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miR-23a inhibits E-cadherin expression and is regulated by AP-1 and NFAT4 complex during Fas-induced EMT in gastrointestinal cancer

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Fas signaling has been shown to induce the epithelial–mesenchymal transition (EMT) to promote gastrointestinal (GI) cancer metastasis, but the involvement of microRNA in this mechanism remains unknown. We found that Fas ligand (FasL) treatment inhibited E-cadherin expression and promoted cell invasion by upregulation of miR-23a, but overexpression of the miR-23a inhibitor could partially block this activity. FasL-induced extracellular signal-regulated kinase/mitogen-activated protein kinase signaling activated the activator protein 1 (AP-1) complex and repressed glycogen synthase kinase-3β activity, which contributed to nuclear translocation of AP-1 and nuclear factor of activated T cells (NFAT4). Nuclear accumulation and interaction of AP-1 and NFAT4 and subsequent binding to the miR-23a promoter led to increased miR-23a expression. Inhibition of Fas signaling by downregulation of the Fas receptor led to a decrease in miR-23a expression and cell invasion ability *in vivo* and *in vitro*, as well as an increase in E-cadherin. Evaluation of human GI precancerous and cancer specimens showed that the expression of FasL and miR-23a increased, whereas the expression of E-cadherin decreased during GI cancer progression. A significant correlation was noted between any two of these three molecules. An EMT phenotype was shown to correlate with an advanced cancer stage and worse prognosis. Taken together, our results show that miR-23a participates in the mechanism of the FasL-induced EMT process and may serve as a potential therapeutic target for cancer metastasis.

Introduction

Fas (APO-1/CD95) is a member of the tumor necrosis factor and normal growth factor transmembrane receptor superfamily and activates caspase-dependent apoptosis in susceptible cells when triggered by its cognate ligand, Fas ligand (FasL)/CD95L (1). However, Fas signaling can also control non-apoptotic events in cells, including the regulation of cell cycle progression (2), cytokine and chemokine expression (3,4), tumor growth (5) and motility (6), through various cellular signaling pathways, such as nuclear factor-κB (6) and mitogen-activated protein kinase (MAPK) (4,6). Recently, we reported that Fas signaling promotes motility and metastasis through the epithelial–mesenchymal transition (EMT) in gastrointestinal (GI) cancer (7).

The ability of cancer cells to undergo EMT serves as a good model for how solid tumors metastasize from the site of origin to a new site, although this theory is still under debate (8). A defining feature of EMT is a reduction in E-cadherin levels and a concomitant production of N-cadherin (9). The central role played by E-cadherin loss in EMT is fully illustrated by the actions of several EMT-inducing transcription factors, such as Snail and Slug (10). Previously, we found that FasL treatment inhibited E-cadherin transcription by upregulation of Snail in GI cancer cells, and that Snail was increased by inhibition of glycogen synthase kinase-3β (GSK-3β) activity through FasL-induced extracellular signal-regulated kinase (ERK)/MAPK signaling (11).

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs that simultaneously regulate levels of multiple proteins, primarily by binding to the 3′ untranslated region (UTR) of targets and inhibiting protein translation (12). Important roles for miRNAs have been shown in multiple types of cancer, including functions in tumor progression by modulating mechanisms of differentiation, proliferation and metastasis (13). miRNAs are also components of the cellular signaling circuitry that regulate the EMT program (10). For example, miR-200 and miR-205 inhibit the repressors of E-cadherin expression, ZEB1 and ZEB2, and thereby help in maintaining the epithelial cell phenotype (14–16). In breast carcinoma, a loss of miR-200 correlates with increased expression of vimentin and a decrease in E-cadherin expression (14–16). Conversely, upregulation of miR-9 (17), miR-10b (18), miR-23a (19) or miR-92a (20) directly inhibits E-cadherin expression and promotes EMT and cancer metastasis. Based on these findings, we speculated if miRNA is involved in the FasL-mediated induction of EMT, such as through regulation of E-cadherin, an important marker in EMT.

In this study, we investigated whether miRNA and which miRNA regulates E-cadherin during Fas-signaling-induced EMT in GI cancer. Primarily, we demonstrate that miR-23a inhibits E-cadherin expression and is regulated by the activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT4) complex during FasL-induced EMT in GI cancer *in vivo* and *in vitro*.

Materials and methods

Cell culture and reagents

Human GI cancer cell lines [colorectal cancer (CRC): SW480, DLD1 and Lovo; gastric cancer (GC): AGS] were obtained from American Type Culture Collection (Manassas, VA) and routinely maintained in our laboratory as described previously (7). Serum-free medium was used in most experiments unless otherwise indicated. FasL was used at a concentration of 12.5 ng/ml as described previously (7). U0126 or PD98095 (data not shown) was added into the culture medium 2 h before FasL treatment to inhibit activation of ERK/MAPK signaling. Detailed information for reagents and antibodies is listed in the Supplementary Information, available at *Carcinogenesis* Online.

Cell line transfection

SW480, DLD1 and AGS cells were transduced with constructs expressing *NFAT4*, *c-Jun* or *c-Fos* small hairpin RNA (shRNA; Santa Cruz Biotechnology, Santa Cruz, CA) for stable knockdown. Another set of the same constructs was also purchased from GeneCopoeia, and similar results for experiments of this study were confirmed using the second set (data not shown). The miR-23a precursor, miR-23a inhibitor or E-cadherin open reading frame clone vector without the 3′ UTR (GeneCopoeia) was stably transfected into SW480, DLD1 and AGS cells. SW480, DLD1 and AGS cells expressing the GSK-3β S9A mutant (Addgene, Cambridge, MA), a constitutively active mutant unable to be inhibited by phosphorylation at Ser 9, was utilized as described previously (11). Lovo cells were stably transduced with constructs expressing miR-23a

Abbreviations: CRC, colorectal cancer; EMT, epithelial–mesenchymal transition; ERK, extracellular signal-regulated kinase; FasL, Fas ligand; GC, gastric cancer; GI, gastrointestinal; MAPK, mitogen-activated protein kinase; miRNAs, microRNAs; NFAT4, nuclear factor of activated T cells; qRT–PCR, quantitative real-time–PCR; shRNA, small hairpin RNA; UTR, untranslated region.

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inhibitor, Fas shRNA and/or miR-23a precursor. The efficacy of gene transfection is shown in Supplementary Figure S1, available at *Carcinogenesis* Online. Detailed information for transfectants is listed in the Supplementary Information, available at *Carcinogenesis* Online. All procedures were conducted according to the manufacturers' protocols.

GI cancer specimens and follow-up

This study was conducted with GI precancerous (CRC: $N = 367$; GC: $N = 419$) and cancer samples (CRC: $N = 135$; GC: $N = 143$) collected from Nanfang Hospital (Guangzhou, China). None of the patients received therapy before the study. All tissues were examined by at least two experienced pathologists and checked for the presence of tumor cells. The research protocol was approved by the ethics committee of Nanfang Hospital and consent was acquired from all patients for the study. Follow-up data were available for all patients with GI cancer. Correlation between clinicopathologic parameters and expression of EMT molecules in GI cancer is shown in Supplementary Tables 1 and 2, available at *Carcinogenesis* Online.

