ANXA2, FLNA, PDIA3, CTSD, TAGLN, VIM, ANXA1, and PEBP1.

C. Western blot analysis of FGFR4, β-actin, p-Tyr, FRS2, p-FRS2, and Erk.

D. Immunoprecipitation (IP) experiment with FGFR4 as bait.

E. Western blot analysis of TAF and FRS2 with TAF and NAF treatment conditions.
Liu et al., Figure 2

A

FGFR1
FGFR2
FGFR3
β-actin
TAF 0 24 48 (h)

B

p-FRS2
FRS2
p-Erk
Erk
TAF − + + + + +

C

D

E

F

G

E-cadherin
Vimentin
Snail
β-actin
TAF − + + + + +
Mock − + + + + +
FGFR4-DN − + + + + +
siNC − + + + + +
siFGFR4 − + + + + +

H

p-FRS2
FRS2
p-Erk
Erk
SU5402 − +

I

p-FRS2
FRS2
p-Erk
Erk

Solo culture TAF
Mock FGFR4-DN siNC siFGFR4

Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 2013 American Association for Cancer Research. Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Liu et al., Figure 4

A diagram showing network interactions involving proteins such as ANXA1, ANXA7, HSPA5, GFAP, CTSD, CCR2, CFL1, CCL2, TAGLN, FLNA, and TFF2.

B. Graph showing CCL2 concentration (pg/ml) with untreated TAF and co-cultured TAF conditions.

C. Western blot analysis of FGFR4 and β-actin in RKO and SW480 cells with CCL2 conditions.

D. Relative luciferase activity with TAF, IgG, anti-CCL2, siNC, and siCCR2 conditions.

E. Top/Flop Flash analysis with p-β-catenin and β-catenin conditions.

F. Relative luciferase activity with FGFR4 conditions.

G. FGF19 concentration (pg/ml) with TAF and IgG conditions.

H. Immunoblot analysis of p-Tyrosine, FGFR4, p-β-catenin, β-catenin, TAF, IgG, and anti-FGF19 conditions.
Liu et al., Figure 7

Non-metastatic tumor

Metastatic tumor

TAFs
CCL2
FGF19

Non-aggressive tumor cell

FGFR4

FGFR4

Ets-1

FGFR2

FGFR1

E-cadherin
α-catenin
β-catenin

Aggressive tumor cell
FGFR4 promotes stroma-induced epithelial to mesenchymal transition in colorectal cancer

Rui Liu, Jingyi Li, Ke Xie, et al.

Cancer Res  Published OnlineFirst August 13, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-4718

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2013/08/13/0008-5472.CAN-12-4718.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.