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ORIGINAL ARTICLE

Fas signaling promotes motility and metastasis through epithelial–mesenchymal transition in gastrointestinal cancer

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Fas signaling was reported to participate in cell apoptosis. However, this pathway has also been shown to promote tumor cell motility, leading to the hypothesis that Fas signaling may induce epithelial–mesenchymal transition (EMT) to promote metastasis. The effects of Fas-ligand (FasL) treatment and inhibition of Fas signaling on colorectal and gastric cancer cells were tested using motility assay, immunofluorescence, RT–PCR and immunoblot analyses. Fas signaling downregulated epithelial markers, upregulated mesenchymal markers and promoted motility in gastrointestinal (GI) cancer cells. FasL treatment also increased the expression of EMT transcriptional factors in the nucleus and induced a spindle shape cell morphology in these cells. Knockdown of Snail or Twist expression significantly decreased FasL-induced motility. The ERK1/2 pathway was activated by Fas signaling and is required for FasL-induced EMT and motility. Moreover, oxaliplatin, a chemotherapeutic agent, induced EMT partly through Fas signaling. Evaluation of human GI clinical specimens showed that FasL expression increased whereas E-cadherin expression decreased during GI cancer progression. Both markers were significantly inversely correlated. Tissue samples with a non-EMT phenotype were mainly distributed in patients with early cancer stages, whereas samples with an EMT phenotype were mostly distributed in patients with advanced cancer stages. A non-EMT phenotype significantly correlated with better prognosis. Altogether, these data indicate that Fas signaling may induce EMT to promote tumor motility and metastasis in GI cancer *in vivo* and *in vitro*.

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Keywords: Fas signaling; metastasis; motility; epithelial–mesenchymal transition

INTRODUCTION

Fas (APO-1/CD95), belongs to the TNF and NGF transmembrane receptor superfamily, and activates caspase-dependent apoptosis in susceptible cells when triggered by its cognate ligand, FasL/CD95L. After Fas-ligand (FasL) binding, the Fas receptor induces recruitment of caspase-8, FADD, and caspase-10 into a complex, which leads to activation of other procaspases, such as caspase-3, and, subsequently cell death.[1] FasL/Fas system can also induce non-apoptotic events in cells, including regulation of cell-cycle progression,[2] cytokine and chemokine expression,[3,4] tumor growth,[5] motility in apoptosis-resistant cancer cells[6] and liver regeneration.[7] A recent study demonstrated that Fas engagement could induce either a survival or death signal depending on the strength of the stimulus.[8] Low-dose FasL treatment is sufficient to induce non-apoptotic signaling events. Moreover, Fas stimulation in apoptosis-resistant cancer cells can activate various cellular signaling pathways to induce non-apoptotic events, including modulation of NFκB,[6] MAPK[4,6] and PI3K/AKT.[9]

Gastrointestinal (GI) cancer is one of the leading causes of cancer death in the United States.[10] The prognosis for advanced colorectal cancer (CRC) remains dismal, mainly because of the propensity for metastasis and resistance to chemotherapy.[11] Chemotherapeutic drugs can cause upregulation of FasL and Fas, which contributes to the elimination of tumor cells by Fas-induced apoptosis.[12] However, many tumor cells are still resistant to Fas-mediated apoptosis after chemotherapy.[12] Progression or metastasis occurred in 26% of CRC patients after chemotherapy.[13] Therefore, it is possible that chemotherapy-mediated upregulation of FasL and Fas induces tumor proliferation and metastasis in a subset of patients that are resistant to treatment.

Epithelial–mesenchymal transition (EMT)and mesenchymal–epithelial transition is a good model to explain how solid tumors metastasize from the site of origin to a new site, although this theory is still under debate.[14] EMT occurs by a series of orchestrated events in which cell–cell and cell–extracellular matrix interactions are altered, and the transition from an epithelial to a mesenchymal phenotype allows for cell movement.[15,16] During cancer progression, advanced stage cancer cells frequently show downregulation of epithelial markers, which leads to loss of epithelial polarity, intercellular junctions and reduced intercellular adhesion, and these alterations are often accompanied by increased cell motility and expression of mesenchymal markers,[17] indicating EMT process may probably be involved in cancer metastasis. Some cytokines and growth factors can trigger the EMT process. Among them, transforming growth factor-β1 is the most common one that can induce EMT in many epithelial cell types.[17] Recently, it has been reported that in addition to enhancing proliferation, Fas signaling may induce the EMT process allowing for invasion and metastasis in murine apoptosis-resistant colon cancer.[18] Although these data were

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informative, it still needs to be carefully verified in human cancer *in vivo* and *in vitro*.

Fas signaling can promote motility in different apoptosis-resistant cancer cells,[6] therefore, in this study we examined whether Fas signaling can promote motility in GI cancer cells and the possible mechanisms required. The novelty of this study is that we use low-dose FasL stimulation and establish the relationship between Fas signaling and the EMT process *in vivo* and *in vitro*. We found that Fas signaling could induce EMT to promote motility and metastasis *in vivo* and *in vitro*. Furthermore, oxaliplatin, a chemotherapeutic agent, induced EMT, at least in part, by Fas signaling. Hence, any treatment aimed at inducing tumor apoptosis through Fas signaling should be administered cautiously, as this pathway may induce aggressive tumor traits.[18]

RESULTS

Expression of FasL and Fas in GI cancer cell lines

FasL and Fas expression were detected by flow cytometry in all GI cell lines maintained in our lab. We also detected the expression of antiapoptotic proteins, including Bcl-2, Bcl-xl, FLIP and XIAP. There was moderate Fas expression but undetectable FasL expression in DLD1,HT29, HCT116, SW480 and AGS cells (Supplementary Table 1). These cell lines were epithelial-like in morphology (data not shown). In contrast, SW620 and LOVO cells had moderate FasL and little Fas expression (Supplementary Table 1), and were fibroblast-like in morphology (data not shown). SW480, DLD1, AGS and SW620 cells were used for further study. Low-dose FasL (12.5 ng/ml) did not affect the growth of SW480, DLD1 and AGS cells (Supplementary Figure 1). However, moderate-dose FasL (25 ng/ml) induced apoptosis in DLD1 and AGS cells but not in SW480 cell whereas high-dose FasL (50 ng/ml) induced apoptosis in three cells (Supplementary Figure 1). Meanwhile, FasL inhibitor, NOK-1, didnot affect the growth of SW620 cells (Supplementary Figure 1).

