

## Original Article

# Ginkgo biloba extract 761 enhances 5-fluorouracil chemosensitivity in colorectal cancer cells through regulation of high mobility group-box 3 expression

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**Abstract:** Although the standard ginkgo biloba extract EGb 761 exhibits antioxidative, anti-apoptotic, and anticancer properties, there is no research focusing on the chemopreventive effects of EGb 761 in colorectal cancer (CRC). The present study investigated whether EGb 761 could increase 5-fluorouracil (5FU) sensitivity in CRC and its potential mechanism. We found that combined EGb 761 and 5FU treatment significantly elevated the chemosensitivity of CRC cells to 5FU in 5FU-resistant (5FUR) CRC cells, whereas no obvious cytotoxicity of EGb 761 was observed in parental cells. Then, real-time PCR and western blotting revealed that EGb 761 notably attenuated drug resistance through inhibition of epithelial-mesenchymal transition (EMT) factors (increased E-cadherin and decreased vimentin). In addition, we found that EGb 761 significantly inhibited 5FU-induced upregulation of high mobility group-box 3 (HMGB3) expression in 5FUR CRC cells both at mRNA and protein levels. Knockdown of HMGB3 effectively reversed 5FU-induced EMT and attenuated 5FU-induced cytotoxicity in 5FUR CRC cells while overexpression of HMGB3 achieved the opposite results. Moreover, we found that knockdown of HMGB3 effectively reversed the EGb 761-induced inhibition of the Wnt/ $\beta$ -catenin pathway. The results of the current study collectively demonstrated that EGb 761 can chemosensitize 5FUR CRC cells by inhibiting an EMT phenotype via regulation of HMGB3 expression, suggesting it to be a novel chemoprotective agent in CRC.

**Keywords:** Colorectal cancer, 5-fluorouracil, ginkgo biloba extracts, chemosensitivity, high mobility group-box 3

## Introduction

Colorectal cancer (CRC) is the third most common cancer in the United States and is also a leading cause of cancer-related mortality worldwide [1]. China has a high incidence of CRC, with 159.3 thousand estimated mortalities in 2012 [2]. The majority of patients with stage I and II colon cancer undergo partial or total colectomy alone, while about two-thirds of those with stage III disease receive chemotherapy [3]. Chemotherapy is becoming an important adjuvant therapy for the treatment of CRC. 5-Fluorouracil (5FU) is widely used to treat CRC and continues to be the main chemotherapeutic agent [4]. 5FU is a precursor of UTP and dTTP that interferes with the metabolism of DNA and RNA. However, prolonged exposure of cancer cells to 5FU may cause chemoresistance, which has become one of the most challenging factors in the effective treatment of CRC [5, 6]. Understanding the key mechanisms underlying drug resistance and finding a novel

agent to effectively sensitize the cells immune to 5FU underlies any rational attempt to increase patients' responses to treatments.

Ginkgo biloba has been used for centuries in Asia as a traditional therapy for a range of diseases [7]. Currently, ginkgo biloba extract (GBE) is one of the most widely used herbal medicinal products without verified adverse drug interactions. EGb 761, a GBE with antioxidant properties, has attracted much attention for its antitumor capability [8]. EGb 761 has been shown to induce cell apoptosis, and suppress tumor proliferation and progression of various cancers [9-11]. EGb 761 could enhance caspase-3 activities, and upregulate p53 and down-regulate bcl-2 to inhibit the progression of CRC cells [12]. A recent study also demonstrated that EGb 761 inhibited the migration and invasion of CRC cells through regulation of the LincRNA-p21/EZH2 pathway [13]. The aforementioned data provided important evidence for tumor prevention and adjuvant therapy using EGb

761. Of note, Liu et al. found that EGb 761 treatment enhanced chemotherapy sensitivity and reversed chemoresistance of gastric cancer cells through suppression of the ERK1/2 pathway [14]. However, whether EGb 761 can enhance the chemotherapeutic sensitivity and reverse chemoresistance of 5FU in CRC remains unclear.

Herein, the effects of EGb 761 on the chemotherapeutic sensitivity of CRC cell lines to 5FU was investigated. High mobility group-box 3 (HMGB3), a member of the HMG superfamily, plays an important role in DNA recombination, replication, repair, and transcription [15]. A recent study demonstrated that HMGB3 was upregulated in CRC tissues and acts as an oncoprotein to promote CRC progression [16]. However, no literature has reported on its role in chemoresistance. Since the epithelial-mesenchymal transition (EMT) was closely correlated with increased cellular motility and chemoresistance [17], we explored the effects of EGb 761 on this phenotypic change in 5FU-resistant (5FUR) CRC cell lines. In addition, we further investigated whether HMGB3 is involved in triggering the occurrence of EMT and plays a role in chemoresistant CRC. The study aimed to characterize the potential role of EGb 761 in 5FUR in CRC and its underlying mechanisms.

