Introduction

Colorectal cancer 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 that not only support growth and survival of the primary tumor, but also facilitate its metastatic dissemination to distant organs (6). Tumor-associated fibroblasts (TAF) have recently been linked to a number of prometastatic capabilities, including induction of epithelial-to-mesenchymal transition (EMT) and remodeling of the extracellular matrix (ECM; ref. 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.

Materials and Methods

Coculture 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 noncancerous colonic tissues from five patients undergoing segmental colonic resection for injuries. To observe the fibroblast–cancer cell communication, we used a noncontact coculture system, as described previously (9).
Cell lines

Human colorectal cancer cell lines RKO, HCT116, LoVo, and SW480 were purchased from and tested by the American Type Culture Collection. All the cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) containing 10% fetal calf serum (Hyclone), penicillin (10^5 U/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 Materials.

Results

FGFR4 is upregulated 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 noncancerous colonic tissues (NAF). Purity of these fibroblasts was confirmed by expression of vimentin and loss of pan-Cytokeratin (Supplementary Fig. S1A). As expected, coculture with TAFs or TAF-Ms, but not NAFs, induced a stem cell-like phenotype (Supplementary Fig. S1B and 1C), enhanced both migratory and invasive capacity (Supplementary Fig. S2A–2C), and triggered significant EMT in colorectal cancer cells (Supplementary Fig. S2D and 2E). 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 cocultured with TAFs, was conducted (Supplementary Fig. S3A). A total of 41 proteins were identified with altered expression (Supplementary 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). Furthermore, 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 more than eightfold in RKO cells cocultured with TAFs compared with the solo-cultured cells (Supplementary Fig. S3B). Overexpression of FGFR4 was further validated in a series of colorectal cancer cell lines of epithelium origin by immunoblot (Fig. 1C and Supplementary Fig. S4A) and/or immunocytofluorescence analysis (Fig. 1D). As RKO and HCT116 cells showed most significant alteration in FGFR4 expression, both of these cell lines were selected for further validation. TAF-induced upregulation of FGFR4 was accompanied by increased phosphorylation of FGFR4 (Fig. 1C and Supplementary Fig. S4B), enhanced phosphorylation of FRS2, a direct substrate of FGFR4, and Erk, the major downstream effector of FGFR4 (Fig. 1C and Supplementary Fig. S4B; ref. 10), and elevated cell sensitivity to FGFR1, which uniquely binds to, and signals through FGFR4 (Fig. 1E and Supplementary Fig. S4C; ref. 11). However, compared with TAFs, NAFs were unable to induce activation of either FGFR4 or its downstream signaling pathways in colorectal cancer cell lines (Fig. 1C–E and Supplementary Fig. S4A–4C). 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 hours of coculture (Fig. 2A and Supplementary 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 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 Supplementary 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 cocultured with TAFs, accompanied with reversion to a more compact epithelium-like morphology (Fig. 2C–E and Supplementary Fig. S4F–4I). 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 and 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. Furthermore, 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 cocultured with TAFs, compared with cells solo-cultured or cocultured with NAFs (Fig. 3A and Supplementary Fig. S5A). Furthermore, repression of Wnt signaling by siRNA targeting β-catenin markedly abrogated TAF-induced EMT in these two cell lines (Fig. 3B and Supplementary Fig. S5B), suggesting that Wnt signaling is required for TAF-induced metastasis of colorectal cancer cells.

Next, we investigated whether FGFR4 plays a role in TAF-induces 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 cocultured with TAFs (Fig. 3C and Supplementary Fig. S5C).

Furthermore, 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 that are likely to be involved. 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, immunoprecipitation detected an FGFR4–β-catenin complex in SW480 cells, and such a complex was accumulated when the cells were cocultured with TAFs (Fig. 3D and E). Furthermore,
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 Supplementary Fig. S5D; ref. 16). Furthermore, coculture with TAFs markedly enhanced β-catenin Y142 phosphorylation, which could be largely abolished on 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 upregulation**

To explore the mechanisms responsible for TAF-induced FGFR4 upregulation, bioinformatics analysis was employed to highlight the key regulatory factors on the basis of 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 upregulation. As shown in Fig. 4B, TAFs showed a markedly elevated expression of CCL2, compared with NAFs. Notably, coculture with colorectal cancer cells further enhanced the CCL2 production in TAFs. Furthermore, addition of CCL2 in the culture induced a dramatic upregulation 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 Supplementary Fig. S5F), as well as the subsequent phosphorylation of β-catenin Y142 (Fig. 4E). In addition,
we investigated whether Ets-1 was involved in FGFR4 upregulation. As shown, siRNA-mediated suppression of Ets-1 dramatically abolished TAF-induced FGFR4 overexpression (Fig. 4F and Supplementary Fig. S5F). These results suggested that TAF-derived CCL2 was required for TAF-induced FGFR4 upregulation in an Ets-1-dependent manner.

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

It has been reported that FGFR4 is a specific FGFR4 activator that has been implicated in colorectal cancer development (5). Therefore, we next examined the expression of FGFR4 in TAFs and NAFs. As shown in Fig. 4G, expression of FGFR4 was enhanced in TAFs compared with NAFs, revealed by both immunoblot and ELISA. Furthermore, inhibition of FGFR4 by a neutralizing antibody decreased TAF-induced phosphorylation of both FGFR4 and β-catenin (Fig. 4H), suggesting that FGFR4 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). Histopathologic analysis confirmed that the liver metastatic nodules were composed of cancer cells, but not fibroblasts (Supplementary 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 and 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 clinicopathologic parameters in clinical samples. Clinicopathologic information for the clinical samples is summarized in Supplementary Table S2. FGFR4 immunoreactivity was more...
intense in cancer tissues compared with noncancerous adjacent tissues \( (P < 0.0001; \text{Fig. 6A}) \). Furthermore, a high level of FGFR4 expression was more likely to be associated with lymph node metastasis \( (\text{Fig. 6B}) \) and poor outcome \( (\text{Fig. 6C}) \). In addition, we analyzed the correlation between the distribution of fibroblasts and the expression of FGFR4. As shown in Fig. 6D, the tumor cells adjoining the stromal fibroblasts displayed strong FGFR4 immunoreactivity, whereas 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 \( (Y142; \text{Fig. 6E and F}) \). 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 cross-talk between tumor cells and TAFs remain largely unknown. In this study, we show that FGFR4 is a potential regulator of this process. FGFR4, but not other FGFRs, was markedly upregulated in colorectal cancer cells cocultured with TAFs. Inhibition of FGFR4 attenuated TAF-induced intracellular signaling cascades, including phosphorylation of Erk and FRS2. Furthermore, 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 the 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 shown 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 with NAFs. Furthermore, treating colorectal cancer cells with CCL2 triggered FGFR4 expression. In contrast, inhibition of the CCL2–CCR2 axis attenuated TAF-induced FGFR4 upregulation and subsequent activation of Wnt signaling, as well as liver metastasis of colorectal cancer cells in a mouse model. In addition, Ets-1, a downstream transcription factor of CCL2, seemed to be involved in this process: siRNA-mediated knockdown of Ets-1 markedly suppressed TAF-induced FGFR4 upregulation. These results suggest that the CCL2 signaling pathway plays crucial roles in early steps of TAF-induced colorectal cancer metastasis by mediating FGFR4 upregulation.

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 show 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 adjacent to the fibroblasts, and was positively correlated with expression of CCL2 or phosphorylated β-catenin \( (Y142) \), respectively. These data suggest that FGFR4 and its associated pathways are 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 (PDGFR). 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.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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