FMNL2 Enhances Invasion of Colorectal Carcinoma by Inducing Epithelial-Mesenchymal Transition

Yufa Li1,2, Xiling Zhu1,4, Yuanfeng Zeng1,3, Jianmei Wang1,3, Xiaojing Zhang1,3, Yan-qing Ding1,3, and Li Liang1,3

Abstract

FMNL2 is a member of diaphanous-related formins that control actin-dependent processes such as cell motility and invasion. Its overexpression in metastatic cell lines and tissues of colorectal carcinoma has been associated with aggressive tumor development in our previous study. But its specific role in cancer is largely unknown. Here we report that FMNL2 is involved in epithelial-mesenchymal transition (EMT) maintenance in human colorectal carcinoma cells. A positive correlation between FMNL2 and vimentin expression and an inverse correlation between FMNL2 and E-cadherin expression were found in colorectal carcinoma cell lines and cancer tissues. Specific knockdown of FMNL2 led to an epithelial-state transition, confirmed by the cobblestone-like phenotype, upregulation of E-cadherin, α-catenin, and γ-catenin, and downregulation of vimentin, snail, slug. Loss of FMNL2 expression lowered the ability of TGF-β to induce cell invasion and EMT, as shown by morphology and the expression levels. Upregulation of vimentin, slug, snail, downregulation of E-cadherin and activation of receptor-Smad3 phosphorylation were observed in M5 and MDCK cells induced by TGF-β, whereas altered expression of these markers was not obvious in FMNL2-depleting M5 cells. High levels of activation of p-MAPK and p-MEK, but not p-PI3K and p-AKT, were observed in SW480/FMNL2+ cells compared with control cells. Treatment with U0126 could abrogate the activation of p-MAPK and p-MEK, whereas LY294002 treatment had no effect on the PI3K/AKT pathway. In conclusion, these findings identify a novel EMT and tumor promoting function for FMNL2, which is involved in TGF-β–induced EMT and colorectal carcinoma cell invasion via Smad3 effectors, or in collaboration with MAPK/MEK pathway.

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pathway involved in EMT during colorectal carcinoma progression.

Materials and Methods

Cell culture

Human CRC cell lines SW620, SW480, and HT29 with differing metastatic abilities were obtained from American Type Culture Collection (ATCC). SW480/M5 cell line is a subline of SW480 with enhanced ability for hepatic metastasis developed after consecutive in vivo selection at our laboratory (20). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (GIBCO) with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) in humidified 5% CO2 atmosphere at 37°C. For TGF-β and inhibitors treatment, 10 μmol/L MEK inhibitor U0126 or 10 mmol/L PI3K inhibitor LY294002 (Cell Signal Technology) was added in the cultured cells every 2 days. The cells were stimulated by 10 ng/mL human recombinant TGF-β1 (Peprotech) diluted with serum-free medium containing 2 g/L bovine serum albumin for time periods of 12, 24, and 48 hours.

Patients and tissue specimens

This study was conducted on a total of 168 paraffin-embedded samples collected retrospectively from archival material stored at the Department of Pathology, Southern Hospital (Guangzhou, China). Samples included 30 cases of normal colorectal mucosa, 93 cases of CRC tissues, 45 cases of metastatic lymph nodes, and 10 cases of metastatic livers. All tissues obtained were reviewed by at least 2 experienced pathologists and examined for the presence of tumor cells. Pathologic diagnosis and classification were made based on the system of the International Union Against Cancer. The research protocol was approved by the Ethics Committee at Nanfang Hospital, and consent was obtained from all patients for the study.