### Materials and methods

#### *Cell and chemicals*

Human CRC cell lines SW480 and SW620 were purchased from the ATCC of the Chinese Academy of Sciences (Shanghai, China). The culture medium was RPMI 1640 (Thermo Fisher Scientific, Wilmington, DE, USA) with 100 U/mL penicillin and 100 µg/mL streptomycin (Solarbio, Beijing, China), containing 10% fetal bovine serum (Gibco, Rockville, MD, USA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and passaged every 3 days. Standard GBE solution (EGb 761) was obtained from Dr. Willmar Schwabe GmbH & Co. KG (Karlsruhe, Germany). 5FU was obtained from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). Antibodies against E-cadherin, vimentin, β-catenin, c-myc, cyclin D1, and GAPDH, and HRP-conjugated goat anti-mouse/rabbit IgG were from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit IgG H&L Alexa Fluor® 488 was from Abcam (Cambridge, MA, USA). HMGB3 antibody was purchased from R&D Systems (Minneapolis, MN, USA).

Western blot related reagents were obtained from Beyotime (Jiangsu, China). Cell counting kit-8 was obtained from BIOSHARP (Hefei, China). The ThermoScript RT-PCR System was from Invitrogen (Carlsbad, CA, USA). SYBR Green PCR Master Mix was from Applied Biosystems (Foster City, CA, USA). The specific primers were obtained from SangonBiotech Co., Ltd. (Shanghai, China). HMGB3 plasmids and siRNA were obtained from GenePharma (Shanghai, China). TOP/FOP flash plasmids and Dual Luciferase Assay Kit was from Promega (Madison, WI, USA).

#### *Cell treatment*

5FUR cell lines were established as previously described [18]. Both the parental and 5FUR cell lines were used to investigate the efficacy of individual and combined 5FU and EGb 761 treatments. The concentration of EGb 761 was determined by referring to our previous study where 500 mg/L EGb 761 significantly inhibited the migration and invasion of CRC cells [13]. The HMGB3 vector (pcDNA3.1/HMGB3) was constructed by sub-cloning the coding sequence of wild-type HMGB3 into pc-DNA3.1. The empty pc-DNA3.1 vector was used as the control. The siRNA specifically targeting HMGB3 and corresponding control RNA oligoribonucleotides were transfected into the cells using Lipofectamine 2000 in accordance with the manufacturer's instructions.

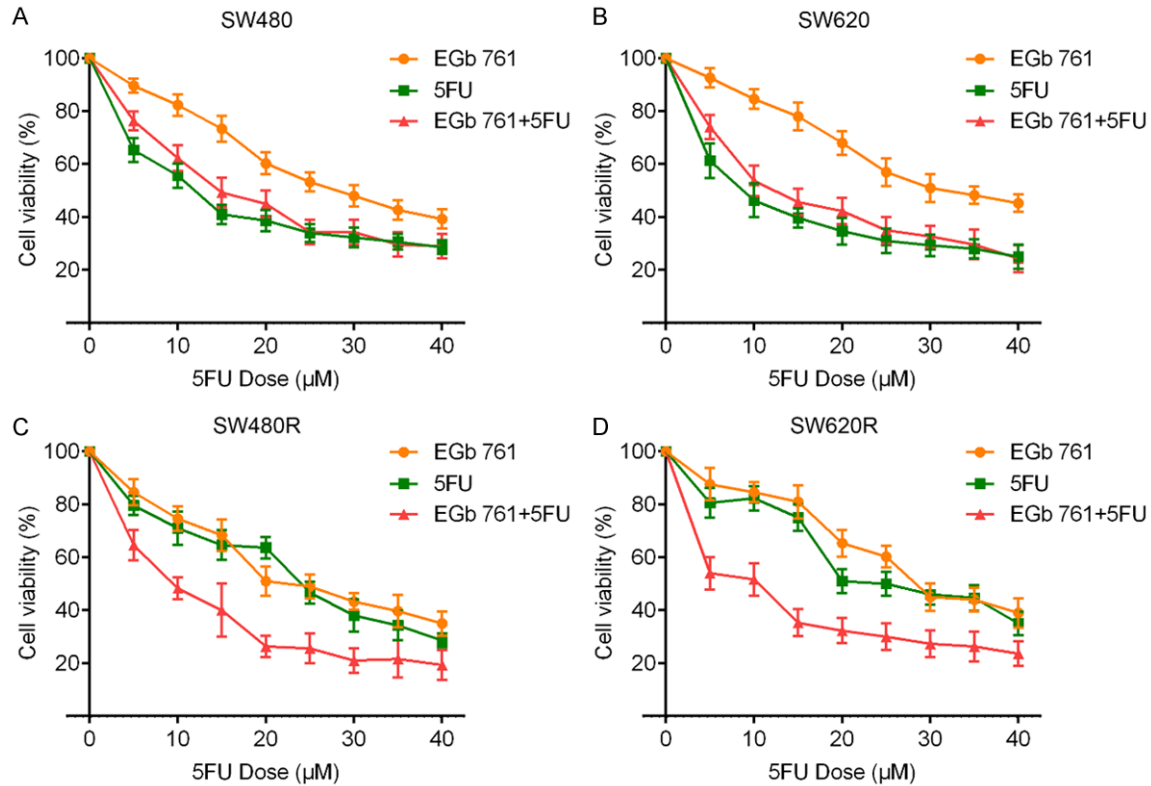
#### *Cell viability assay*

Cells ( $5 \times 10^3$ ) were seeded in 96-well plates overnight and treated with the indicated drugs. A CCK8 assay was performed after incubation for 72 h. The absorbance (optical density, OD) of cells was read at a wavelength of 450 nm. The cell viability rate was calculated as follows: (OD treated/OD control) × 100%. Three biological replicates were prepared for each treatment and each assay was performed in triplicate.