Fas signaling promotes migration and invasion in GI cancer cell lines

As Fas signaling promotes motility in apoptosis-resistant cell lines,[6] we determined whether low-dose FasL (12.5 ng/ml)[19] treatment would promote motility in GI cancer cell lines. Our results show that FasL treatment promotes invasion in SW480 (Figure 1a), DLD1 (Figure 1b) and AGS (Supplementary Figure 2A) cells. In addition, pretreatment with NOK-1 inhibits the FasL-induced motility of SW620 cells (Figure 1e). When Fas was stably knocked-down in SW480 (Figure 1c), DLD1 (Figure 1d) and AGS (Supplementary Figure 2B) cells, FasL treatment could no longer enhance motility. Knockdown of FasL by shRNA also significantly decreased the invasion capability of SW620 (Figure 1e) compared with control cells. Migration assays were also conducted and results were consistent with those of the invasion assay (Supplementary Figure 3). Moreover, cell growth curves were performed under the same experimental conditions and no differences in cell proliferation rates were found (data not shown), excluding any differences in proliferation rates contributing to the above results.

Tumor cells most likely encounter FasL in two different forms *in vivo*: soluble FasL (sFasL) in the serum and membrane-bound FasL (mFasL). mFasL can be found *in vivo* on different types of cells, including tumor-infiltrating lymphocytes. To test whether human mFasL can also induce an increase in motility, we incubated DLD1 or AGS cells with fixed SW620 cells, which express human mFasL, and performed invasion chamber assays. Contact with cells expressing mFasL significantly increased the motility of both DLD1 (Figure 1f) and AGS (Supplementary Figure 2C) cells, and this activity could be fully inhibited by NOK-1 pretreatment, indicating only Fas signaling functions to promote DLD1 and AGS cells motility, not other cytokines secreted by SW620 or other mechanism caused by SW620.

Fas signaling induces EMT to promote motility in GI cancer cell lines

The EMT process can promote motility in tumor cells by downregulating epithelial (CDH1) and upregulating mesenchymal markers (Vimentin).[17] To test whether Fas signaling can induce EMT, we performed RT–PCR(Supplementary Table 2, Supplementary Figure 4) and immunoblot (Figure 2) to detect the expression of EMT markers in GI cancer cells after Fas activation. Both assays showed that Fas signaling inhibits epithelial markers (E-cadherin, Villin and Occludin) and enhances mesenchymal markers (Vimentin and Snail) in SW480 (Figure 2a), DLD1 (Figure 2b) and AGS (Supplementary Figure 5A) cells. Whereas inhibition of FasL by pretreatment with NOK-1 (Figure 2c) or FasL shRNA expression (data not shown) in SW620 cells induced upregulation of epithelial markers and downregulation of mesenchymal markers. The expression of matrix metalloproteinases (MMP9 and MMP2), which promote cell invasion, increased significantly in the culture supernatant after FasL treatment (Figure 2d and Supplementary Figure 5B). When Fas expression was downregulated by shRNA, FasL treatment could not induce EMT in SW480, DLD1 or AGS cells (data not shown). Additionally, immunofluorescence analysis confirmed the changes in expression of EMT markers in CRC cells (Figure 3a). SW480 and DLD1 cells changed from a cobblestone-like morphology to a spindle shape in a monolayer culture after FasL treatment for 3 days (Figure 3b). Altogether, these data indicate that Fas signaling can induce EMT in GI cancer cells.

Next, we determined whether the FasL-induced increase in motility requires EMT in GI cancer cells. EMT is primarily mediated by transcriptional factors that inhibit epithelial markers and/or increase mesenchymal markers. Snail or Twist was stably knocked-down using a lentiviral shRNA-expressing construct in SW480, DLD1 and AGS cells. FasL treatment couldnot induce expression of Snail (Figure 4a, Supplementary Figure 6A) or Twist (Figure 4b, Supplementary Figure 6B) in cells expressing the respective shRNA. The motility of cells expressing *Snail* or *Twist* shRNA was significantly less than cells transduced with control nonsense shRNA after FasL stimulation (Figures 4c and d, Supplementary Figures 6C and 6D). Moreover, a significant increase in motility was noted in FasL-treated cells expressing *Snail* or *Twist* shRNA compared with untreated cells expressing *Snail* or *Twist* shRNA. Therefore, Fas signaling-induced EMT seems required for increasing the motility of GI cancer cells by different transcriptional factors.

Fas signaling activates ERK1/2 to induce EMT and promote motility in GI cancer cells

Fas signaling may trigger various cellular pathways to induce non-apoptotic events.[4,6,9] Therefore, we performed immunoblot analysis to determine whether the MAPK, NFkB or PI3K/AKT pathways are activated in GI cancer cells after FasL treatment. We found that ERK1/2 and p38 were activated in SW480 cells after FasL stimulation (Figure 5a), but only ERK1/2 were activated in AGS cells (Supplementary Figure 7A). Cells were then pretreated with U0126 (10 μM) before FasL treatment to inhibit ERK1/2 activation. Immunoblot and immunofluorescence results show that ERK1/2 inhibition suppresses Fas-induced EMT (Figure 5b and d, and Supplementary Figure 7B) and motility (Figure 5c and Supplementary Figure 7C) in SW480 and AGS cells. These data suggest that activation of ERK1/2 is indispensable for FasL-induced EMT and promotes motility in GI cancer cells. However, inhibition of p38 activation by pretreatment with SB203580 (10 μM) in SW480 cells did not affect FasL-induced EMT and motility (Supplementary Figure 8A and 8C). Pretreatment with an NFkB inhibitor (BAY 11-7082, 10 μM) also had no effect on