Quantitative real-time–PCR

RNA was extracted from cells and tissues using the miRNeasy Mini Kit according to the protocol (Qiagen, Valencia, CA). Quantitative real-time–PCR (qRT–PCR) primers for miR-23a, small nuclear RNA U6, E-cadherin and FasL were purchased from GeneCopoeia. qRT–PCR for E-cadherin and FasL was conducted using the All-in-One™ qPCR Mix (GeneCopoeia), whereas miR-23a and small nuclear RNA U6 qRT–PCR was performed with the All-in-One™ miRNA qRT–PCR Reagent Kit (GeneCopoeia) according to the manufacturer's instructions. PCR amplification was performed with the same parameters as indicated in the protocol using an Applied Biosystems Cyclers 7500 (PerkinElmer Corp., Wellesley, MA). Relative quantification of FasL, miR-23a or E-cadherin level in lesion samples was calculated using the comparative Ct method and the formula $2^{-\Delta\Delta Ct}$ (21), and with corresponding levels in adjacent normal epithelium used as controls (22). For FasL and miR-23a, ranking of lesion samples compared with normal epithelium was shown as –, +, ++ and +++ (0≤–<1-fold, 1≤+<3-fold, 3≤++<5-fold, 5-fold ≤ +++), whereas for E-cadherin, the ranking was presented as –, +, ++ and +++ (0≤–<0.1-fold, 0.1≤+<0.3-fold, 0.3≤++<0.5-fold, 0.5≤+++< 1-fold). PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining using the Chemidoc XRS Imager (Bio-Rad).

Luciferase reporter assay

The TRE reporter construct (SABiosciences, Valencia, CA) containing AP-1 binding sites was introduced into GI cancer cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Luciferase activity was detected with the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI), as described previously (23). The miR-23a promoter reporter or E-cadherin 3′ UTR miRNA target clone (GeneCopoeia), a dual-reporter system with *Gaussia* Luciferase (GLuc) and secreted alkaline phosphatase, was introduced into GI cancer cells using Endofectin™ (GeneCopoeia). The predicted miR-23a binding sites (seed sequence) of E-cadherin 3′ UTR region from E-cadherin 3′ UTR miRNA target clone (GeneCopoeia) was mutated using Quick Change Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Luciferase activity was assessed with the Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia) according to the manufacturer's instructions as described previously (11).

Chromatin immunoprecipitation

Chromatin immunoprecipitation assay was performed with a commercial kit (Millipore, Bedford, MA), according to the protocol. The purified DNA was used as template and amplified with the following primer set, which is specific for the miR-23a promoter: 5′-TTGAGCCACCAGGTGCAACTGT-3′ and 5′-GAAAGCCCGAGTAAGCCGA GT-3′ (24).

Animal models

About 4- to 6-week-old athymic BALB/c nu/nu mice were purchased from the Shanghai Slaccas Laboratory Animal Co. Ltd (Shanghai, China). All mice were housed and manipulated in the specific pathogen-free breeding room at the animal laboratory center of Nanfang Hospital. All protocols for animal studies were reviewed and approved by the institutional animal care and use committee of Nanfang Hospital. For the orthotopic metastasis assay, nude mice (8 week old) were anesthetized and their cecum was exteriorized by laparotomy ($N = 15$ per group). Lovo cells (1×10^6) with or without transfectants were injected into the cecum wall (25). The animals were killed 8 weeks after injection or when they had become moribund, and the primary tumors were weighed. The livers from the mice were removed and fixed in Bouin's solution for 24 h to differentiate neoplastic lesions from the liver parenchyma (25). Liver metastases were calculated and determined by two independent pathologists. Liver metastasis rate in each group was defined as number of mice with liver neoplastic lesions/total number of mice ($N = 15$ per group), whereas the number of liver metastatic sites was defined as the number of neoplastic lesions in the liver.

Immunoprecipitation, immunofluorescence, immunoblot and cell invasion assay

Immunoprecipitation, immunofluorescence, immunoblot and cell invasion assays were performed as described previously (7,11).

Statistical analysis

Statistical analysis was conducted using SPSS 17.0 (SPSS, Chicago, IL). Difference in EMT molecule expression, EMT phenotype and liver metastasis rate was calculated using Chi-square test. Significance between changes in different groups detected by qRT–PCR, luciferase reporter assay and transwell assay was evaluated by one-way analysis of variance, whereas least-significant difference test was used for multiple comparisons. Correlation coefficient was calculated by the Spearman method. Survival curves were generated according to follow-up data with Kaplan–Meier method, and comparison between cumulative survival rates was performed using log-rank test. P-values <0.05 were considered statistically significant.

Results

miR-23a is upregulated upon FasL-induced ERK/MAPK activation, and inhibits E-cadherin expression and promotes metastasis

To date, several miRNAs have been proposed to target the E-cadherin 3′ UTR and inhibit its translation, such as miR-9 (17), miR-10b (18), miR-23a (19) and miR-92a (20). qRT–PCR was used to detect the expression of these miRNAs after FasL treatment in SW480, DLD1 and AGS cells. Among these four miRNAs, we found that only miR-23a was upregulated and dependent on FasL-induced ERK/MAPK activation (Figure 1A and B; Supplementary Figure S2A, available at *Carcinogenesis* Online). The miR-23a precursor significantly inhibited E-cadherin 3′ UTR luciferase activity and protein expression, and promoted cell invasion, whereas the miR-23a inhibitor showed the reverse effects (Figure 1C and D; Supplementary Figure S2B and S3, available at *Carcinogenesis* Online). When miR-23a binding site (seed sequence) in the 3′ UTR region of E-cadherin was mutated, miR-23a precursor could not decrease the luciferase activity, indicating E-cadherin is direct target of miR-23a (Supplementary Figure S4, available at *Carcinogenesis* Online). Furthermore, FasL treatment could block E-cadherin 3′ UTR luciferase activity and protein expression, and promote cell invasion, but this activity could partially be blocked by the miR-23a inhibitor (Figure 1E and F, Supplementary Figure S2C and S5, available at *Carcinogenesis* Online), implying that miR-23a may be involved in the mechanism of FasL-induced EMT. Moreover, exogenous expression of E-cadherin partially inhibited the cell invasion ability induced by FasL or the miR-23a precursor (Supplementary Figure S6, available at *Carcinogenesis* Online), indicating that downregulation of E-cadherin is required for increased cell invasiveness. However, other mechanisms besides E-cadherin regulated by Fas signaling may be involved as FasL stimulation could still promote invasion in GI cancer cells expressing E-cadherin vector.