Immunohistochemistry

Sections were deparaffinized, rehydrated, and endogenous peroxidase was inhibited with 0.3% H2O2 methanol. For antigen retrieval, slides were boiled in 0.01 mol/L (pH 6.0) sodium citrate buffer for 15 minutes in a microwave oven. After blocking with 5% normal goat serum, primary anti-FMNL2 monoclonal antibody (Abnova Corporation), anti–E-cadherin (BD Corporation), and anti-vimentin (Thermo Fisher Corporation) in blocking buffer (1:50) were applied and the slides were incubated at 4°C overnight. Following incubation with biotinylated secondary antisera, the streptavidin-biotin complex/horse radish peroxidase was applied. Finally, the visualization signal was developed with 3,3'-diaminobenzidine tetrahydrochloride, and slides were counterstained with hematoxylin. The immunohistochemically stained tissue sections were reviewed and scored separately by 2 pathologists blinded to the clinical parameters. The scoring approach used in the assessment of immunostaining was in accordance with a relatively simple and reproducible protocol (21). The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). Extent of staining was scored as 0 (0%), 1 (1% to 25%), 2 (26% to 50%), 3 (51% to 75%), and 4 (76% to 100%), according to percentages of the positive staining areas in relation to the whole cancer area or entire section for the normal samples. The sum of the intensity and extent score was used as the final staining score. Tumors having a final staining score of 3 or more were considered positive. The staining of FMNL2 was assessed as follows: (−) meant a final staining score of less than 3; (+) a final staining score of 3; (+ +) a final staining score of 4; and (+ + +) a final staining score of 5 or more.

Real-time PCR assays

Total RNA was extracted using Trizol reagent (Invitrogen) and cDNA was synthesized by oligo dT primed reverse transcription from 2 μg of total RNA using an access RT system (Promega). Reverse transcription was carried out for 20 minutes at 42°C. Real-time PCR was performed using Mx3000P Real-time PCR System (Stratagene) and SYBR PremixEx TaqTM (TaKaRa), using the following thermal cycling profile: predenaturation at 95°C for 10 seconds, followed by 30 cycles of amplification (95°C for 30 seconds, predicted TM value for 30 seconds, 72°C for 30 seconds). The dissociation curve analysis was used to validate the amplification of a single product. Each reaction consisted of 12.5 μL SYBR Premix ExTaq, 2 μL cDNA template, 0.5 μL each primer set (10 μmol/L), and 9.5 μL nuclease-free water. Relative quantity of target transcripts in each sample was expressed as n-fold differences relative to control, or 1 × sample, and according to the equation: ΔΔCt = [Ct(target gene) – Ct(GAPDH)]experimental group – [Ct(target gene) – Ct(GAPDH)]control group. Each sample was tested 3 times and all other quantities were expressed as an n-fold difference relative to the corresponding control group. Gene-specific primers were as follows: FMNL2 (sense, 5'-TGG CAT CGT GTA TCG AGG CTA A-3', 175bp); E-cadherin (sense, 5'-TTA AAC TCC TGG CCT CAA GCA ATC-3', 139bp); vimentin (sense, 5'-TGA GTA CCG GAG ACA GGT GCA G-3', 119bp).

Western blot

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice in RIPA buffer (1 × PBS, 1% NP-40, 0.1% SDS, 5 mmol/L EDTA, 0.5% sodium deoxycholate and 1 mmol/L sodium orthovanadate) with protease inhibitors and quantified by bichinonic acid method. Protein lysates (50 μg) were resolved on 6% SDS polyacrylamide gel, electrotransferred to polyvinylidene fluoride membranes (ImmobilonP; Millipore) and blocked in 5% nonfat dry milk in Tris-buffered saline, pH 7.5 (100 mmol/L NaCl, 50 mmol/L Tris, and 0.1% Tween-20). Membranes were immunoblotted overnight at 4°C with anti-FMNL2 monoclonal antibody (Abnova Corporation), anti–α-catenin and anti–β-catenin (BD Corporation); anti-slug and anti-snail (Abcam Corporation); anti–E-cadherin and anti–β-catenin (BD Corporation); anti–N-cadherin (BD Corporation); anti–integrin αv and anti–integrin β1 (BD Corporation); anti–fibronectin (BD Corporation); anti–vitronectin (BD Corporation); anti–vitamin D receptor (Abcam Corporation); anti–insulin-like growth factor-1 receptor (BD Corporation); anti–platelet-derived growth factor receptor (BD Corporation); anti–c-Met (Abcam Corporation); anti–epidermal growth factor receptor (BD Corporation); and anti–caspase-3 (BD Corporation).
anti-MAPK, anti–p-MAPK, anti-MEK, anti–p-MEK, anti-PI3K, anti–p-PI3K, anti-AKT, and anti–p-AKT (Cell Signaling Technology, Inc.); anti-GAPDH antibody (Santa Cruz Biotechnology), followed by their respective horse-radish peroxidase-conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence (Pierce).