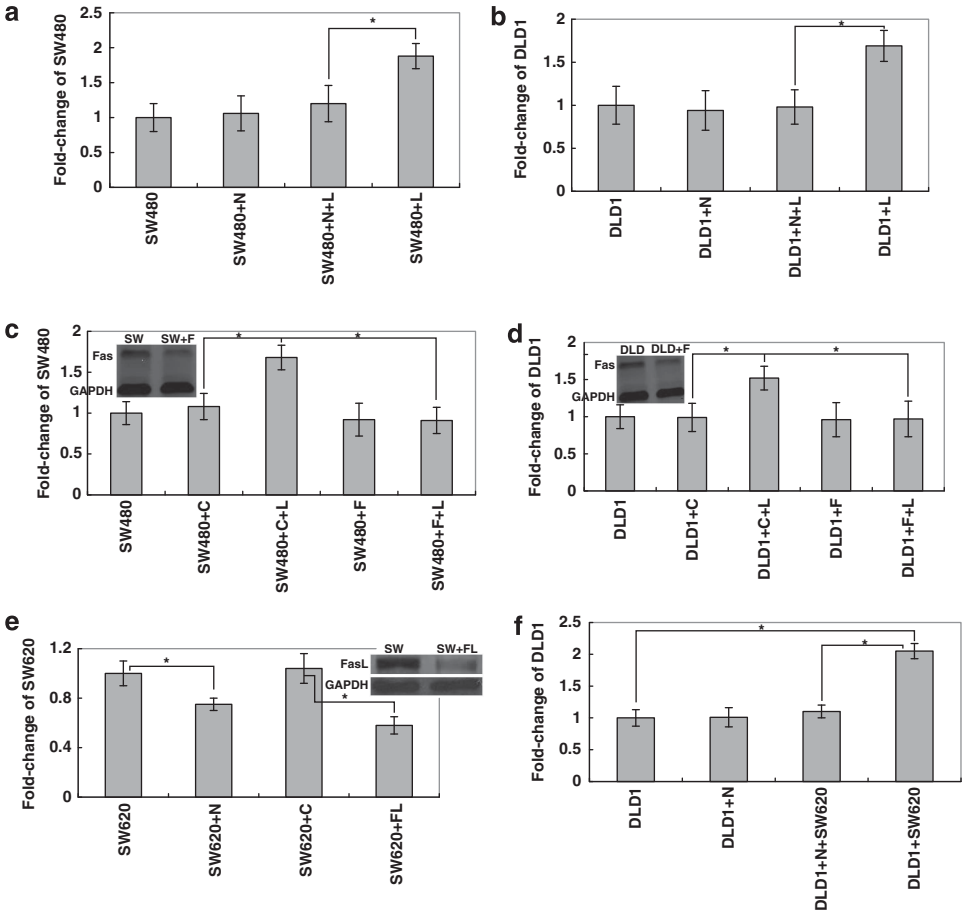

Figure 1. Fas signaling promotes invasion in CRC cell lines. SW480 (**a**) or DLD1 (**b**) cells were incubated with sFasL(12.5 ng/ml) for 3 days or treated with NOK-1 before sFasL treatment, and invasion assays were performed. sFasL promoted invasion in both cell lines and this activity was inhibited by NOK-1 pretreatment. SW480 (**c**) or DLD1 cells (**d**) were transduced with control or *Fas* shRNA-expressing constructs, treated with sFasL and invasion assays were performed. Expression of *Fas* shRNA prevented sFasL-mediated invasion in both cell lines. (**e**) SW620 cells were transduced with control or *FasL* shRNA-expressing constructs, or treated with NOK-1 for 3 days. NOK-1 treatment and FasL knockdown inhibited invasion in SW620 cells. (**f**) SW620 cells, which contain mFasL, and DLD1 cells (control, do not contain mFasL) were fixed in 4% paraformaldehyde and then individually mixed with live/unfixed DLD1 cells at a ratio of 2:1 (SW620 (fixed):DLD1 (live) = 40 000:20 000, DLD1 (fixed):DLD1 (live) = 40 000: 20 000) and invasion assays were performed. The expression of mFasL on SW620 cells promoted invasion of DLD1 cells. Treatment of SW620 cells (fixed) with NOK-1 before incubation with DLD1 cells (live) prevented the increased invasion. All data are represented as fold-change ± s.d. compared with control cells. Experiments were performed in triplicate. C, control shRNA (non-coding shRNA); F, *Fas* shRNA; FL, *FasL* shRNA; L, sFasL; N, NOK-1. *$P < 0.05$.

FasL-induced EMT and invasiveness (Supplementary Figure 8B and 8D). Moreover, a broad caspase inhibitor (Z-VAD-FMK, 20 μM) didnot inhibit FasL-induced ERK1/2 activation and induction of EMT (data not shown).

Oxaliplatin treatment induces EMT partly through Fas signaling in CRC cells

Chemotherapy is commonly used in CRC patients before and after surgery, which can sensitize cancer cells to apoptosis by upregulating Fas and FasL.[12] However, progression or metastasis may also occur in some CRC patients after chemotherapy.[13] Therefore we examined whether chemotherapeutic drugs reported to enhance expression of FasL and Fas, specifically oxaliplatin,[20] can induce EMT through Fas signaling. We treated SW480 and HT29 cells with oxaliplatin (2 μM, the plasma concentration in patients) for 3 days and found that expression of Fas and FasL increased (Figures 6a and b) and EMT markers expression changed accordingly (Figures 6c and d). Pretreatment with NOK-1 partially inhibits oxaliplatin-induced EMT (Figures 6c and d), indicating that oxaliplatin can induce EMT by Fas signaling but that this is not the only mechanism by which oxaliplatin induces EMT in these cells. Furthermore, oxaliplatin can induce EMT in SW480 cells stably expressing *Fas* shRNA (Figure 6e), further indicating that other mechanisms besides Fas signaling are required for oxaliplatin-induced EMT.

Fas signaling may induce EMT and promote metastasis in GI cancer cells *in vivo*

To investigate whether Fas signaling can induce EMT and promote metastasis *in vivo*, paraffin-embedded samples from 188 CRC and 143 gastric cancer (GC) patients were analyzed by immunohistochemistry (IHC). Immunoreactivity scoring showed FasL expression increased, whereas E-cadherin expression decreased during CRC and GC progression (Figure 7a, Supplementary Table 3 and 4). Expression of these two markers was significantly inversely correlated (Figure 7b, Supplementary Table 5), suggesting that FasL might induce EMT *in vivo*. As the expression of E-cadherin, an important EMT marker, decreases during the EMT process, we defined FasL(−/+)/E-cadherin(++/+++) as non-EMT phenotype samples and FasL(++/+++)/E-cadherin(−/+)