AP-1 and NFAT4 complex promotes miR-23a expression, depending on FasL-induced ERK/MAPK activation

Expression of miR-23a is regulated by the NFAT4 transcription factor (24), which often coordinates with AP-1 (the c-Jun/c-Fos complex) to augment its activity (26). To test possible involvement of NFAT4 and AP-1, we first assessed the expression of NFAT4, c-Jun and c-Fos proteins in nuclear extracts after FasL stimulation. We found that the nuclear expression of NFAT4, phospho-c-Jun (Ser 63, active form), c-Jun, phospho-c-Fos (Ser 374, active form) and c-Fos increased upon FasL treatment, and that this increase was dependent on FasL-induced ERK/MAPK activation (Figure 2A and B; Supplementary Figure S7A, available at *Carcinogenesis* Online). Total c-Fos expression increased, whereas total NFAT4 and c-Jun remained unchanged (Supplementary Figure S8, available at *Carcinogenesis* Online). FasL stimulation led to binding of NFAT4 to the miR-23a promoter region and subsequent miR-23a expression, but this activity was significantly inhibited by lentiviral constructs expressing *NFAT4* shRNA, *c-Jun* shRNA or *c-Fos* shRNA, to various degrees (Figure 2C–F; Supplementary Figure S7B and C, available at *Carcinogenesis* Online). Consistent with these results, FasL-induced activation of the miR-23a promoter activity was

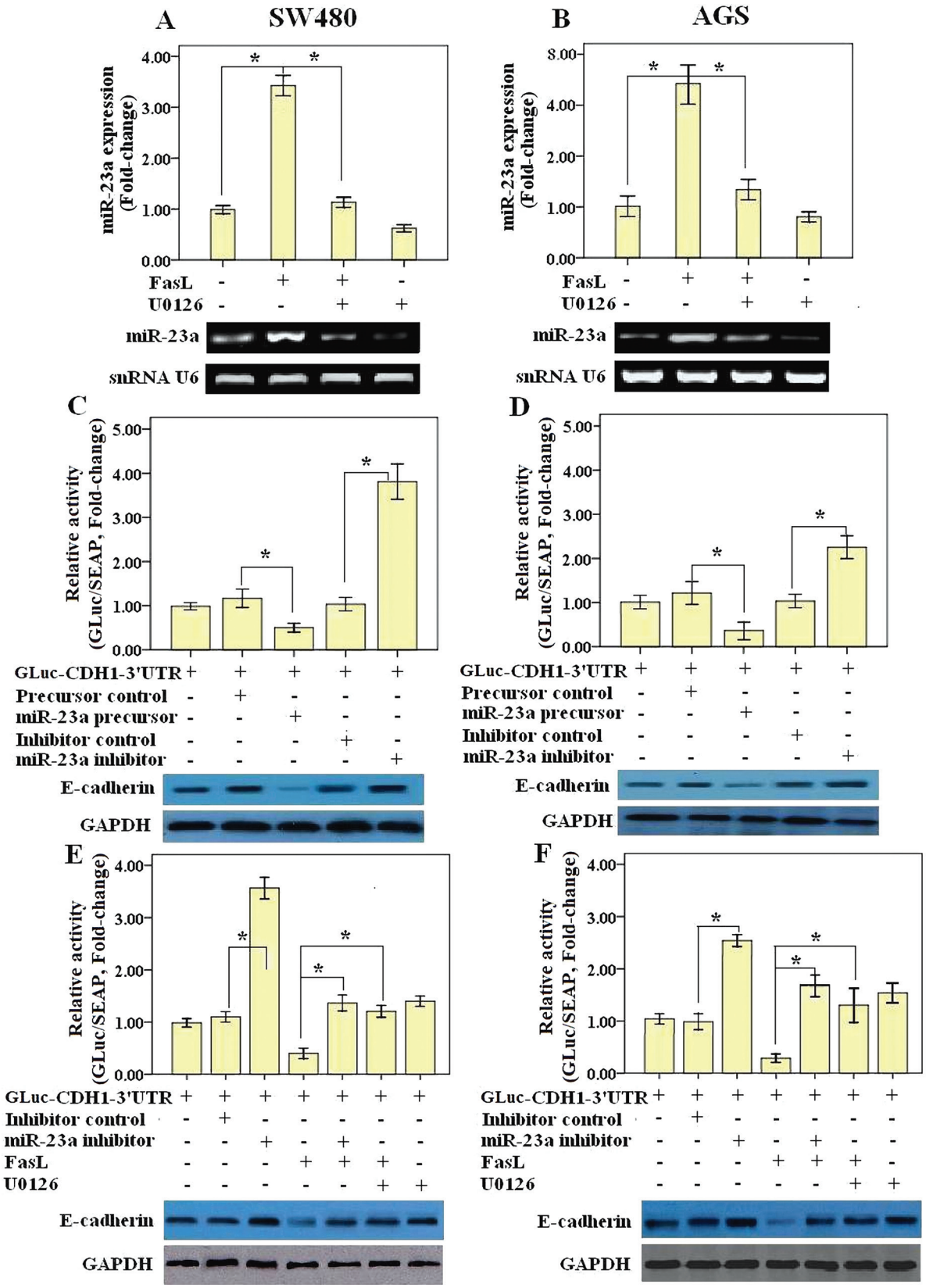

Fig. 1. miR-23a is upregulated after FasL-induced ERK1/2 activation and inhibits E-cadherin expression. SW480 (**A**) and AGS (**B**) cells were treated with FasL (12 h) and/or U0126, and qRT–PCR was performed. miR-23a upregulation is dependent on FasL-induced ERK1/2 activation. A GLuc-CDH1-3′ UTR reporter was transduced into SW480 (**C**) and AGS (**D**) cells stably expressing either the miR-23a precursor or the miR-23a inhibitor, and luciferase activity was assessed. Immunoblot was performed in parallel. miR-23a targets the CDH1-3′ UTR and inhibits E-cadherin expression. SW480 (**E**) and AGS (**F**) cells expressing the miR-23a inhibitor were transduced with the GLuc-CDH1-3′ UTR reporter and stimulated with FasL for 12 h. The miR-23a inhibitor reverses FasL-induced reduction of GLuc-CDH1-3′ UTR reporter activity. Immunoblot confirmed similar results (FasL treatment for 72 h). U0126 was added 2 h before FasL stimulation. All data are represented as fold-change ± SD compared with control cells. Experiments were performed in triplicate. $^{*}P < 0.05$.