RNA interference

We designed 2 different siRNA duplexes of FMNL2 (GenBank NM_001904): siRNA1 (sense, 5′-GAT CCG CTG GAG CGA TTT GCC ATT CAA GAC GTG GCA AAT CCG TTC TTC AGT TTT TTG TCG ACA-3′); siRNA2 (sense, 5′-GAT CCG ACC TCC TGA CAA AGC CTT CAA GAC GGG CTT TGT CAG GAT GTA GGT TTT TTG TCG ACA-3′). The 2 pairs of double-stranded DNA were cloned into plasmid vector Pgenesil-1. DNA was purified with a Mini Plasmid Purification Kit (Qiagen Co. Ltd.) and digested with appropriate restriction enzyme, SalI. Fragments were electrophoresed on 1% agarose to verify the insertion of sequences. For transfection, SW480/M5 and SW480 cell lines were divided into negative control group and 2 test groups. Approximately 1 × 10⁶ cells per well were plated in 6-well plates in medium containing 10% FBS to grow overnight to 90% confluency. All cells were transfected with 3 μg of plasmids using Lipofectamine2000 according to instructions (Invitrogen). After 2 days, cells were selected for stable transfection by 800 ng/mL neomycin (800 ng/mL). Independent colonies resistant to neomycin were picked up and subcultured in 14 days of G418 selection. Western blot was performed to examine the knockdown of FMNL2 expression.

Construction and transfection of FMNL2 recombinant plasmids

Because the coding region of FMNL2 is very long with the length of 3279bp, which makes transfection difficult, we constructed the functional FMNL2-CT vector. Miralles et al. (22) reported that the FH2 domain of Dia1 could promote stress fiber formation and transcriptional activation of the MAL/SRF pathway through its actin-polymerizing activity. The functional FH1-FH2-DAD (CT) domain of the MAL/SRF pathway through its actin-polymerizing activity. The functional FH1-FH2-DAD (CT) domain of FMNL2 was amplified by nested PCR and then inserted into the pcDNA3/Flag (Invitrogen). Outer primers: forward 5′-CAT TGT CCT CCA TGG CAG CAG AAG T-3′, reverse 5′-GTC AGT TTC ATT CAC GCA CA-3′; inner primers: forward 5′-AAC GGT ACC CCC TGG CCC CCT CCT CCA CC-3′, reverse 5′-CCA CTC GAG TCA CAT TGT TAT TTC GGC ACC-3′. The PCR conditions were as follows: 95°C for 3 minutes, followed by 30 cycles of amplification (94°C for 30 seconds, 55°C for 40 seconds with outer primers or 68°C for 40 seconds with inner primers, 72°C for 2 minutes). The correct coding regions of all plasmids were confirmed by sequencing. Transient transfection was performed using Lipofectamine (Invitrogen) as vehicle according to the manufacturer’s instructions. The plate was incubated for 48 hours until it was ready for further assay.

In vitro invasion assay

In vitro invasive ability was tested by Boyden Chamber assay. Invasion Chamber has 8-μm pores in the polyethylene terephthalate (PET) membrane, which is coated with matrigel (BD Biosciences). First, the invasion chambers were rehydrated with RPMI 1640 (serum free) for 2 hours at 37°C in 50 mL/L CO₂ atmosphere. RPMI 1640 with 100 mL/L FBS was added to the lower compartment as the chemotactic factor. Then 1.5 × 10⁵ tumor cells in serum-free Dulbecco’s Modified Eagle’s Medium were added to the upper compartment of the chamber. Each cell group was plated in 3 duplicate wells. After incubation for 48 hours, the noninvasive cells were removed with a cotton swab. Cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed with methanol and stained with hematoxylin. Finally, the cells in lower compartment of the chamber that had invaded the lower sides of the PET membrane were counted under a light microscope in 5 random visual fields (×200).

Statistical analysis

Statistical analysis was carried out using SPSS 13.0 for Windows. Results were presented as mean ± SD. Statistical differences of immunohistochemistry (IHC) were calculated using Kruskal-Wallis and Mann-Whitney test. The independent samples t test was used for real-time RT-PCR. Statistical differences between groups were evaluated by 1-way analysis of variance. Least-significant difference (LSD test) was used for multiple comparisons. All P values for multiple testing were corrected by Bonferroni correction. P < 0.05 was considered statistically significant.