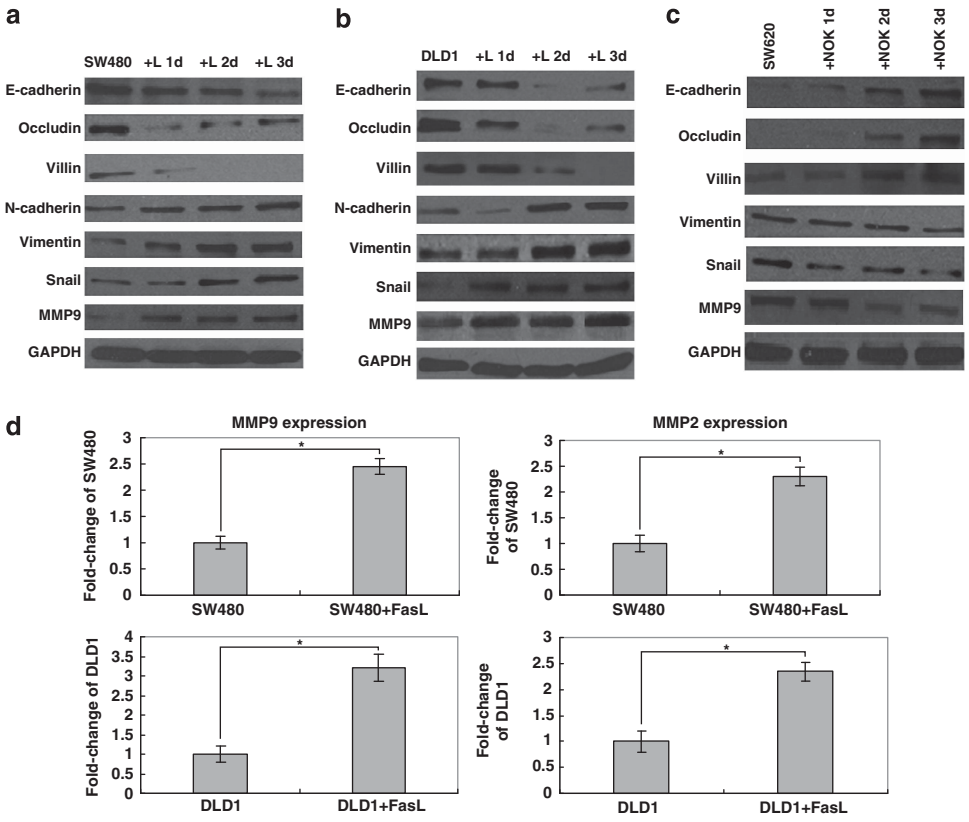

Figure 2. Fas signaling induces EMT in CRC cells. (**a**, **b**) Cells were serum-starved for 24 h before stimulation and then treated with FasL for the indicated time points and western blot analyses were performed. Expression of epithelial markers decreased in SW480 (**a**) and DLD1 (**b**) cells, whereas expression of mesenchymal markers increased. (**c**) SW620 cells were seeded at 50% confluence and cultured with NOK-1 for 3 days. Inhibition of Fas signaling in SW620 cells induced expression of epithelial markers and downregulated expression of mesenchymal markers. (**d**) Cells were serum-starved for 24 h then treated with FasL for 3 days. Cell supernatant was collected and ELISA was performed to detect levels of MMP2 and MMP9 secretion. MMP2 and MMP9 secretion significantly increased in SW480 and DLD1 cells. All data are represented as fold-change ± s.d. compared with control cells. Experiments were performed in triplicate. L, FasL. $^*P < 0.05$.

as EMT phenotype samples. Coincidently, non-EMT samples were mainly distributed in the early cancer stages (CRC: Dukes' stage A and B, GC: pStage I) whereas EMT samples were mainly distributed in the advanced stages (CRC: Dukes' stage C and D, GC: pStage II–IV, Figure 7c, Supplementary Table 6), implying that FasL-induced EMT might promote metastasis *in vivo*. Moreover, expression of EMT markers in clinical samples from GI cancer patients was also detected by immunoblot. We found that, in general, FasL and Vimentin expression increased whereas E-cadherin expression decreased during cancer progression (Figure 7d, Supplementary Figure 9 and 10), which was consistent with the IHC results. Finally, we wanted to determine whether EMT marker expression can indicate prognosis. Better prognosis was significantly noted in patients with non-EMT phenotype CRC (Figure 7e) and GC cancers (Supplementary Figure 11). FasL(−/+)/E-cadherin(−/+) or FasL(++/+++)/E-cadherin(++/+++) scoring was not able to provide prognostic information. No difference in CRC Dukes' stage (data not shown) or prognosis in CRC patients (Figure 7f) or GC patients (data not shown) was observed between these two groups.

DISCUSSION

In addition to its ability to induce apoptosis, Fas signaling can also induce non-apoptotic events in tumor cells, such as regulation of tumor growth[5] and motility.[6] In this study, we aimed to investigate whether Fas signaling could enhance motility in GI cancer cells and the possible mechanism by which Fas signaling promotes motility. In summary, we found that Fas signaling-induced EMT to promote motility *in vivo* and *in vitro*.

As reported, high-dose FasL (25–50 ng/ml) can induce apoptosis while low-dose FasL can induce non-apoptotic signal events, such as proliferation in gastric adenocarcinoma cells and rat gastric mucosa cells.[8,19] However, high-dose FasL was also shown to promote motility in apoptosis- and chemotherapy-resistant cancer cells.[6,21] In this study, we used low-dose FasL to promote motility in GI cancer cells (SW480, DLD1 and AGS). Altogether, both sFasL and mFasL could promote motility in these cell lines that are not apoptosis or chemotherapy resistant.

Fas signaling can promote motility, which leads to metastasis, in GI cancer cells but the mechanism is still unknown. The EMT and mesenchymal–epithelial transition model is a good way to explain how solid tumors metastasize from the site of origin to a new site, although this theory is controversial.[14] Whether FasL, like transforming growth factor-β1,[17] can induce EMT in human GI cancer cells to promote metastasis needed further investigation. To test this hypothesis, we treated SW480, DLD1 and AGS cells that were cobblestone-like in cell culture with FasL and found that epithelial markers decreased whereas mesenchymal markers increased over time.This change was Fas-dependent. Fas signaling is also required to maintain an EMT phenotype in SW620 to some extent. These data suggest that Fas signaling can induce EMT in GI cancer cells. Transcriptional factors, such as Snail and Twist, have an important role in induction of EMT.[17] Herein, we noticed that Fas-signaling induced