#### *RNA isolation and real-time PCR*

Cells were treated with 500 mg/L EGb 761 and/or 15 µM 5FU for 24 h. Subsequently, total RNA was isolated from cells in accordance with the manufacturer's protocol. Subsequently, cDNA was synthesized from RNA and real-time PCR was then performed on an ABI 7300 Real-Time PCR system (Applied Biosystems). The reactions were an initial incubation at 95°C for 2 min, followed by 40 cycles at 95°C for 5 s,

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**Figure 1.** EGb 761 enhanced 5FU sensitivity in 5FU resistant colorectal cancer cells. A, B. Cell viability of both SW480 and SW620 cells were detected via CCK8 assay and the results showed that 500 µM EGb 761 alleviated 5FU-induced cytotoxicity while EGb 761 alone had minor cytotoxicity on SW480 and SW620 cells. C, D. Cell viability of both 5FU resistant SW480 cells (SW480R) and 5FU resistant SW620 cells (SW620R) cells treated with 10-40 µM 5FU were detected via CCK8 assay and the results showed that co-treatment with EGb 761 and 5FU enhanced 5FU sensitivity in 5FU resistant colorectal cancer cells. \*P<0.05 compared to 5FU.

60°C for 60 s and 72°C for 30 s. Primers for HMGB3 were: forward 5'-CAGCTTGATACCTGTGAATGGG-3', reverse 5'-TATCTGTGGTCGTGTGGACT-3'. Primers for E-cadherin were: forward 5'-AAAGGCCCATTTCTCTAAAAACCT-3', reverse 5'-TGCGTTCTCTATCCAGAGGCT-3'. Primers for vimentin were: forward 5'-AGTCCACTGAGTACCGGAGAC-3', reverse 5'-CATTTACGCATCTGGCGTTC-3'. GAPDH (forward, 5'-ACAACCTTGGTATCGTGAAGG-3' and reverse, 5'-GCCATCACGCACAGTTTC-3') was used as an internal control for calculating the relative expression levels of the above genes via the  $2^{-\Delta\Delta Ct}$  method.

### Western blot

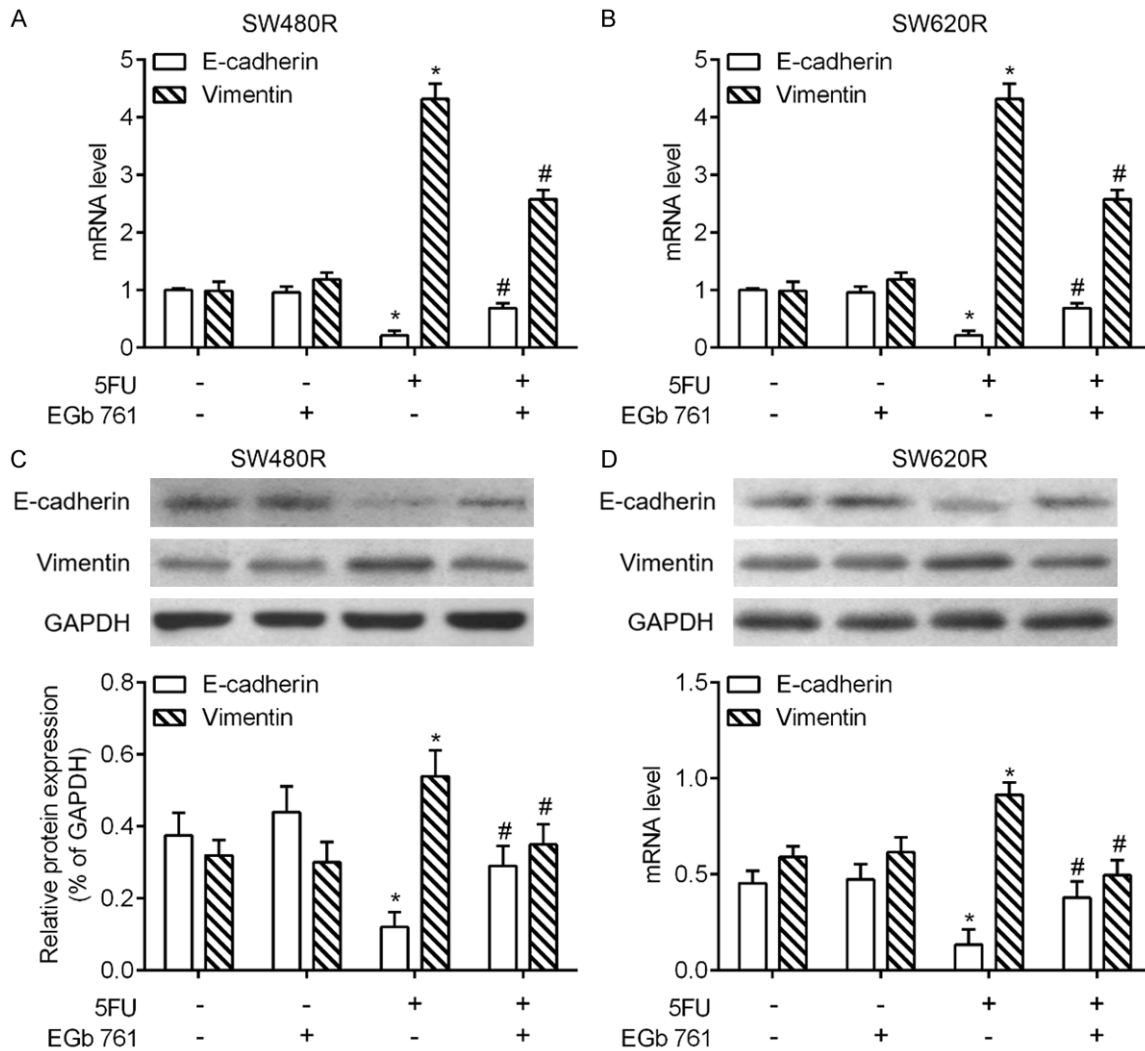
Following treatment, the cells were lysed in RIPA lysis buffer supplemented with phenylmethanesulfonyl fluoride for 15 min at 4°C and after centrifugation at 15,000 × g for 10 min at 4°C, protein concentrations were quantified using the BCA method. Equal amounts of proteins were separated by 8-12% SDS-PAGE. After being transferred to polyvinylidene difluoride