Results

Expression status of FMNL2, E-cadherin, and vimentin in CRC cell lines and tissues

To determine the involvement of FMNL2 in EMT of CRC cell lines, we first examined the expressions of FMNL2, EMT epithelial maker E-cadherin, and mesenchymal maker vimentin in 4 human CRC cell lines with different metastatic abilities. Among the cells studied in Figure 1A, SW620 cells were originally established from the lymph node metastasis in a patient with colon adenocarcinoma, whereas SW480 cells were derived from primary colon adenocarcinoma. SW480/M5 cells were established by in vivo selection and had the high hepatic metastatic ability (20). Results of real-time PCR indicated that the expression of FMNL2 and vimentin in highly metastatic SW620 and SW480/M5 cell lines was higher than in lowly metastatic SW480 and HT29 cells, whereas E-cadherin was inversely expressed (P < 0.05; Fig. 1A). Western blot analysis confirmed the similar changes in these cell lines (Fig. 1B). Results of cell IHC showed that in the lowly metastatic SW480 and HT29 cell lines, FMNL2 and vimentin were expressed at low levels. But highly metastatic SW620 and SW480/M5 cells had increasing expression of vimentin and reduced expressions of E-cadherin (Fig. 1C).
To investigate the involvement of FMNL2 in EMT in vivo, we then examined the expression status of FMNL2, E-cadherin, and vimentin in archived paraffin-embedded human CRC tissues, normal tissues, metastatic lymph nodes and livers. The expression of FMNL2 was gradually increased in normal mucosa, CRC tissues, metastatic lymph nodes and livers. The expression of FMNL2 in CRC tissues was higher than in normal mucosa \((P < 0.01)\) and high levels of FMNL2 expression were observed in metastatic lymph nodes and livers compared with those in CRC tissues \((P = 0.017; \text{Fig. 2A, Table 1})\). Expression of E-cadherin was mostly found in normal mucosa, whereas there seemed to be very low levels of FMNL2 in the control tissues. The expression of E-cadherin was decreased or even lost in CRC tissues and lymphatic and hepatic metastases. E-cadherin was expressed more highly in normal mucosa than in CRC tissues \((P < 0.01)\). But there was no significant difference in E-cadherin expression between CRC tissues and metastatic lymph nodes or livers \((P = 0.187; \text{Fig. 2B and C; Table 2})\). Vimentin was found dominantly in stromal cells around the carcinoma cells. Occasionally, positive signal of vimentin was observed in CRC tissues with poor differentiation and lymphatic and hepatic metastases. The expression of vimentin in CRC tissues was higher than in normal mucosa \((P < 0.01)\). Higher levels of vimentin in metastatic lymph nodes and livers were seen

![Figure 1. Expression status of FMNL2, E-cadherin, and vimentin in colorectal carcinoma cell lines. A, real-time RT-PCR analyses of FMNL2, E-cadherin, and vimentin in highly metastatic SW620, SW480/M5 cells and less metastatic SW480, HT29 cells. The relative mRNA levels with the use of control SW480 were normalized to 1. Values were given as mean and SD from more than 3 independent experiments when compared with control cells. *, \(P < 0.05\). B, Western blot analyses of FMNL2, E-cadherin, and vimentin in 4 cell lines. GAPDH was shown as a control. C, immunohistochemical analyses of FMNL2, E-cadherin, and vimentin in 4 cell lines.](image-url)
Figure 2. Expression status of FMNL2, E-cadherin, and vimentin in colorectal carcinoma tissues by immunohistochemistry. Serial sections of normal mucosa (A), colorectal carcinoma tissues (B), metastatic lymph nodes (C), and metastatic livers (D). The expression of FMNL2 was gradually increased in normal mucosa, CRC tissues, metastatic lymph nodes, or livers. Expression of E-cadherin was mostly found in normal mucosa, and decreased or even lost in colorectal carcinoma tissues and lymphatic or hepatic metastases. Vimentin was found dominantly in stromal cells around the carcinoma cells. Occasionally, positive signal of vimentin was observed in colorectal carcinoma tissues with poor differentiation and lymphatic or hepatic metastases. (3,3'-Diaminobenzidine, ×200)

Table 1. Expressions of FMNL2 in normal mucosa, CRC tissues, and metastatic lymph nodes and livers