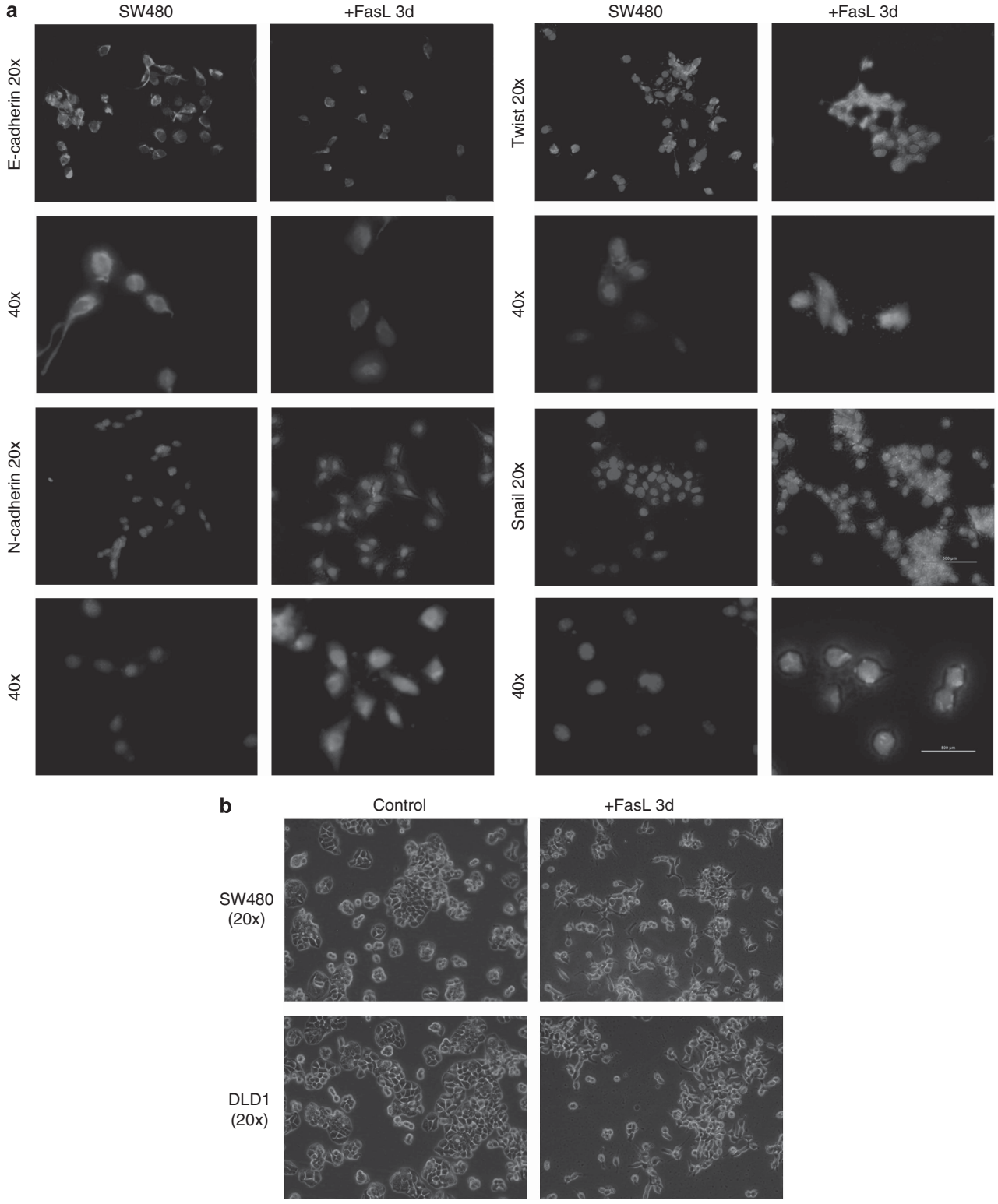

Figure 3. Fas signaling induces EMT and morphological changes in CRC cells. Cells were serum-starved for 24 h and then treated with FasL for 3 days. (**a**) Immunoflourescent analysis was performed to determine expression of epithelial and mesenchymal markers. The expression of N-cadherin increased in the membrane and cytoplasm, expression of E-cadherin decreased, and Snail and Twist expression increased in the nucleus after FasL treatment. (**b**) Microscopic analysis of cells after FasL treatment. Both SW480 and DLD1 cells transformed from a cobblestone-like appearance to a spindle and fibroblast shape in a monolayer culture after FasL treatment.

EMT and promoted motility through different EMT transcriptional factors. This model is similar to the transforming growth factor-β-induced EMT process.[22]

Fas stimulation in cancer cells may activate various cellular signaling pathways, such as NFκB,[6] MAPK[4–6] and PI3K/AKT,[9] to induce non-apoptotic events. In this study, FasL treatment

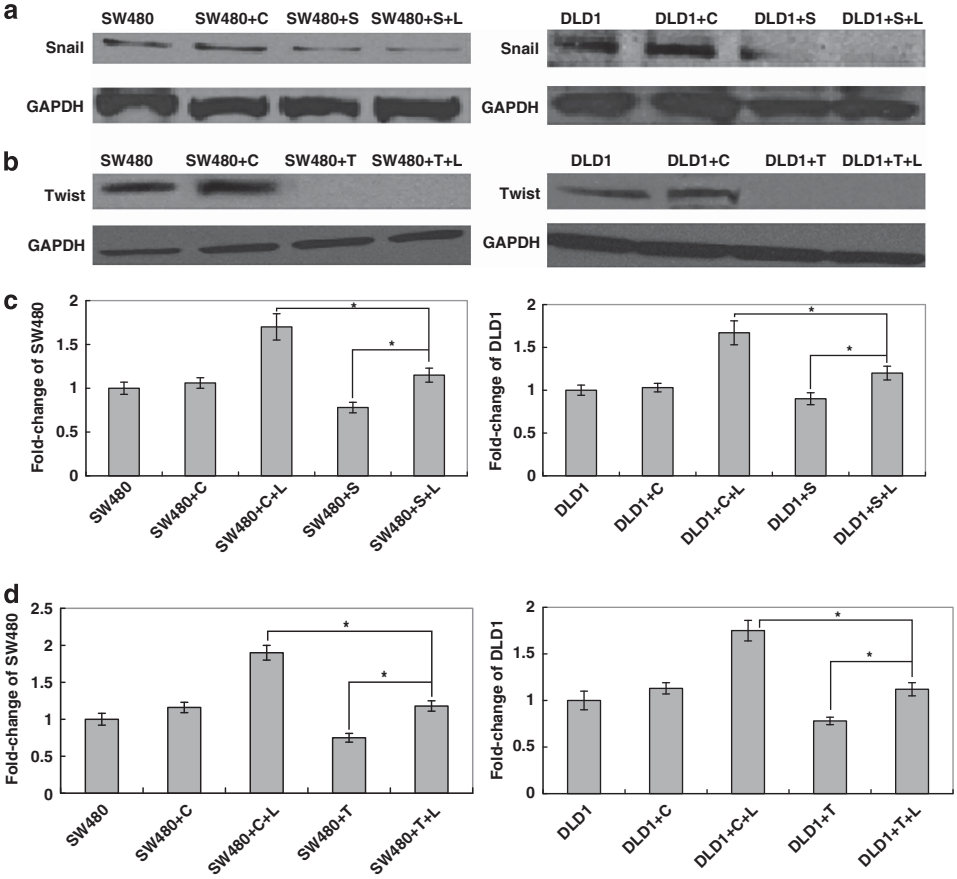

Figure 4. Loss of Snail or Twist expression prevents FasL-induced EMT and cell invasion in CRC cells. *Snail* (**a**, **b**) or *Twist* (**b**, **d**) was stably knocked-down by lentiviral shRNA expression in CRC cells, cells were treated with FasL for 3 days, and western blot analysis or invasion assay was performed. (**a**, **b**) Western blot analysis showed that transduction of cells with shRNA constructs prevented protein expression of the respective gene. (**c**, **d**) Invasion assays showed that there was a significant increase in motility after FasL treatment in cells expressing *Snail* or *Twist* shRNA compared with untreated cells (SW480 + S, DLD1 + S, SW480 + T and DLD1 + T).However, FasL-induced motility was significantly less in cells expressing *Snail* and *Twist* shRNA compared with cells expressing control non-coding shRNA. (**c**, **d**) Data are represented as fold-change ± s.d. compared with control cells. Experiments were performed in triplicate. C, control shRNA; L, FasL; S, *Snail* shRNA; T, *Twist* shRNA. $^*P < 0.05$.