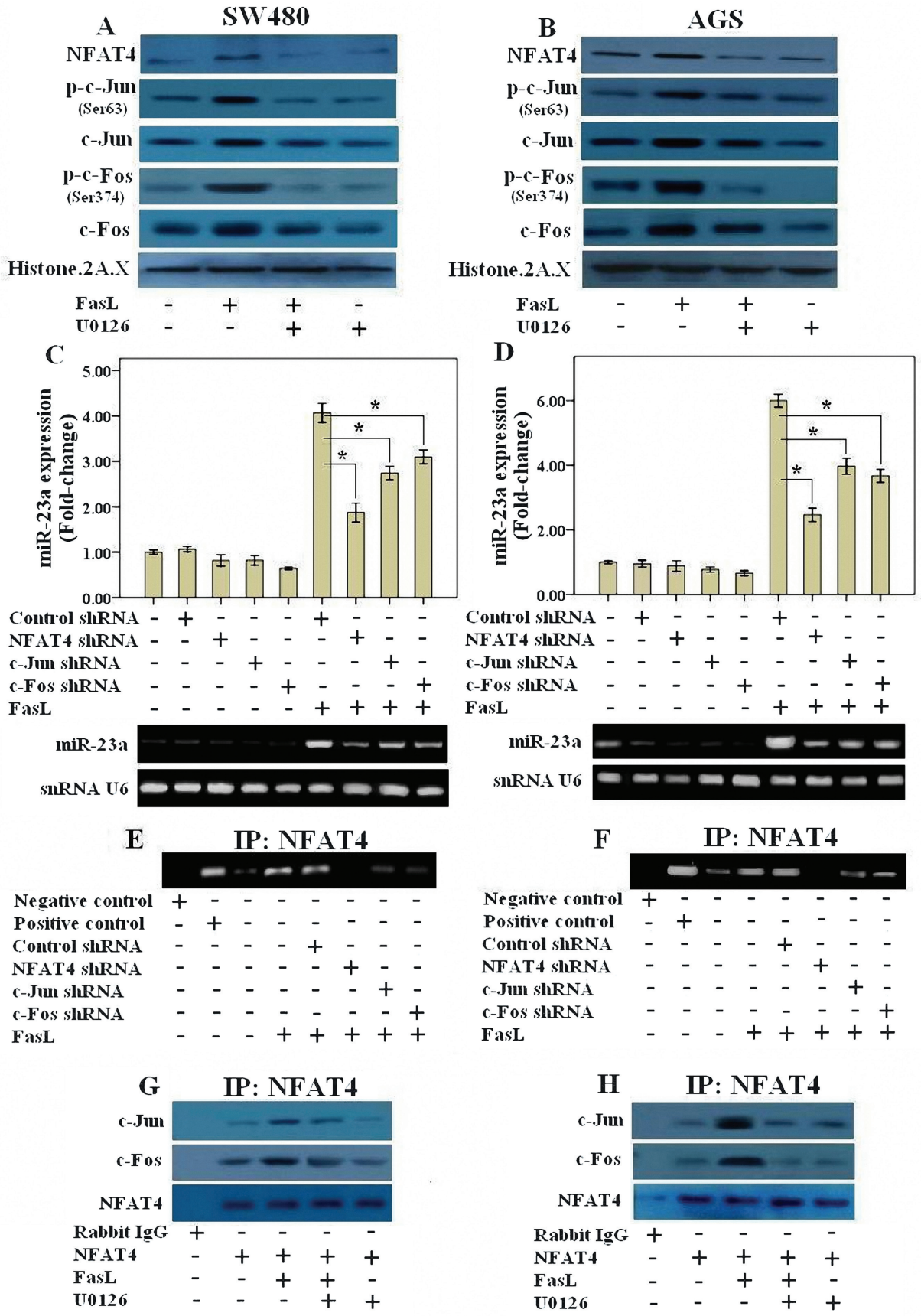

Fig. 2. The AP-1 and NFAT4 complex promotes miR-23a expression and is dependent on FasL-induced ERK1/2 activation. SW480 (**A**) and AGS (**B**) cells were treated with FasL for 1 h and immunoblot analysis was performed using nuclear extract. NFAT4, phospho-c-Jun (Ser 63), c-Jun, phospho-c-Fos (Ser 374) and c-Fos levels increased noticeably, and were blocked by U0126. SW480 (**C**) and AGS (**D**) cells were stably transduced with the *NFAT4* shRNA, *c-Jun* shRNA or

partly inhibited by knockdown of *NFAT4*, *c-Jun* or *c-Fos*, whereas the TRE promoter (containing AP-1 bind sites) activity was partly repressed by downregulation of either *c-Jun* or *c-Fos* (Supplementary Figure S9, available at *Carcinogenesis* Online). FasL treatment also promoted the interaction between NFAT4 and AP-1, and this interaction was dependent on ERK/MAPK activation (Figure 2G and H, Supplementary Figure S7D, available at *Carcinogenesis* Online). These data suggest that FasL-induced miR-23a expression relies on the activation of the NFAT4 and AP-1 complex.

Inhibition of GSK-3β by Fas signaling promotes nuclear import and interaction between AP-1 and NFAT4 and increases their transcriptional activity

Previously, we demonstrated that Fas signaling reduces GSK-3β activity by ERK/MAPK activation, leading to nuclear accumulation of Snail, and, subsequently, decreased transcription of E-cadherin (11). Active GSK-3β could promote nuclear export of NFAT4 and inactivate c-Jun by site-specific phosphorylation on Thr 239, and thus decrease their transcriptional activity (27,28). When the constitutively active GSK-3β S9A mutant was transduced into SW480, DLD1 and AGS cells, nuclear expression of NFAT4 and c-Jun induced by FasL stimulation was completely blocked (Figure 3A and B; Supplementary Figure S10A, available at *Carcinogenesis* Online), whereas c-Fos remained unaffected (data not shown). Activities of miR-23a and TRE (containing an AP-1 binding site) promoters induced by FasL treatment were partially inhibited by overexpression of the GSK-3β S9A mutant (Figure 3C–F; Supplementary Figure S10B and C, available at *Carcinogenesis* Online). Consistent with this result, the FasL-induced interaction between NFAT4 and AP-1 was also repressed by the GSK-3β S9A mutant (Figure 3G and H; Supplementary Figure S9D, available at *Carcinogenesis* Online).

Inhibition of Fas signaling decreases miR-23a expression and cancer metastasis

As Lovo cells express sufficient amounts of FasL and miR-23a (7,22) and lower levels of Fas receptor (7), we created a stable knockdown of Fas expression to block Fas signaling, and examined the expression of miR-23a and E-cadherin, as well as cell invasion ability. miR-23a expression decreased, whereas E-cadherin increased upon Fas inhibition by lentiviral shRNA (Figure 4A), along with decreased cell invasion ability (Figure 4B). The miR-23a inhibitor could depress the cell invasion ability and liver metastasis in a mouse model, whereas the miR-23a precursor could, in part, reverse this effect even without Fas signaling (Figure 4C and D), indicating that Fas signaling promoted cell invasion partly through miR-23a. Similar to another report (22), the weight of primary tumors and the number of liver metastatic sites showed no difference among all groups (data not shown).

Fas signaling may inhibit E-cadherin expression by upregulation of miR-23a in vivo

To investigate whether Fas signaling inhibits E-cadherin expression by upregulation of miR-23a *in vivo*, fresh GI precancerous and cancer samples were obtained and analyzed by qRT–PCR. The expression of FasL and miR-23a increased smoothly among precancerous samples, but showed a sharp increase in cancer in various stages. Levels of E-cadherin decreased, showing the same trends (Figure 5; Supplementary Tables 3, 4 and Figure S11, available at *Carcinogenesis* Online). These data suggest that FasL-induced miR-23a represses E-cadherin expression to allow cancer cells metastasis from original site in late stage of GI cancer. Moreover, a positive correlation between FasL and miR-23a was noted, whereas a negative correlation between E-cadherin and FasL or miR-23a was found to a medium degree (Supplementary Tables 5 and 6, available at *Carcinogenesis* Online), implying that Fas signaling might inhibit E-cadherin expression by upregulation of miR-23a *in vivo*. As expression of these three molecules correlates with GI cancer progression, we defined FasL(−/+)/miR-23a(−/+), FasL(−/+)/E-cadherin(++/+++) and miR-23a(−/+)/E-cadherin (++/+++) as non-EMT phenotypic samples and the reverse expression as EMT phenotypic samples. Notably, non-EMT samples were mainly distributed in the early cancer stages (CRC: Dukes' A, GC: p-Stage I), whereas EMT samples were mostly distributed in the advanced stages (CRC: Dukes' D; GC: p-Stage II–IV; Supplementary Tables 7 and 8, available at *Carcinogenesis* Online), indicating that FasL-induced EMT may promote metastasis *in vivo*. Moreover, the EMT phenotype CRC (Figure 6A, C and E) and GC (Figure 6B, D and F) patients showed worse prognosis than patients with the non-EMT phenotype. Samples in which expression of EMT-associated molecules were paradoxical, such as FasL(−/+)/miR-23a(++/+++) or FasL(++/+++)/miR-23a(−/+), were not able to predict cancer staging or prognosis (data not shown).