<table>
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<td>0</td>
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<td>LN metastasis</td>
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<td>22</td>
<td>6</td>
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<td>Hepatic metastasis</td>
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<td>3</td>
<td>4</td>
<td>3</td>
<td>10\textsuperscript{d}</td>
</tr>
</tbody>
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\textsuperscript{a}Z = −5.700, P = 0.000.
\textsuperscript{b}Z = −5.243, P = 0.000 (Mann-Whitney Test); χ^2 = 48.559, V = 4, P = 0.000 (Kruskal-Wallis Test).
\textsuperscript{c}Z = −2.990, P = 0.017.
\textsuperscript{d}Z = −3.230, P = 0.026.
\textsuperscript{e}Z = −2.455, P = 0.014.
than in CRC tissues \( (P = 0.037; \text{Fig. 2D; Table 3}) \). Two-

variance of Pearson correlation analysis showed that the

expression of FMNL2 was negatively correlated with that of

e-cadherin \( (r = -0.338, P = 0.000) \), but positively

correlated with that of vimentin \( (r = 0.349, P = 0.000) \).
The above results suggest that enhanced expression of

FMNL2 is at least partly correlated with EMT in CRC

cell lines and tissues.

Requirement of FMNL2 for maintaining the

mesenchymal state of CRC cells

To determine whether FMNL2 plays a contributory

role in CRC invasion by inducing EMT, we knocked
down FMNL2 using 2 specific siRNAs. Western blot

analysis confirmed that FMNL2 were depleted by the

specific knockdown in SW480/M5 and SW480 cells

(Fig. 3A). Cell death, which was assessed by trypan blue

staining, did not increase in these knockdown cells

(Fig. 3B). We observed that SW480/M5 and SW480

cells developed morphologic changes under the FMNL2

knockdown (Fig. 4A). As shown by phase contrast image,

the FMNL2-depleting CRC cells appeared with a cob-

blestone-like phenotype, whereas SW480/M5 and

SW480 cells, maintained their spindle shaped and fibro-

blastic appearance in monolayer culture. We observed

frequent appearance of multiple clusters of adherent cells

under FMNL2 knockdown. The morphologic changes

imply that the FMNL2-depleting cells have undergone

transdifferentiation from mesenchymal cells to epithelial

cells (MET).

Then we detected the expressions of MET markers

following FMNL2 silencing. E-cadherin was upregulated

in both FMNL2-depleting cells compared with mock cells,

whereas vimentin was downregulated in FMNL2-depleting

cells by real-time RT-PCR \( (P < 0.05) \), suggesting a switch to

the epithelial state under FMNL2 knockdown (Fig. 3C and

D). Western blot analysis further showed that the expres-
sion of E-cadherin was increased in FMNL2-depleting M5

and SW480 cells, whereas that of vimentin was decreased.

Another 2 EMT epithelial markers \( \alpha \)-catenin and \( \gamma \)-catenin

were also found to be increasingly expressed in FMNL2-
depleting cells. In addition, FMNL2 knockdown caused decrease of 2 EMT-stimulating tran-
scription factors Snail and Slug in FMNL2-depleting cells.

Table 2. Expressions of E-cadherin in normal mucosa, CRC tissues, and metastatic lymph nodes and livers

<table>
<thead>
<tr>
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<td>0</td>
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\(^a\) \( Z = -6.585, P = 0.000 \).

\(^b\) \( Z = -6.213, P = 0.043 \) (Mann-Whitney Test); \( \chi^2 = 146.243, V = 4, P = 0.000 \) (Kruskal-Wallis Test).

\(^c\) \( Z = -2.019, P = 0.043 \).

\(^d\) \( Z = -5.875, P = 0.031 \).

\(^e\) \( Z = -5.320, P = 0.000 \).

Table 3. Expressions of vimentin in normal mucosa, CRC tissues, and metastatic lymph nodes and livers

<table>
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\(^a\) \( Z = -5.630, P = 0.000 \).

\(^b\) \( Z = -6.693, P = 0.000 \) (Mann-Whitney Test); \( \chi^2 = 59.896, V = 4, P = 0.000 \) (Kruskal-Wallis Test).

\(^c\) \( Z = -2.086, P = 0.037 \).

\(^d\) \( Z = -3.792, P = 0.000 \).

\(^e\) \( Z = -4.382, P = 0.017 \).
These data suggest that FMNL2 is actively involved in maintaining EMT in CRC cells.