activated ERK1/2 and p38 in SW480 cells and ERK1/2 in AGS cells. This result is similar to the finding that Ras-RAF-MAPK pathway has an important role in EMT mediated by receptor tyrosine kinases induced by growth factors, such as HGF, VEGF and EGF.[23,24] It has been reported that the PI3K/AKT signaling pathway can control hypoxia-induced EMT in hepatocellular cancer[25]and promote Fas-induced invasion in apoptosis-resistant glioblastoma cell,[9] whereas the NFκB pathway was shown to be activated by Fas signaling to promote motility in apoptosis-resistant breast cancer cells.[6] Furthermore, Fas signaling-induced JNK activation contributed to tumor growth in mouse and human samples.[5] It seems that FasL induces different signaling pathways in different tumor cells. In addition, a broad caspase inhibitor did not inhibit ERK1/2 activation and the subsequent EMT process. These results are consistent with reports that activation of ERK1/2 by Fas is independent of its death domain.[8,26]

Most chemotherapeutic drugs can sensitize cancer cells to apoptosis by upregulating Fas and/or FasL expression (mFasL and sFasL), including 5-Fu, oxaliplatin, cisplatin and mitomycin.[12,27] Furthermore, oxaliplatin treatment was shown to induce EMT in CRC cells.[21,28] It was reported oxaliplatin-chemoresistant colon cancer cells could increase expression of Fas and FasL and acquire a more aggressive phenotype compared with their parental cells.[21,28] This aggressive phenotype was dependent on Fas signaling. However, the authors didnot confirm the relationship between Fas signaling and EMT in these cells. Moreover, whether the invasive phenotype mediated by chemotherapeutics was a direct or indirect effect is still unknown; it is probably an integrated effect. To address this issue in our model, we used NOK-1 or Fas shRNA to inhibit Fas signaling in CRC cells before oxaliplatin treatment and found that oxaliplatin-induced EMT was partly dependent on Fas signaling. These data indicate the possible mechanism by which chemotherapies promote tumor progression and metastasis.

To verify our results *in vivo*, IHC was used to analyze expression of FasL and E-cadherin in GI cancerparaffin-embedded samples. Some reports have shown FasL expression increased[29] and E-cadherin decreased[30]during CRC progression, however, this is the first reported study where the expression of these two markers were examined in the same GI cancer samples. We found that both markers inversely correlated in GI cancer specimens, suggesting FasL might induce EMT *in vivo*. The Fas receptor is frequently downregulated during cancer progression, but complete loss of Fas is rarely observed in human cancers.[5,31] In fact, many cancer cells express large quantities of Fas (SW480, DLD1 and AGS) and are highly sensitive to Fas-mediated apoptosis *in vitro*.[5] In addition, tumors from cancer patients frequently have elevated levels of FasL.[5] All of these data raise the possibility that Fas signaling can serve functions to induce non-apoptotic events *in vivo*, such as EMT.

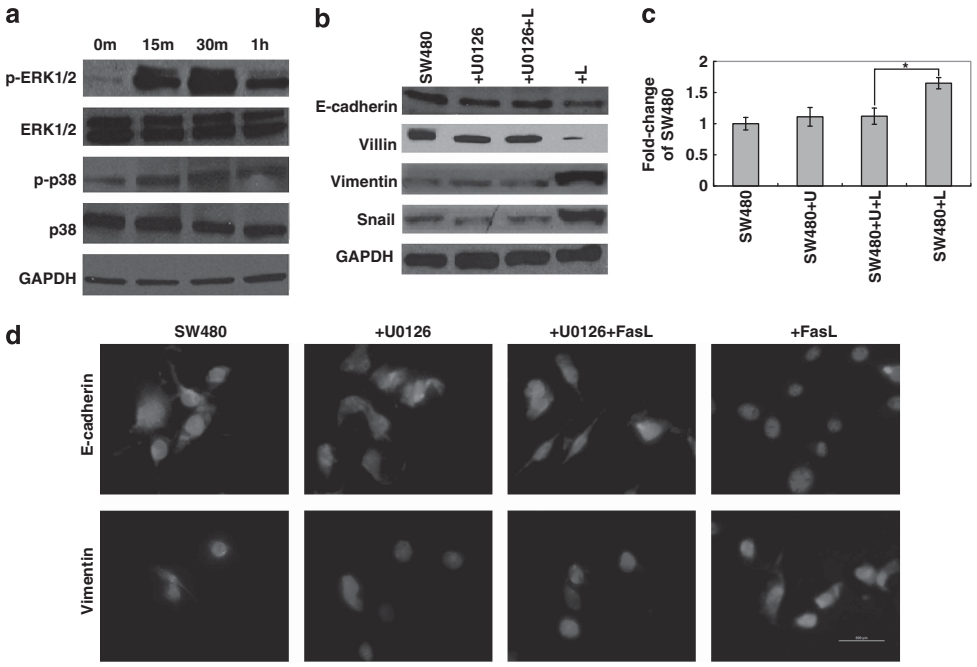

Figure 5. FasL-mediated activation of ERK1/2 is required to induce EMT and promote motility in CRC cells. SW480 cells were serum-starved for 24 h, treated with FasL for 3 days and western blot (**a**, **b**), invasion assay (**c**) or immunoflourescence (**d**, $\times$ 400) analysis was performed. ERK1/2 and p38 were activated by FasL treatment (**a**). Pretreatment with U0126(10 μM) inhibited EMT (**b**, **d**) and cell motility (**c**). Invasion assay results (**c**) are represented as fold-change ± s.d. compared with control cells. Experiments were performed in triplicate. L, FasL; U, U0126. *$P < 0.05$.