Discussion

In addition to its ability to induce apoptosis, Fas signaling can also induce non-apoptotic events in tumor cells, such as proliferation, inflammation, metastasis and EMT (2–7). In this study, we investigated the possible miRNA(s) involved in FasL-induced EMT, and demonstrated that miR-23a inhibits E-cadherin during FasL-induced EMT and is regulated by the AP-1 and NFAT4 complex in GI cancer (Figure 6G). These results suggest that miR-23a may be utilized as a possible therapeutic target for cancer metastasis.

The occurrence of EMT during tumor progression allows benign tumor cells to infiltrate surrounding tissue and ultimately metastasize to distant sites. This EMT is accompanied by loss of E-cadherin, a hallmark of metastatic carcinoma (9,29). Recent reports showed that several miRNAs play a crucial role in the regulation of EMT in several cancers, including the miR-200 family and miR-205 targeting ZEB1 and ZEB2 (14–16) repressor of E-cadherin, as well as miR-9, miR-10b, miR-23a and miR-92a targeting E-cadherin (17–20). However, the mechanism by which miRNA contributes to Fas-signaling-induced EMT has been largely unknown.

Herein, we demonstrated that miR-23a is upregulated in response to Fas signaling to inhibit E-cadherin. These results are similar to one study that miR-23a regulates transforming growth factor-β-induced EMT by targeting E-cadherin in lung cancer (19). Previously, we found that snail was upregulated during FasL-induced EMT (7). Snail represses E-cadherin transcription by binding to its promoter (11), whereas miR-23a inhibits E-cadherin translation through interaction with its messenger RNA 3′ UTR region. Both Snail and miR-23a are downstream targets of GSK-3β that is inactivated by FasL-induced ERK1/2 MAPK. These data indicate that Fas signaling regulates different kinds of molecules that cooperate with each other to induce EMT.

miR-23a belongs to the miR-23a/24/27a cluster located on chromosome 19p13.12 and can be induced by transforming growth factor-β (30). This cluster functions as an oncogenic miRNA in several human cancers (30) and has diverse effects, including in cell proliferation, differentiation and metastasis (31–33). The three miRNAs of this cluster

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c-Fos shRNA constructs and then treated with FasL for 12 h. miR-23a was assessed using qRT–PCR. Knockdown of *NFAT4*, *c-Jun* or *c-Fos* partially blocked FasL-induced increase of miR-23a expression. Chromatin immunoprecipitation assay confirmed that NFAT4 binding to the miR-23a promoter was blocked in SW480 (**E**) and AGS (**F**) cells stably expressing any one of the three constructs, when stimulated with FasL for 1 h. E-cadherin antibody was used as negative control, whereas products amplified from the miR-23a promoter were used as positive control. SW480 (**G**) and AGS (**H**) cells were treated with FasL for 1 h and immunoprecipitation was performed with total extract using NFAT4 antibody. NFAT4 could bind both c-Jun and c-Fos, and binding was inhibited by U0126. Similar results were found with immunoprecipitation using either c-Jun or c-Fos antibody (data not shown). U0126 was added 2 h before FasL stimulation. (C–D) Data are represented as fold-change ± SD compared with control cells. Experiments were performed in triplicate. $*P < 0.05$.

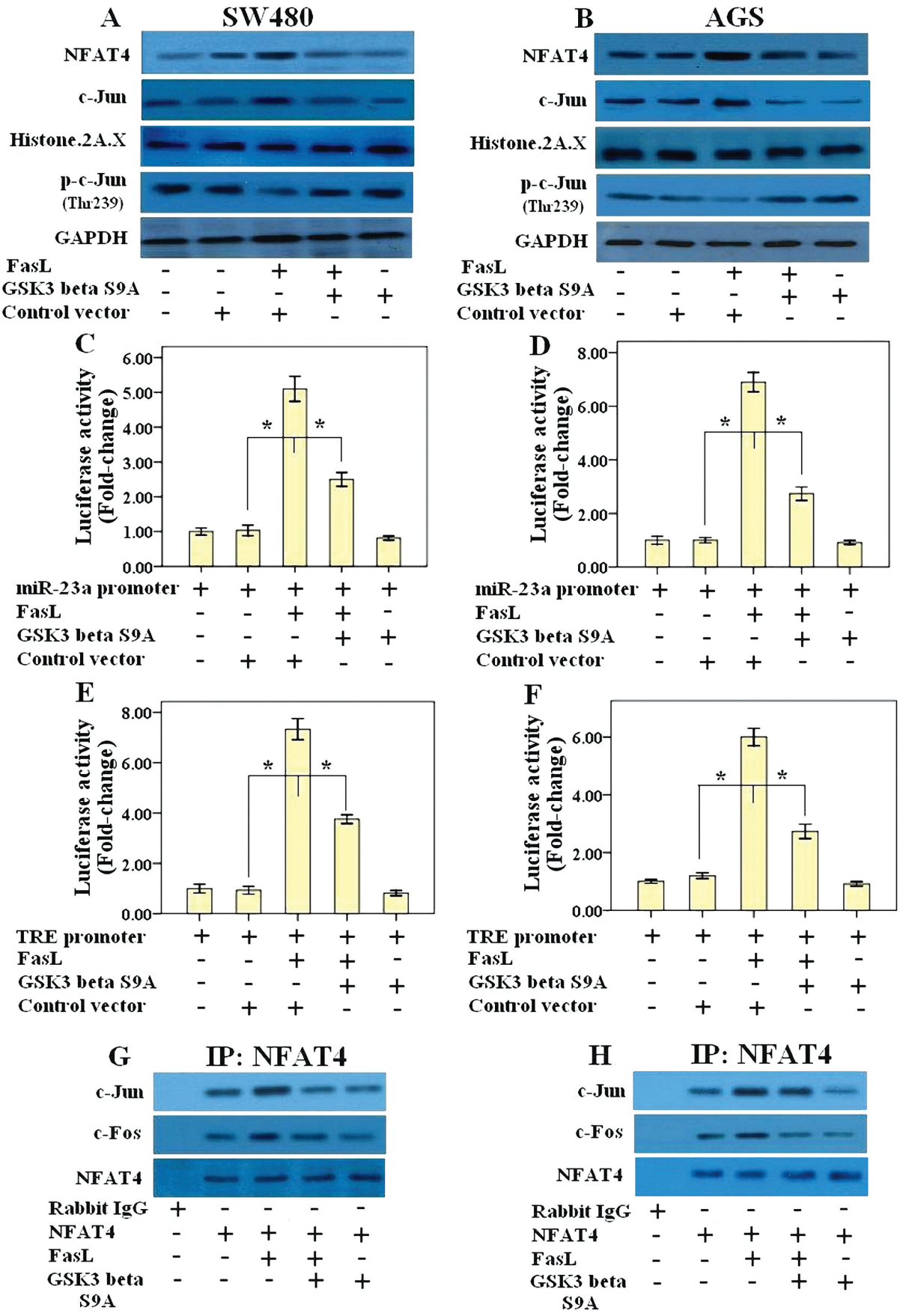

Fig. 3. Inhibition of GSK-3β by Fas signaling promotes nuclear import of and interaction between AP-1 and NFAT4, and increases their transcriptional activity. SW480 (**A**) and AGS (**B**) cells were transduced with either GSK-3β S9A or control vector. Immunoblot assay showed exogenous expression of GSK-3β