(PVDF) membranes, the proteins were blocked with 5% skimmed milk in TBST at room temperature for 1 h. After three washes with PBST, the membranes were incubated with primary antibodies against HMGB3 (1:1000), E-cadherin (1:1000), vimentin (1:500), β-catenin (1:1000), c-myc (1:1000), cyclin D1 (1:1000), or GAPDH (1:2000) at 4°C overnight, and further incubated in horseradish peroxidase conjugated secondary antibodies (1:10000) at room temperature for 2 h. The protein bands were visualized by enhanced chemiluminescence (ECL System; Millipore) and sections were exposed to X-ray film (Kodak). Quantity One software (Bio-Rad) was used to quantify western blotting data.

### TOP/Flash activity assay

SW480R and SW620R cells were planted in a 24-well cell culture plate and serum-starved overnight. Cells were then co-transfected with 0.1 µg pRL-TK plasmid and either 0.2 µg TOP flash plasmid or 0.2 µg FOP flash plasmid us-

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**Figure 2.** EGb 761 reversed 5FU induced epithelial-mesenchymal transition (EMT) in 5FU resistant colorectal cancer cells. A, B. Relative expression levels of E-cadherin and Vimentin mRNA in were significantly upregulated in 5FU treated SW480R and SW620R cells, which were remarkably downregulated in cells treated with both 5FU and EGb761. C, D. 5FU treatment caused increased in E-cadherin and Vimentin protein levels as measured through western blotting analysis. while addition of EGb761 significantly decreased their expression levels in both SW480R and SW620R cells. \*P<0.05 vs. 5FU and EGb 761 negative group; \*\*P<0.05 vs. 5FU positive and EGb 761 negative group.

ing Lipofectamine 2000. The Dual Luciferase Assay Kit was used to determine the activities of both firefly and *Renilla* luciferase reporters 48 h after transfection. The TOP-Flash reporter activity is presented as the relative ratio of luciferase activity to *Renilla* activity. The results were shown as the ratio of TOP/FOP Flash activity. The experiment was carried out in triplicate.

### Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. Cells were

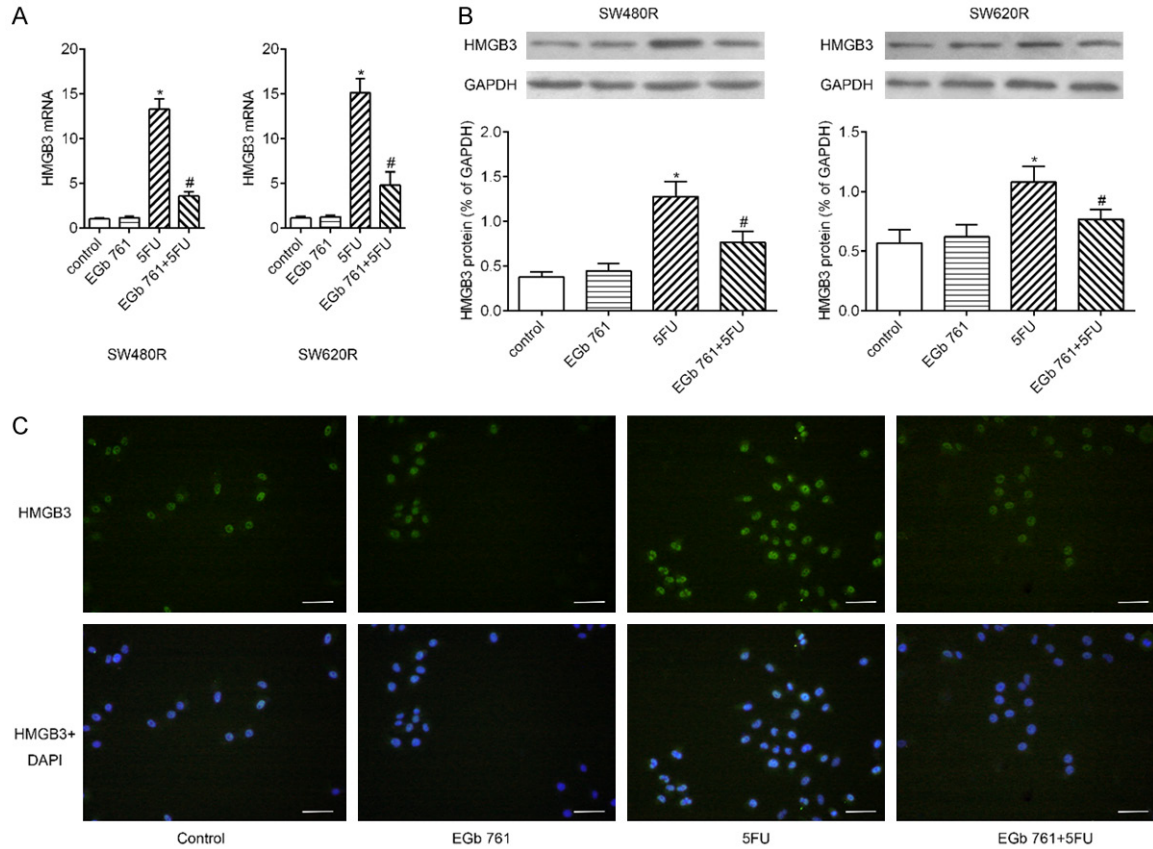
then blocked with 5% BSA for 30 min at room temperature followed by incubation with the HMGB3 antibody (1:200) at 4°C overnight. After washes with PBST, cells were incubated with secondary antibody (1:500) at room temperature for 1 h in the dark. Finally, nuclei were stained with DAPI for 5 min. The cells were examined under a Nikon fluorescence microscope (Image Systems, Columbia, MD) at 200 × magnification.