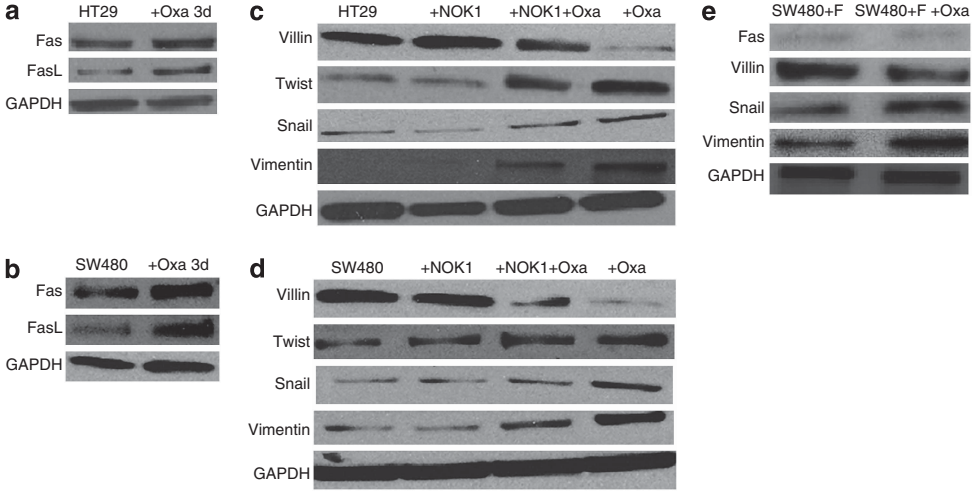

Figure 6. Oxaliplatin treatment induces EMT partly through Fas signaling in CRC cells. HT29 (**a**) and SW480 (**b**) cells were treated with oxaliplatin (2 μM) for 3 days. Oxaliplatin increased the expression of Fas and FasL in these cells. HT29 (**c**) and SW480 (**d**) cells were pretreated with NOK-1 and then treated with oxaliplatin. NOK-1 pretreatment partially inhibited the oxaliplatin-induced EMT process. NOK-1 pretreatment did not affect the expression of EMT markers. (**e**) SW480 cells were stably transduced with a *Fas* shRNA-expressing vector. Oxaliplatin was able to induce EMT in these cells when Fas was knocked-down. Experiments were performed in triplicate. F, *Fas* shRNA; Oxa, Oxaliplatin.

In summary, we demonstrated that Fas signaling can induce EMT to promote motility in GI cancer. Further investigation should be focused on whether Fas signaling can induce EMT and how Fas signaling regulates EMT in other tumor types.

MATERIALS AND METHODS

Cell culture and reagents

All human GI cancer cell lines (Supplementary Table 1) were obtained from American Type Culture Collection (Manassas, VA) and routinely maintained in our lab. These cell lines were cultured in RPMI 1640 with 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum in a humidified 5% CO_2 atmosphere at 37 °C. Serum-free medium was used in most experiments unless otherwise indicated. FasL (Alexis, San Diego, CA, USA) was used at 12.5 ng/ml and the FasL inhibitor, NOK-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 10 μg/ml. Inhibitors were added into the culture medium 2 h before treatment. All detailed information for reagents and antibodies is listed in the Supplementary Information.

Cell line transfection

Lentiviral shRNA vectors (Santa Cruz Biotechology) targeting human Snail, Twist, Fas and FasL were utilized for stable knockdown in GI cancer cells

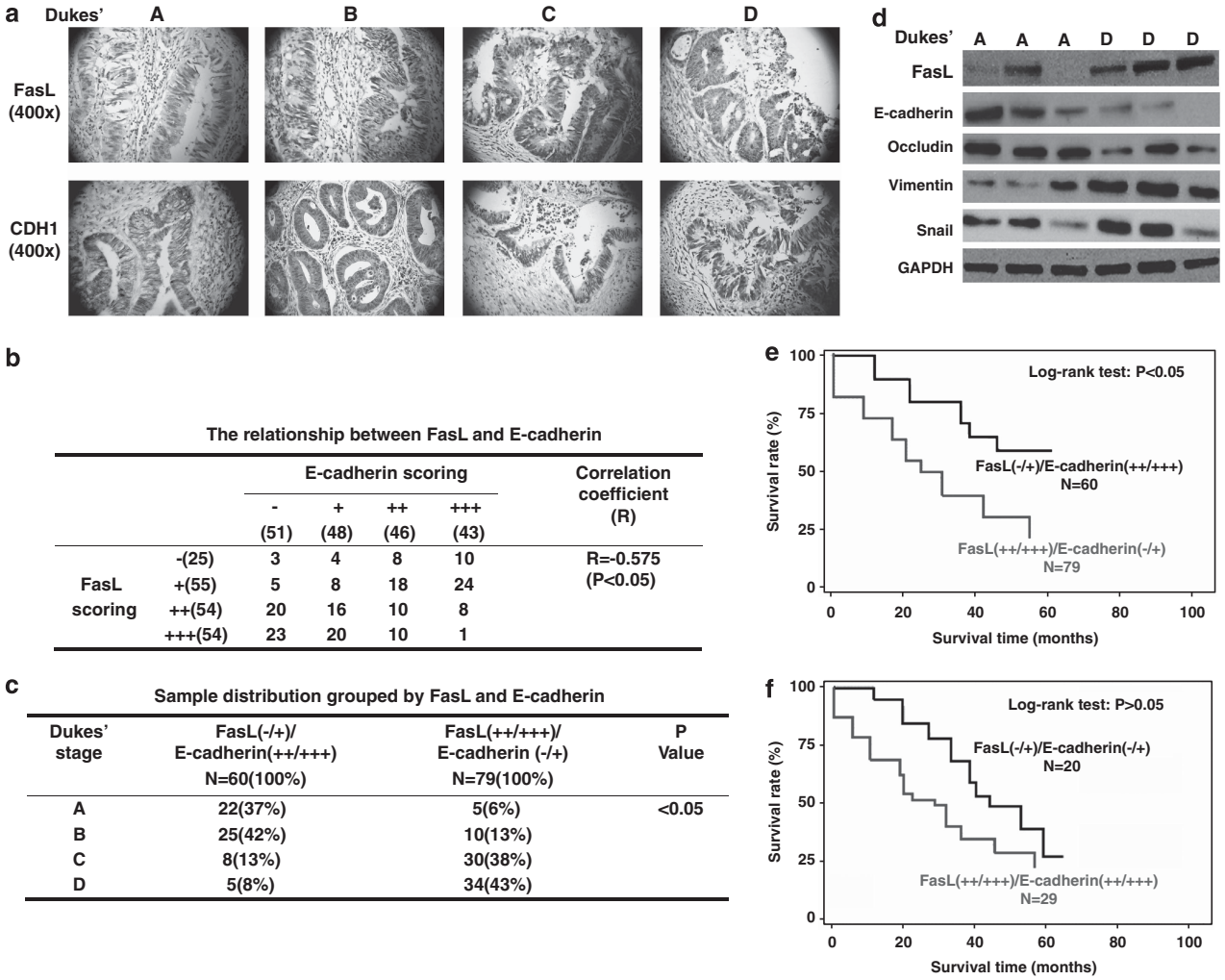

Figure 7. Fas signaling may induce EMT and promote metastasis in CRC cells*in vivo*. (**a**) Human CRC samples were obtained and IHC was performed. FasL expression increased whereas E-cadherin expression decreased during CRC progression. (**b**) FasL and E-cadherin expression were inversely correlated. (**c**) EMT phenotype samples were mostly distributed in Dukes' stage C and D, whereas non-EMT phenotype samples were mainly distributed in Dukes' stage A and B. (**d**) Immunoblot analysis showed that FasL and mesenchymal markers(Vimentin and Snail) were increased in clinical samples during CRC progression whereas epithelial markers (E-cadherin and Occludin) decreased. (**e**) A non-EMT phenotype in patients correlated with a better prognosis compared with an EMT phenotype. (**f**) No difference in survival was observed between the FasL($-/+$)/E-cadherin($-/+$) and FasL($++/+++$)/E-cadherin($++/+++$) groups.