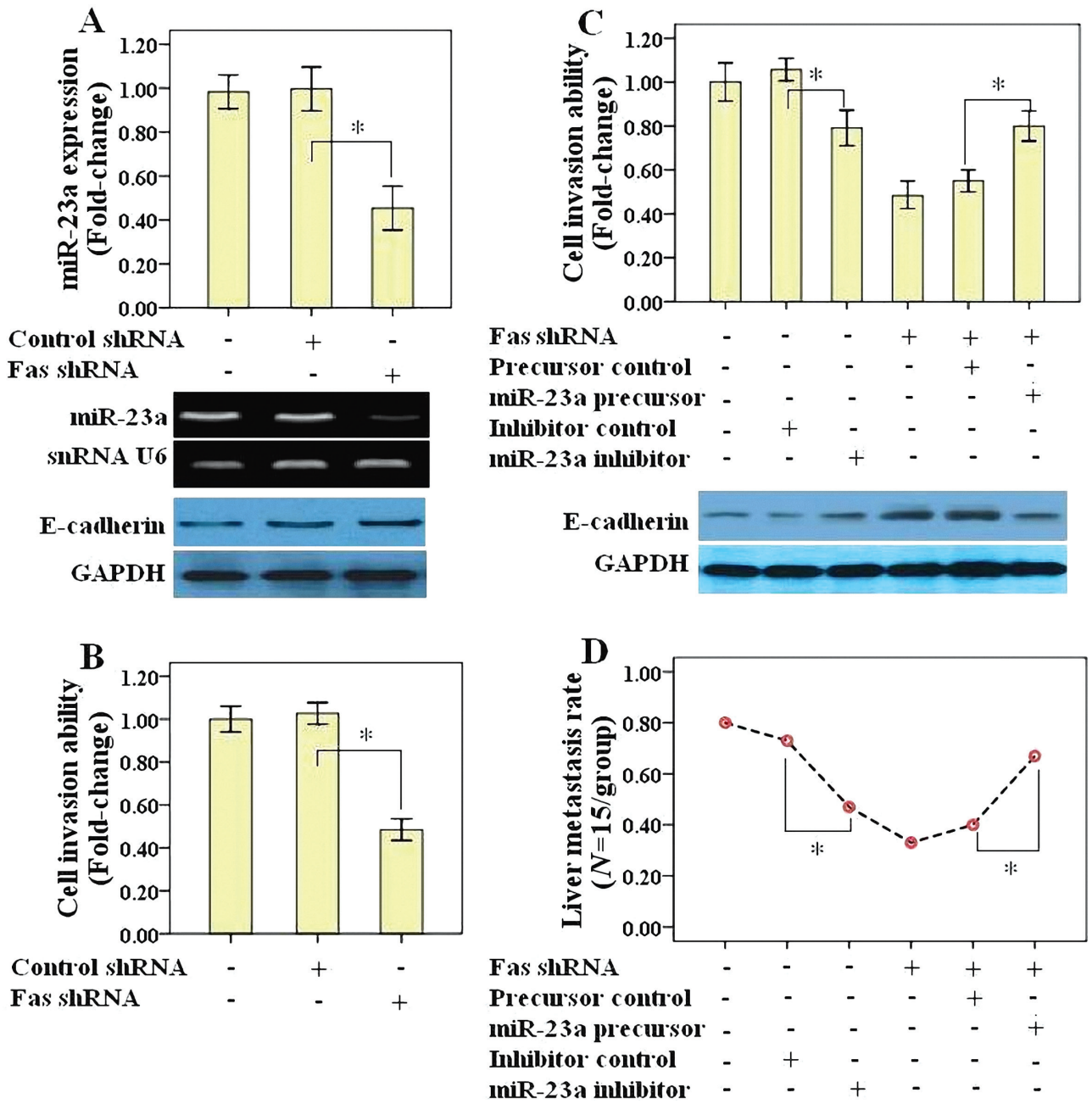

Fig. 4. Inhibition of Fas signaling decreases miR-23a expression and promotes cancer cell metastasis. Lovo cells were transduced with the lentiviral Fas shRNA construct for stable knockdown of Fas signaling. qRT–PCR (**A**), immunoblot (A) and transwell invasion assays (**B**) were conducted. miR-23a expression and cell invasion ability decreased, whereas E-cadherin increased in Lovo cells transduced with Fas shRNA compared with cells transduced with control shRNA. The miR-23a precursor was transfected into Fas shRNA-expressing Lovo cells, and miR-23a inhibitor was stably transduced into Lovo cells. Cells were used for a transwell invasion assay (**C**) and construction of an *in vivo* mouse model by cancer cell injection into the cecum (**D**). miR-23a inhibitor could reduce the cell invasion ability and liver metastasis in the mouse model, whereas expression of the miR-23a precursor could, in part, reverse this effect, even without Fas signaling. (A–C) Data are represented as fold-change ± SD compared with control cells, and experiments were performed in triplicate. $*P < 0.05$.

are derived from a single primary transcript, but the levels of each vary due to posttranscriptional processing (32). We found that miR-24 and miR-27a were not induced upon FasL stimulation. Furthermore, metastasis suppressor 1 is a direct miR-23a target that can interact directly with cortactin to promote filopodia formation and upregulate Src signaling (34). Reduced metastasis suppressor 1 levels promote CRC cell and cancer stem cell metastasis (22). Our unpublished data also indicate that miR-23a inhibits metastasis suppressor 1 expression

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S9A prevented the increased nuclear expression of NFAT4 and c-Jun (nuclear extract), and the reduced phosphorylation of c-Jun on Thr 239 (total cell extract) in response to FasL stimulation for 1 h. SW480 (**C** and **E**) and AGS cells (**D** and **F**) expressing the GSK-3β S9A mutant were transduced with the miR-23a promoter or TRE promoter reporter, and stimulated with FasL for 12 h. FasL-induced NFAT4 and AP-1 transcriptional activities were partially inhibited by exogenous expression of GSK-3β S9A. Similarly, immunoprecipitation with total extract showed that FasL-induced binding between NFAT4 and AP-1 was also inhibited by the GSK-3β S9A mutant in SW480 (**G**) and AGS (**H**) cells. (C–F) Data are represented as fold-change ± SD compared with control cells. Experiments were performed in triplicate. $*P < 0.05$.