### Statistical analysis

All calculations were performed using GraphPad Prism 5.5 (GraphPad Software, San Diego, CA,



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**Figure 3.** EGb 761 attenuated 5FU induced upregulation of HMGB3 in 5FU resistant colorectal cancer cells. **A.** 5FU treatment leading to the increase in the expression levels of HMGB3 mRNA in SW480R and SW620R cells and EGb 761 could significantly reversed this alteration. **B.** Relative expression levels of HMGB3 protein in SW480R and SW620R cells were increased by 5FU treatment but this increase was inhibited by EGb 761 treatment. **C.** Nuclear HMGB3 expression in SW480R cells treated with 5FU and/or EGb 761 was detected by immunofluorescence staining and the results illustrated that EGb761 could attenuated 5FU induced inhibition of HMGB3 expression. (Scar bar: 50  $\mu$ m). \* $P$ <0.05 vs. control; # $P$ <0.05 vs. 5FU.

USA). Data for continuous variables were presented as the median  $\pm$  standard deviation of data. Differences between groups were analyzed using the Student's t-test. The one-way analysis of variance (ANOVA) was used to determine the significant difference among multiple groups. It was considered to be statistically significant when the  $P$  value <0.05.

### Results

#### *EGb 761 enhances 5FU sensitivity of CRC cells*

To explore the role of EGb 761 in CRC chemoresistance, we measured the cytotoxicity of 5FU and EGb 761 individually and in combination on parental and SW480R and SW620R cells. As shown in **Figure 1A** and **1B**, 5FU caused greater cytotoxicity than EGb 761 in both parental SW480 and SW620 cells. Although the combi-

nation of EGb 761 and 5FU showed a minor enhancement in cytotoxicity, it was not statistically significant. On the contrary, in the 5FUR cell lines, 5FU showed similar cytotoxicity to EGb 761 upon treatment with increasing 5FU concentrations. Intriguingly, combined treatment with EGb 761 and 5FU led to a significant synergistic enhancement in cytotoxicity (**Figure 1C** and **1D**). The above findings suggest that EGb 761 could attenuate 5FU resistance in 5FUR cell lines.

#### *EGb 761 reverses 5FU-induced EMT in CRC cells*

To understand the potential mechanism of EGb 761 in sensitizing CRC cells to 5FU, we assessed the effects of EGb 761 on the change in EMT, which may serve as a mediator of colon cancer chemotherapy resistance. As expected,

the expression of E-cadherin mRNA was reduced, while the expression of vimentin mRNA was increased by 5FU treatment alone; the addition of EGb 761 reversed the above alteration in both SW480R (**Figure 2A**) and SW620R cells (**Figure 2B**). Accordingly, 5FU significantly reduced E-cadherin protein expression and increased vimentin protein expression while the above changes were abolished upon treatment with the combination of EGb 761 and 5FU in both SW480R (**Figure 2C**) and SW620R cells (**Figure 2D**).

### *Egb 761 regulates 5FU-induced EMT via suppression of HMGB3*

We then investigated the potential mechanisms underlying the role of EGb 761 in chemotherapy resistance. Interestingly, by using qPCR, we found that the levels of HMGB3 mRNA were notably higher in the 5FU group than in the control group. Co-treatment with 5FU and EGb 761 significantly reversed the change of HMGB3 mRNA expression in CRC cell lines. However, EGb 761 alone had no effect on HMGB3 expression (**Figure 3A**). Similarly, 5FU caused upregulated HMGB3 protein expression, while EGb 761 alone had no effects on HMGB3 expression. Combined EGb 761 and 5FU treatment attenuated the increase caused by 5FU treatment in SW480R and SW620R cells (**Figure 3B**). We next measured the expression and localization of HMGB3 using immunofluorescence in SW480R cells. With the treatment with 5FU, nuclear HMGB3 expression was significantly increased. However, HMGB3 expression was decreased with combined EGb 761 and 5FU treatment (**Figure 3C**). Rescue assays were performed to validate whether HMGB3 was involved in controlling the EMT in 5FU CRC cells. Intriguingly, by using plasmid-mediated overexpression of HMGB3 (**Figure 4A**), the suppressed EMT phenotype was apparently reversed. As illustrated in **Figure 4B**, EMT markers were modulated by HMGB3, E-cadherin was down-regulated and vimentin was up-regulated. We then used HMGB3 siRNA to downregulate HMGB3 expression to determine whether it was involved in the inhibition of EGb 761 on the EMT. As shown in **Figure 4C**, HMGB3 siRNA3 caused a sufficient knockdown effect, and thus was chosen for subsequent tests. In SW480R and SW620R cells, knockdown of HMGB3 increased E-cadherin protein expression and decreased vimentin protein expres-