FMNL2 is involved in TGF-β–induced EMT and CRC cell invasion

Multiple signal transduction pathways have been identified to be involved in the induction of EMT. Previous reports showed that TGF-β signaling promotes the metastasizing and invasive properties of some cancers, presumably by inducing EMT of the cells (23). TGF-β elicits EMT and in vivo metastasis via Smads and complementary non-Smad effectors, such as Rho GTPases and p38 MAPK (24). In CRC, it was reported that TGF-β itself is overexpressed (25). To elucidate the relationship between FMNL2 and TGF-β signaling in CRC cells, we analyzed the effect of recombinant TGF-β on SW480/M5, SW480, and their corresponding FMNL2-depleting cells. First, we examined the potential of TGF-β to stimulate M5 cell invasion by Boyden chamber assays. Invasion through the ECM is an important step in tumor metastasis. The results showed that at the time of 24 or 48 hours, the cultured M5/FMNL2− cells penetrating the artificial basement membrane (28.34 ± 8.72 or 47.44 ± 5.42) were less than in M5 (83.02 ± 5.739 or 100.12 ± 8.865; P < 0.01; Fig. 4B). TGF-β stimulation can enhance invasive abilities of both cells (Fig. 4B). There was a 2.5-fold induction of tumor cell invasion in M5/FMNL2− cells and an approximate 6-fold induction in M5 cells by TGF-β (Fig. 4C). It indicates that repression of endogenous FMNL2 reduces CRC cell invasion and strongly diminishes the stimulatory effects of TGF-β.

Furthermore, TGF-β treatment for a period of 48 hours induced an EMT in the canine kidney derived epithelial cell line (MDCK) and M5 and SW480 cells, as illustrated by the acquisition of a fibroblast-like phenotype. MDCK is commonly used in EMT study and can undergo typical morphologic changes of EMT induced by TGF-β (26). MDCK cells appeared spindle-shaped and fibroblastic-like instead of the cobblestone-like phenotype induced by TGF-β. FMNL2-depleting cells represented mixed fibroblast-like
and epithelial cells, and obviously had less fibroblast-like cells than mock cells (Fig. 4A). Loss of FMNL2 expression lowered the ability of TGF-β to induce a spindle-like cell shape.

Second, we detected the altered expressions of EMT markers induced by TGF-β. At the mRNA level, TGF-β–induced EMT over a period of 24 or 48 hours was reflected by upregulation of vimentin and downregulation of E-cadherin in M5 and MDCK cells compared with those without TGF-β stimulation (P < 0.05; Fig. 5A and C). TGF-β stimulation resulted in a dramatic increase of FMNL2 expression in M5 cells (P < 0.05; Fig. 5A). In contrast, the FMNL2-depleting M5 cells showed no obvious expression changes of EMT under treatment of TGF-β (P > 0.05; Fig. 5B). At the protein level, there was low expression of E-cadherin and high expression of vimentin, transcription factor snail, and slug in M5 and MDCK cells on TGF-β stimulation. These cells also showed rapid and sustained activation of receptor–Smad3 phosphorylation whereas the expression levels of Smad3 was not affected by TGF-β treatment, even after 48 hours postapplication (Fig. 5D). However, the expression changes of these markers were not obvious in M5/FMNL2– cells as compared with those in M5 and MDCK cells (Fig. 5D). Reduced cancer cell migration and invasion were associated with reduced EMT, and loss of FMNL2 expression lowered the ability of TGF-β to induce EMT, as shown on the morphologic and the expression levels. This evidence makes it reasonable to presume that FMNL2 is involved in TGF-β–induced mesenchymal state in CRC cells.