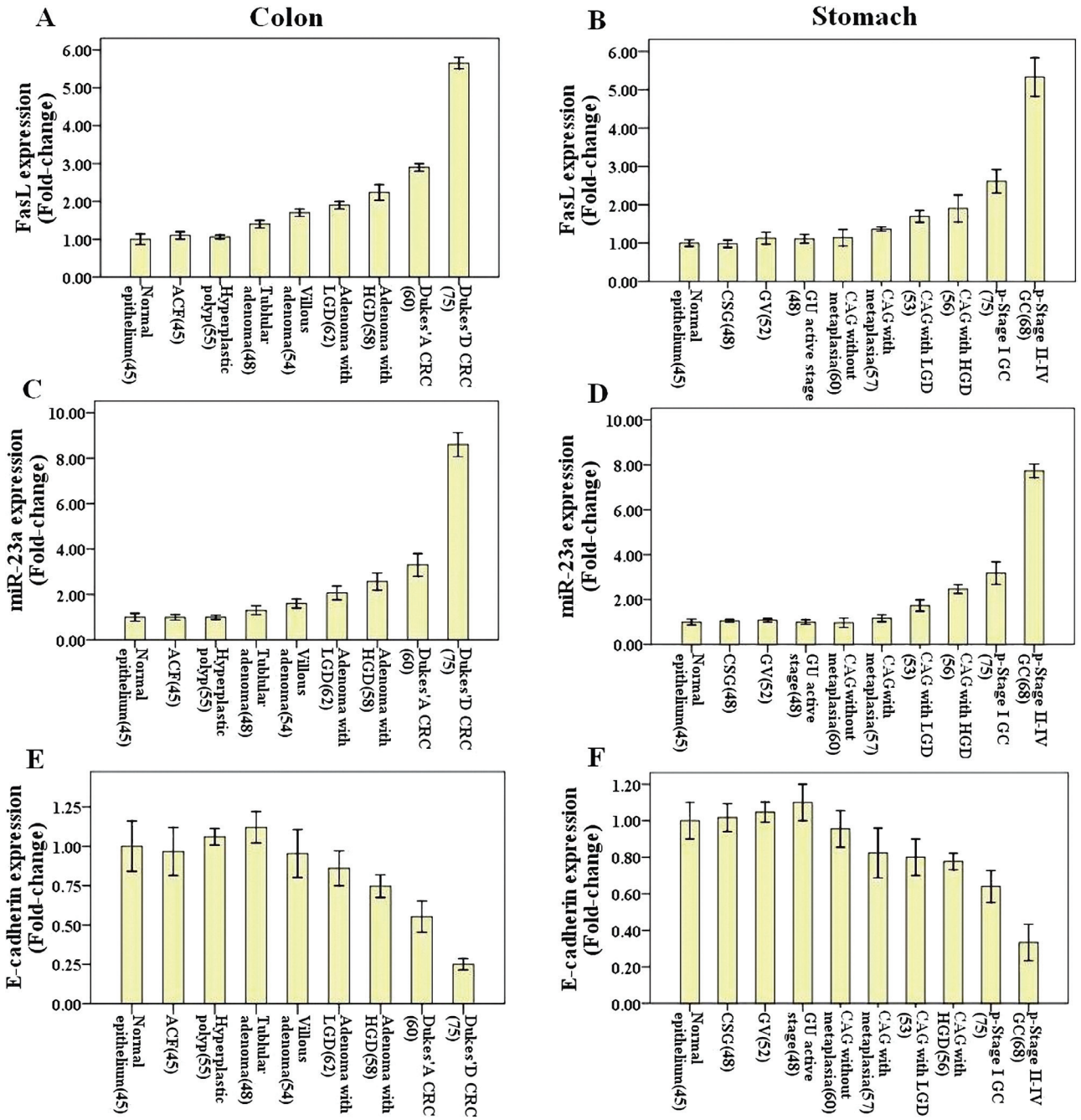

Fig. 5. Expression of FasL, miR-23a and E-cadherin in human tissues. Human GI precancerous (CRC: $N = 367$; GC: $N = 419$) and cancer samples (CRC: $N = 135$; GC: $N = 143$) were analyzed by qRT–PCR. Sample number is indicated in parentheses. The expression of FasL and miR-23a increased, whereas E-cadherin decreased during GI cancer progression. All data are represented as fold-change ± SD compared with the control group (normal epithelium). ACF, aberrant crypt foci; LGD, low-degree dysplasia; HGD, high-degree dysplasia; CSG, chronic superficial gastritis; GV, gastritis verrucosa; GU, gastric ulcer; CAG, chronic atrophic gastritis.

in GI cancer cells, depending on FasL-induced ERK/MAPK activation. This may explain why exogenous expression of E-cadherin partially inhibited cell invasion ability induced by the miR-23a precursor.

As our results showed that miR-23a is increased by FasL-induced ERK/MAPK activation, the mechanism by which ERK/MAPK signaling regulates miR-23a expression was investigated next. Lin *et al.* (24) reported that NFAT4 could directly activate miR-23a expression through the transcriptional machinery. In humans, the NFAT family is comprised of five distinct gene products (NFAT1-5) that are ubiquitously expressed in mammalian cells and tissues (26,35). In resting cells, NFAT is phosphorylated and localized in the cytosol, and has low affinity for DNA binding. In response to calcium mobilization, activated calcineurin dephosphorylates NFAT, resulting in nuclear import and increased transcription of NFAT-regulated genes. Several findings have pointed to important roles for NFATs in modulating tumor progression. NFAT isoforms are overexpressed in human solid tumors and hematological malignancies, and seem to have roles in tumor motility and angiogenesis (36–38). Mechanistically, Sanna *et al.* (39) showed that MEK1-ERK1/2 signaling enhances NFAT-dependent gene expression through an indirect mechanism involving the induction of the activity of AP-1, which functions as a necessary NFAT-interacting partner. AP-1 is a family of basic leucine zipper transcription factors, composed of members of the Jun, Fos or activating transcription factor families that form homodimer or heterodimer protein complexes