sion (**Figure 4D**). Notably, EGb 761+HMGB3 siRNA significantly sensitized 5FU CRC cells to 5FU as compared with EGb 761+si-NC. Overexpression of HMGB3 in cells with combined EGb 761 treatment pronouncedly attenuated 5FU induced cytotoxicity compared with cells treated with EGb 761 alone (**Figure 4E** and **4F**). The collective findings suggested that EGb 761 regulated HMGB3 expression to inhibit EMT.

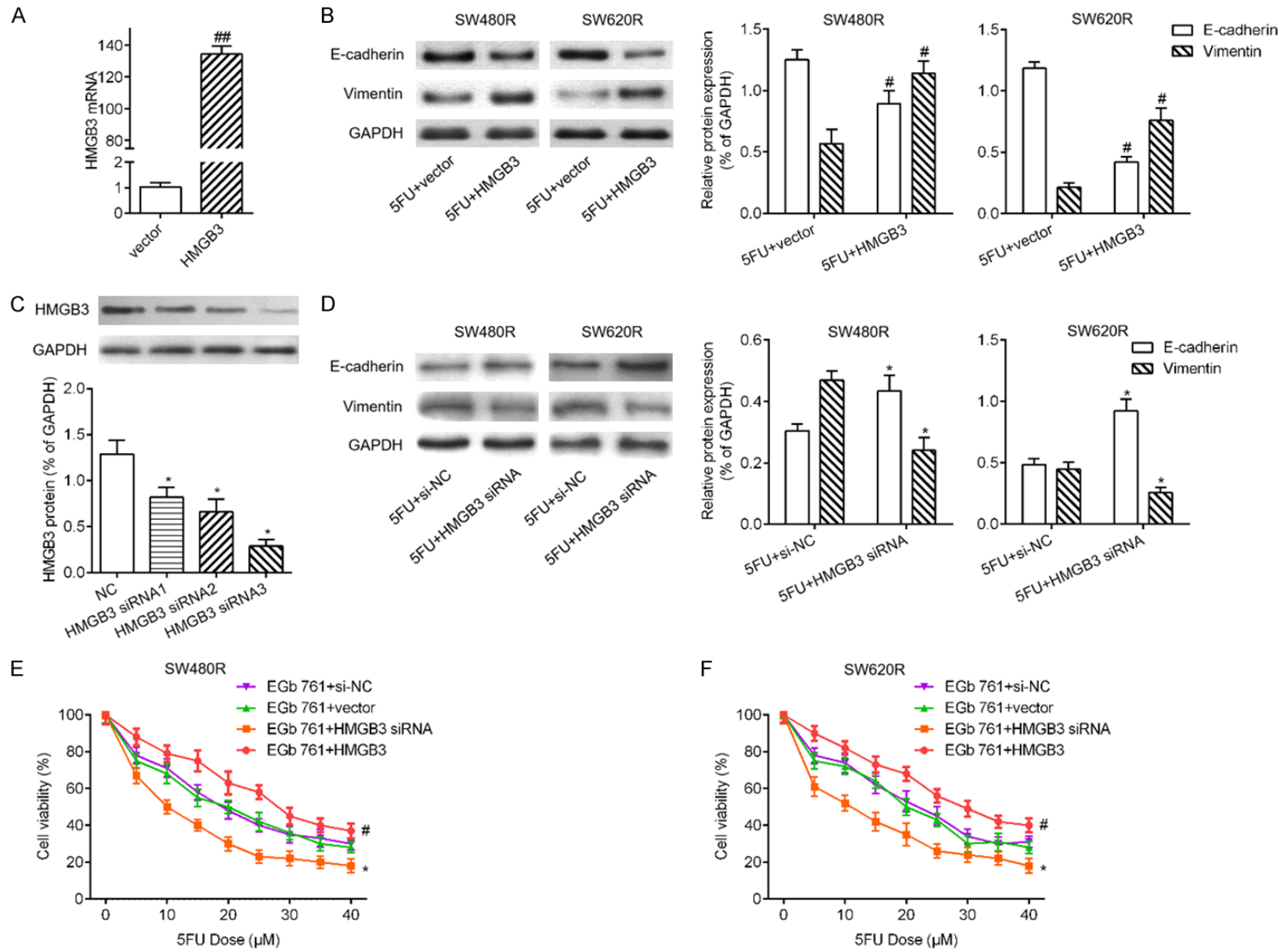
### *HMGB3 activates the Wnt/ $\beta$ -catenin pathway in CRC cells*