(Supplementary Figure 12). Procedures were conducted according to the manufacturer's protocol. Cells resistant to puromycin (10 μg/ml) were selected and passaged for further study.

GI tissue specimens and follow-up

This study was conducted with a total of 188 CRC and 143 GC paraffin-embedded samples collected from Nanfang Hospital (Guangzhou, China). All patients did not receive therapy before the study. All tissues were examined by at least two experienced pathologists and checked for the presence of tumor cells. Pathological diagnosis and classification were performed based on the criterion of the International Union Against Cancer. The research protocol was approved by the Ethics Committee of Nanfang Hospital and consents were acquired from all patients for the study. Follow-up data was available for all patients.

IHC

Paraffin-embedded samples were processed using routine IHC procedures as previously described.[32] Stained tissue sections were reviewed and scored separately by two experienced pathologists blinded to the clinical parameters. The scoring method used to evaluate immunostaining was similar to a relatively simple and reproducible protocol.[33] Anti-FasL (Santa cruz, sc-823, N-20) and E-cadherin (BD Bioscience, San Jose, CA, USA BD610181) were used for IHC assay.

Immunoblot

Total protein was extracted in RIPA lysis buffer with protease and phosphotase inhibitors (Roche, Nutley, NJ, USA) and quantified using the BCA method (Thermo Scientific, Waltham, MA, USA). Total protein (50 μg) was resolved by 4–20% SDS polyacrylamide gel electrophoresis (Bio-Rad, Philadelphia, PA, USA) and transferred to nitrocellulose membrane. Membranes were incubated with various antibodies in specific concentrations according to the manufacturer's instructions. Protein expression was detected by ECL (Thermo Scientific).

RT–PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized by oligo dT primed reverse transcription (Promega, San Luis Obispo, CA, USA) from 2 μg of total RNA. Primers for EMT markers are shown in Supplementary Table 2. PCR was performed using an ABI thermocycler (Applied Biosystems Inc., Carlsbad, CA, USA). cDNA (1 μl) and primer mix (1 μl) were added into PCR master mix (Promega) for amplification. PCR conditions were as follows: 95 °C for 5 min; 35 cycles

of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; then 72 °C for 5 min. Samples were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining using the Chemidoc XRS Imager (Bio-Rad).

Flow cytometry

Cells were harvested and resuspended in PBS with 2% BSA. After incubation with Fas-PE or FasL-PE for 30 min at 4 °C, cells were washed twice with PBS and analyzed by Flow Cytometry (BD FACS Calibur, San Jose, CA, USA). Mouse IgG-PE was used as a negative control. MMP inhibitor (GM6001, 10 μM) was used to protect both markers from cleavage by MMP.

Cell invasion and migration assay

Invasion chambers (Corning Inc., Corning, NY, USA) were rehydrated at 37 °C and then 50 000 cells (serum-starved for 24 h before the assay) were added to the top chamber in serum-free medium (100 μl) and the bottom chamber was filled with medium containing 10% fetal bovine serum (chemicals were added to both chambers of each well if necessary). Then cells were cultured for 72 h at 37 °C in a 5% CO_2 humidified incubator. To quantify invasion, cells were removed from the top-side of the membrane using a cotton-tipped swab and invading cells attached to bottom of the membrane were fixed with 4% paraformaldehyde and stained with DAPI solution. Cell number from 10 representative fields was counted for each insert using fluorescent microscopy. The average cell number/field from the control group was set as the baseline (onefold), and was compared with the treatment groups (fold-change = treatment group cells/field÷control group cells/field). For some experiments, cells were cultured with inhibitor 2 h before treatment. To test the effect of mFasL,[6] SW620 (mFasL-expressing cells) were fixed in 4% paraformaldehyde and washed extensively before the assay. Then 50 μl of fixed SW620 (40 000 cells, with or without NOK-1 treatment, in serum-free medium) were mixed with 50 μl of live/unfixed DLD1 cells (20 000 cells in serum-free medium). The cell mixture was added into the top chamber and the bottom chamber was filled with 10% fetal bovine serum medium. SW620 cells were fixed in order to only study the migration of DLD1 cells. It has been previously shown that FasL on fixed cells can still activate the receptor on unfixed cells.[6] As a control, fixed DLD1 cells (do not express mFasL; control for SW620 cells) were mixed with live/unfixed DLD1 cells as described above. For the migration assay, transwell insert chambers (Corning Inc.) were used and all procedures were performed as the invasion assay with the exception of membrane rehydration and the incubation time (18 h, Supplementary Figure 13). Cell proliferation assays were conducted as previously described.[6]

Immunofluorescence

Cells grown in chambered coverglass (Thermo Scientific) were fixed with 4% paraformaldehyde and nonspecific binding was blocked by 5% bovine serum albumin in PBS. The cells were probed with primary antibody, washed and then probed with Texas Red goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody (Santa Cruz). After mounting, the slips were visualized by fluorescence microscopy (Olympus, Tokyo, Japan).

ELISA assay

MMP9 and MMP2 (pro- and active-form) secretion in cell culture supernatant was detected by Human Quantikine ELISA Kit (R&D System, Minneapolis, MN, USA) according to the instruction. The average MMP expression from the control group was set as the baseline (one fold) and was compared with the treatment groups (fold-change = treatment group expression÷control group expression).

Statistical analysis

Statistical analysis was conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Difference in IHC scoring was calculated by Kruskal–Wallis test, whereas significance between changes in different groups was evaluated by one-way analysis of variance. Least-significant difference test was used for multiple comparisons. Correlation coefficient was calculated by the Spearman's correlation method. A P-value <0.05 was considered statistically significant.

ABBREVIATIONS

CRC, colorectal cancer; EMT, epithelial–mesenchymal transition; GC, gastric cancer; GI, gastrointestinal; IHC, immunohistochemistry.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)