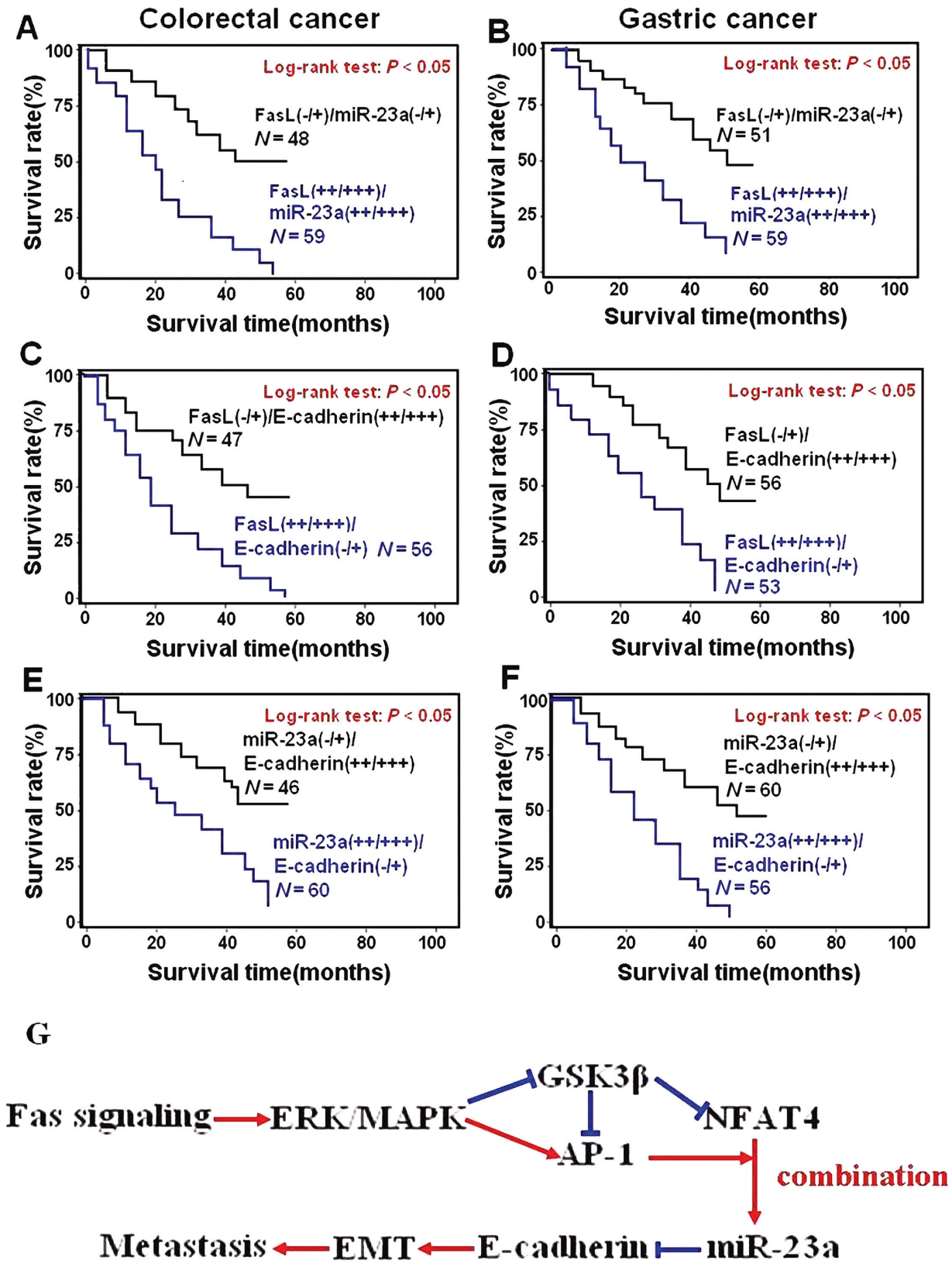

Fig. 6. Survival analysis of GI cancer patients with or without the EMT phenotype. As FasL may induce miR-23a expression, which inhibits E-cadherin expression *in vivo*, we defined FasL(−/+)/miR-23a(−/+), FasL(−/+)/E-cadherin(++/+++) and miR-23a(−/+)/E-cadherin(++/+++) as non-EMT phenotypic samples and the reverse expression as EMT phenotypic samples. Survival analysis and comparison were conducted using Kaplan–Meier method and log-rank test. Patients with a non-EMT phenotype had significantly better prognosis. CRC (**A**, **C** and **E**) and GC (**B**, **D** and **F**). Schematic model of the proposed mechanism for Fas-signaling-induced EMT in GI cancer (**G**). When low concentrations of FasL bind with the cognate Fas receptor, ERK/MAPK is activated, and subsequently represses GSK-3β activity and activates the AP-1 complex. Inhibition of GSK-3β activity further promotes nuclear translocation of NFAT4 and AP-1. NFAT4 and AP-1 accumulate in the nucleus, associate with one another and bind the promoter of miR-23a, thereby enhancing its expression. miR-23a targets E-cadherin messenger RNA 3′ UTR and prevents its translation and expression, leading to EMT and cancer metastasis.

(40). AP-1 activity is strictly regulated at both transcriptional and post-transcriptional levels. The primary posttranscriptional mechanism of AP-1 regulation is phosphorylation. Phosphorylation of c-Jun/AP-1 at Ser 73 and Ser 63 by ERK1/2 and c-jun N-terminal kinase/stress-activated protein kinases increases its stability and ability to activate transcription (28). ERK1/2-mediated phosphorylation of c-Fos/AP-1 on multiple residues (such as Ser 374) results in a remarkable increase in transactivating activity (41). Similarly, our data showed that FasL-induced ERK/MAPK activation contributed to active phosphorylation of c-Jun (Ser 63) and c-Fos (Ser 374) and subsequently enhanced the interaction between AP-1 and NFAT4, ultimately leading to upregulation of miR-23a. However, either NFAT4, c-Jun or c-Fos silencing partially depressed FasL-induced miR-23a expression, suggesting that alternative transcriptional factors are involved in regulation of miR-23a activation.

Previously, we found that FasL treatment inhibited E-cadherin transcription by upregulation of Snail, which was increased by inhibition of GSK-3β through FasL-induced ERK/MAPK signaling (11). Unlike most protein kinases, GSK-3β is active in resting epithelial cells and can be inactivated by various signaling mechanisms, including the PI3K/AKT and ERK1/2 MAPK pathways (28,42). Most of the substrates are functionally inhibited through phosphorylation by GSK-3β, which appears to act as a general repressor, keeping its targets switched off or inaccessible under resting conditions (42). GSK-3β has over 40 substrates with roles in a wide spectrum of cellular processes, including cell differentiation, proliferation and invasion (42). Among these substrates, c-Jun/AP-1 and NFAT4 are two common targets that are inhibited by GSK-3β in resting cells (27,28). Boyle *et al.* (43) reported that GSK-3β phosphorylated c-Jun *in vitro* (Thr 239, Ser 243 and Ser 249) and decreased its DNA binding activity. Conversely, lithium treatment, a GSK-3β inhibitor, increased AP-1-driven transcription, likely due to blockade of the inhibitory phosphorylation by GSK-3β of the c-Jun protein (44). Rephosphorylation of NFAT is required for nuclear export upon termination of calcium-calcineurin signaling. Two studies reported that GSK-3β rephosphorylated NFAT2 in the nucleus, leading to its nuclear export (45), whereas one study showed that inhibition of GSK-3β increased translocation of NFAT3 and NFAT4 to the nucleus *in vivo* (27). In this study, we found similar results, showing that inhibition of GSK-3β activity by FasL-induced ERK/MAPK signaling decreased the expression of inactive c-Jun (phospho-c-Jun Thr 239) and increased nuclear expression of NFAT4 and active c-Jun (phospho-c-Jun Ser 63), leading to upregulation of their transcriptional activity and mutual interaction; expression of the GSK-3β S9A mutant could reverse these effects.

To verify our results *in vivo*, qRT–PCR was used to analyze the expression of FasL, miR-23a and E-cadherin in fresh GI precancerous (CRC: $N = 367$; GC: $N = 419$) and cancer samples (CRC: $N = 135$; GC: $N = 143$). Primarily, we show that expressions of FasL and E-cadherin are consistent with a previous study (7) and that miR-23a levels increase during GI cancer progression. These three EMT-associated molecules significantly correlate with each other and can predict prognosis in GI cancer when combined with each other accordingly. All these data suggest that Fas signaling may inhibit E-cadherin partly by inducing miR-23a expression *in vivo*. However, miR-23a expression in cancer samples may be contradictory with another report (22). The study showed that miR-23a levels were upregulated during the evolution of mouse intestinal adenomas to adenocarcinomas and, importantly, were specifically upregulated during the transition from preinvasive (adenomas and carcinoma *in situ*) to locally invasive (stage I/II) primary CRC tumors (22). Subsequently, miR-23a levels decreased in primary CRCs from patients with cancer cells that had metastasized outside the colorectum (stage III/IV) (22). Conversely, another report suggested that upregulation of miR-23a expression was associated with an advanced clinical stage, depth of invasion and lymph node metastasis (46). Our data support the latter study. Possible reasons for discrepancies with the previous study may be sample size and the difference in race. More samples are needed in a further study to draw a conclusion.

Taken together, we have demonstrated that Fas signaling inhibits E-cadherin expression through induction of miR-23a, which is regulated by the NFAT4 and AP-1 complex. Further investigation will focus on whether other miRNAs are involved in the mechanism of Fas-signaling-induced EMT, such as in the regulation of N-cadherin and vimentin.

Supplementary material

Supplementary Information, Tables 1–8 and Figures S1–S11 can be found at http://carcin.oxfordjournals.org/

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