The Wnt/ $\beta$ -catenin pathway is one of the major signaling pathways that mediates the EMT by transactivating EMT-related genes in malignant tumors. We thus measured the expression levels of total  $\beta$ -catenin in CRC cells with and without treatment of EGb 761. As shown in **Figure 5A** and **5B**, the expression levels of  $\beta$ -catenin and Wnt/ $\beta$ -catenin pathway target genes c-myc and cyclin D1 were markedly increased after 5FU treatment as compared to those of the EGb 761 or control groups. However, addition of EGb 761 to 5FU CRC cells obviously inhibited 5FU-induced activity of the Wnt/ $\beta$ -catenin signal. Subsequently, we investigated the role of HMGB3 in the Wnt/ $\beta$ -catenin signal pathway and demonstrated that HMGB3 knockdown could inhibit Wnt/ $\beta$ -catenin signaling by examining TOP/FLASH-driven luciferase activity (**Figure 5C**). Moreover, we identified that overexpression of HMGB3 significantly revised the inhibition of Wnt/ $\beta$ -catenin signal pathway caused by EGb 761 treatment by detecting  $\beta$ -catenin, c-myc, and cyclin D1 expression via western blot analysis both in SW480R and SW620R cells (**Figure 5D**). Taken together, we confirmed that HMGB3 played an important role in the inhibition of the Wnt/ $\beta$ -catenin signaling pathway by EGb 761 treatment in 5FU CRC cells.

## Discussion

5FU is one of the most commonly used chemotherapeutic drugs in CRC in both adjuvant and metastatic settings. Previous 5FU-based clinical studies have validated the benefits of chemotherapy to prolong the survival of patients with CRC [19]. Nevertheless, about half of the patients with CRC are resistant to 5FU-based chemotherapy [20]. Although great efforts have been made, intrinsic and acquired resistance remains a major setback to 5FU clinical efficacy [21].

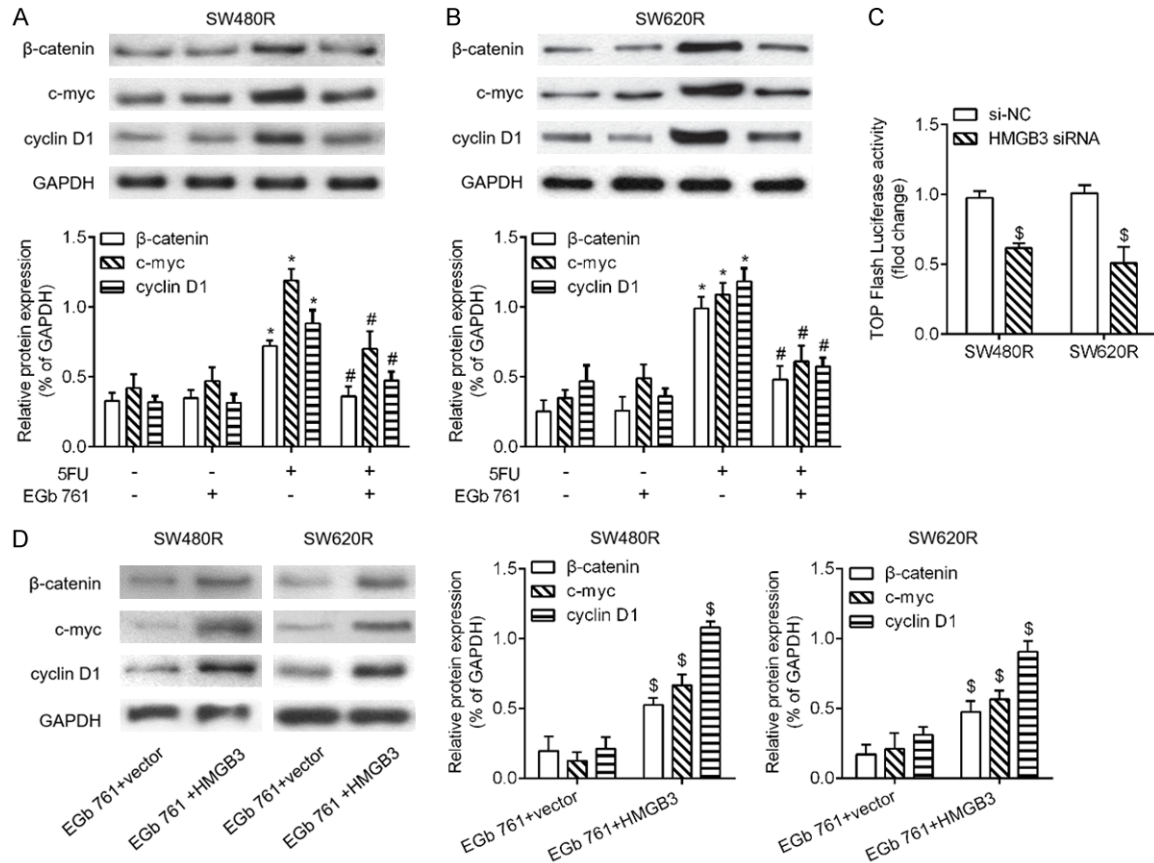
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**Figure 4.** Egb 761 suppressed HMGB3 to chemosensitizes colorectal cancer cells to 5FU. A. The mRNA level of HMGB3 was dramatically upregulated after transfected with HMGB3 vector as compared to the empty vector group as measured using real-time qPCR assay. B. Overexpression of HMGB3 reduced E-cadherin protein

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expression and increased Vimentin protein expression in SW480R and SW620R cells under 5FU condition. C. The expression levels of HMGB3 were significantly decreased in groups transfected with specific HMGB3 siRNAs as compared to those with negative control as evaluated via Western blot. D. Knockdown of HMGB3 increased E-cadherin protein expression and decreased Vimentin protein expression in SW480R and SW620R cells under 5FU condition. E, F. CCK8 assay showed that overexpression of HMGB3 abolished the sensitization of EGb 761 to 5FU while knockdown of HMGB3 enhance the sensitization of EGb 761 to 5FU both in SW480R and SW620R cells treated with 10-40  $\mu$ M 5FU. #P<0.05 vs. 5FU+vector group; ##P<0.05 vs. vector group; \*P<0.05 vs. EGb 761+si-NC group.