**Ras-MAPK pathway is involved in FMNL2-induced EMT**

In our previous study, the gene expression profile between FMNL2-depleting M5 cells and M5 cells revealed significant downregulation of PI3K, AKT, MAPK, and MEK genes in FMNL2-depleting cells. Analysis of the signaling network on the chip data indicated that PI3K/
AKT and MAPK/MEK signaling pathways may be involved in the downstream regulation of FMNL2 (data not shown). To test the involvement of the 2 pathways in EMT induced by FMNL2, we prepared exogenous FMNL2-overexpressing SW480 cells, in which FMNL2 expression was at low level, and then treated them with MEK inhibitor U0126 or PI3K inhibitor LY294002 for 2 days. Compared to SW480/mock cells, the expression of FMNL2 mRNA was higher in SW480/FMNL2 cells, but lower in FMNL2-depleting SW480 cells as compared by real-time RT-PCR (*P < 0.05; Fig. 6A). Western blot analysis showed that high levels of activations of p-MAPK and p-MEK, but not p-PI3K and p-AKT, were observed in SW480/FMNL2 cells compared with control cells, with no change in the amount of total protein. Knockdown of FMNL2 gene in SW480 cells resulted in the reduced expressions of the 4 phosphorylated proteins, whereas the expression levels of total protein were not affected. We also detected upregulation of vimentin and downregulation of E-cadherin in SW480/FMNL2 cells compared with control cells, whereas FMNL2 knockdown showed the opposite effects (Fig. 6B). Treatment of SW480/FMNL2 cells with U0126 could abrogate the activation of p-MAPK and p-MEK and the expression changes of EMT marker vimentin and E-cadherin, whereas LY294002 treatment had no effect on the PI3K/AKT pathway (Fig. 6B). The results suggest that Ras-MAPK pathway is involved in FMNL2-induced EMT.

Discussion

FMNL2 is a member of the DRFs that are key regulators of the actin cytoskeleton exerted through their forming homology (FH2) domains and act as effectors of Rho family GTPases (15). Recently, DRFs have been shown to be involved in essential cellular processes such as cytokinesis, cell movement, and polarity, which are frequently deregulated during pathologic situations such as tumor cell transformation and metastasis (27). Three members of the DRF family (DRF1 – DRF3) are reported to be required for invadopodia formation and invasion of breast tumor cells (28). FMNL2 was identified as a candidate metastasis associated gene in our previous study (19). However, the precise role of FMNL2 in mediating tumor cell invasion and metastasis has not been investigated.

Figure 5. FMNL2 is involved in TGF-β–induced EMT of colorectal carcinoma. Real-time RT-PCR analyses of FMNL2, E-cadherin, and vimentin in TGF-β–induced EMT at the time periods of 24 and 48 hours. The data were represented with SW480/M5 (A), FMNL2-depleting M5 (B), and MDCK cells (C). The relative mRNA levels with the use of control cells were normalized to 1. Values were given as mean and SD from more than 3 independent experiments when compared with control cells. *, *P < 0.05. D, Western blot analyses of vimentin, E-cadherin, p-Smad3, Smad3, snail, and slug in TGF-β–induced EMT at the time periods of 24 and 48 hours. SW480/M5, FMNL2-depleting M5, and MDCK cells were used. GAPDH was shown as a control.
EMT is characterized by the disassembly of cell-cell contacts, reorganization of the actin cytoskeleton, and cell–cell separation by reduced E-cadherin expression and β-catenin relocalization. As defined by Hay (29), EMT results in the complex loss of epithelial traits (including loss of E-cadherin, plakoglobin, and cytokeratins, and the dismantling of adherens junctions and desmosomes), accompanied by the total acquisition of mesenchymal characteristics, including expression of vimentin, N-cadherin, fibronectin, and an elongated “fibroblast-like” morphology with capacity to execute invasive motility (30). E-cadherin expression is irreversibly lost in invasive lobular breast cancer (31). The cytoplasmic/nuclear relocalization of β-catenin from the adherens and tight junctions is a common process in the EMT associated with tumor invasion (32). This transition is considered to be an important event during malignant tumor progression and metastasis (33, 34).

To determine the involvement of FMNL2 with EMT of CRC, we first detected the expressions of FMNL2, E-cadherin, and vimentin in 4 metastatic CRC cell lines and CRC tissues. In highly metastatic SW620 and SW480/M5 cells, FMNL2 and vimentin were expressed at higher levels, whereas E-cadherin was inversely expressed. The expression of FMNL2 was gradually increased in normal mucosa, CRC tissues, metastatic lymph nodes, and livers. Two-variance of Pearson correlation analysis showed that the expression of FMNL2 in normal mucosa, CRC tissues, and lymph node metastases was negatively correlated with those of E-cadherin and CK, whereas positively correlated with that of vimentin. These observations suggest that enhanced expression of FMNL2 is at least partly correlated with EMT in CRC cell lines and tissues.

Then we showed that FMNL2 conferred EMT to CRC epithelial cells, with gain of biological features consistent with neoplastic transformation and invasiveness. Knockdown of FMNL2 in SW480/M5 cells could induce the conversion of spindle-shape mesenchymal morphology to cobblestone-like epithelial morphology. Consistent with the morphologic change, E-cadherin, α-catenin, and γ-catenin were increased whereas vimentin was reduced or even lost during the transition. Transcriptional factors such as snail (35), slug (36), and Twist (37) were unveiled as key regulators in induction of EMT. We discovered that FMNL2 knockdown also inhibited the expression of snail and slug.

Several growth factors such as TGF-β, hepatocyte growth factor (HGF), and matrix metalloproteinase (MMP-3) have been reported to induce EMT and to be associated with a more invasive phenotype in pancreatic cancer (38-40). TGF-β was first described as inducer of EMT in normal MECs (5) and is now recognized as a master regulator of EMT in a variety of cell types and tissues (6). In various tumor cell line, TGF-β signaling induces snail, slug, and SIP1, which may then proceed to repress the expression of E-cadherin (35).

Our study reveals that FMNL2, in association with the TGF-β signaling pathway, is required for maintenance of invasive ability and the mesenchymal state in CRC cells. TGF-β stimulation can enhance the invasive abilities of SW480/M5 and M5/FMNL2- cells. Repression of
FMNL2 reduced M5 cell invasion under unstimulated conditions and strongly diminished the stimulatory effects of TGF-β. Reduced cancer cell migration and invasion were associated with reduced EMT. TGF-β treatment for a period over 24 and 48 hours induced an EMT changes in MDCK and SW/480/M5 cells, as illustrated by the acquisition of a fibroblast-like phenotype, downregulation of E-cadherin, upregulation of vimentin, and activation of receptor–Smad3 phosphorylation. But the expression changes of these markers were not obvious in M5/FMNL2− cells compared with those in M5 and MDCK cells. Loss of FMNL2 expression lowered the ability of TGF-β to induce EMT, which suggests that FMNL2 contributes to the acquisition of a mesenchymal and highly migrating phenotype in CRC cells induced by TGF-β.

The transduction pathway involved in EMT is very complex. Tyrosine kinase receptors get activated and mediate downstream signaling after binding to various ligands such as EGF and TGF-β. EMT occurs by hyperactivation of 1 pathway or, more probably, by simultaneous activation of more pathways, which either lead to Snail-mediated downregulation of the E-cadherin gene, as Ras–Raf–MEK–MAPK, PI3K–Akt, TGF–β–Smads, and Wnt–β-catenin signaling do, or directly affects cell adhesion and/or the cytoskeletal dynamics, as accomplished by Src, TGF–β–Par6–Smurf1, and Rho GTPases. Thereby, activation of a single molecular pathway can lead to 1 or more features of EMT (41). PI3K/Akt-dependent signaling pathways served to regulate hypoxia-induced EMT of hepatocellular carcinoma cells (42). Also well documented was the fact that the Ras-Raf-MAPK pathway played an indispensable role in EMT induced by activation of receptor tyrosine kinase of growth factors such as HGF, VEGF, EGF, and bFGF (18, 43). Finally, we determined the signaling pathways that FMNL2 may participate in EMT according to our previous cDNA chip data. Only activation of p-MAPK and p-MEK, but not p-PI3K and p-AKT, were observed in SW480/FMNL2 cells, with no change in the amount of total protein. Upregulation of vimentin and downregulation of E-cadherin were also seen in SW480/FMNL2− cells, whereas FMNL2 knockdown showed the opposite effects. Treatment with U0126 could abrogate the activation of p-MAPK and p-MEK, and the expression changes of EMT marker vimentin and E-cadherin, whereas LY294002 treatment had no effect on the PI3K/AKT pathway. The results suggest that Ras-MAPK pathway is involved in FMNL2-induced EMT.

Taken together, our studies indicate that (1) the enhanced expression of FMNL2 is at least partly correlated with EMT in CRC cell lines and tissues; (2) FMNL2 is required for maintaining the mesenchymal state of CRC cells; (3) FMNL2 is involved in TGF–β–induced EMT and CRC cell invasion via Smad3 effectors; (4) Ras–MAPK pathway is also involved in FMNL2-induced EMT. These findings identify a novel EMT and tumor promoting function for FMNL2 in metastasis of colorectal carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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