**Figure 5.** HMGB3 is required for the inhibition of Wnt/ $\beta$ -catenin signaling pathway by EGb 761 treatment in 5FU CRC cells. (A, B) EGb 761 significantly inhibited 5FU caused activation of Wnt/ $\beta$ -catenin signaling pathway. Relative expression as evidence by the increased levels of  $\beta$ -catenin, c-myc and cyclin D1 protein in (A) 5FU SW480R (SW480R) cells and (B) 5FU SW620R (SW620R) cells with or without EGb 761 treatment, as detected by Western blot. (C) CRC cells were transfected with TOP-Flash or control FOP-Flash reporter to determine reporter activities 48 h later. The bar chart represented the ratios of TOP/FOP and the differences between independent experimental groups were analyzed using Student' t-test. The results demonstrated that knockdown of HMGB3 inhibited the activation of Wnt/ $\beta$ -catenin signaling. (D) Overexpression of HMGB3 revised the inhibition of Wnt/ $\beta$ -catenin signal pathway caused by EGb 761 as evidenced by the increased  $\beta$ -catenin, c-myc, and cyclin D1 protein expression in SW480R and SW620R cells. \*P<0.05 vs. control group; #P<0.05 vs. 5FU group; \$P<0.05 vs. EGb 761+vector group.

Natural compounds and their potential use in clinical practice have undoubtedly many advantages. With regard to anticancer properties, they possess chemoprotective and pro-apoptotic properties that affect proliferation, angiogenesis, tumor metastasis, or chemosensitivity [22, 23]. GBE contains over 60 biologically active substances, the most important of which

are terpenes, flavonoids, carboxylic acids, and L-ascorbic acid. They have been widely investigated for their roles in human disease and health [24]. Simultaneous administration of a conventional chemotherapy drug together with one or more natural bioactive agents tend to be safer and more tolerated [25]. EGb 761 could protect against cisplatin-induced ototox-



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icity and be served as a chemoprotective agent [26]. It could reduce the cisplatin-induced oxidative stress to chemosensitize gastric cancer cells to cisplatin [14]. It has also been reported that EGb 761 has chemopreventive effects in estrogen receptor-independent breast cancer through anti-proliferative and apoptosis-inducing activities [27]. Importantly, researchers had identified a good benefit-risk ratio of the combined 5FU and EGb 761 therapy as second-line treatment in metastatic CRC and an improvement was observed in some patients despite the failure of the conventional 5FU pretreatment [28]. However, to date, no literature has reported on the chemopreventive effects of EGb 761 on 5FUR CRC cells. We, for the first time, showed that EGb 761 treatment significantly reduced 5FU resistance of CRC cells. However, we found that EGb 761 does not enhance the chemotherapeutic potential of 5FU in parental cell lines. These findings suggest the potential therapeutic usefulness of EGb 761 as an adjunctive treatment to 5FU in patients with CRC.

EMT has been considered to be a key program associated with the invasion and metastasis of CRC [29]. Studies have also suggested that the EMT is associated with the acquisition of chemotherapeutic resistance, at least in experimental models [30]. Notably, in the presence of 5FU, CRC cells undergo ultrastructural changes and exhibit a mesenchymal phenotype, with loss of E-cadherin and disruption of cell-cell junctions [30]. Consistent with previous studies, we showed that 5FUR CRC cells processed higher E-cadherin expression and lower vimentin expression than parental CRC cells. These changes could be partly reversed by combination treatment with 5FU and EGb 761. However, how EGb 761 controls the 5FU-induced EMT remains unclear.

HMGB3 has a HMG-box, and hence it can regulate gene transcription by participating in the formation of enhancosomes. Current studies reported that HMGB3 participates in the progression of many cancers including prostate cancer, breast cancer, gastric cancer, and CRC [31-33]. HMGB3 was also identified as a biomarker detected in peripheral blood in lung cancer [34]. Furthermore, Li et al. demonstrated that overexpression of HMGB3 promoted cell proliferation and migration and is associated with poor prognosis in urinary bladder can-

cer patients [35]. In CRC cells, knockdown of HMGB3 expression significantly inhibited cell growth and attenuated cell migrative ability [16]. Importantly, a recent study found that silencing of HMGB3 expression in gastric cancer cells reduced tolerance to paclitaxel and oxaliplatin but increased tolerance to oxaliplatin [36]. We thus hypothesized that HMGB3 may also influence the chemosensitivity of CRC cells to 5FU. As expected, we found that 5FU treatment caused upregulated HMGB3 expression in 5FUR cells. Combined treatment with 5FU and EGb 761 effectively decreased HMGB3 expression. Our further experiments found that knockdown of HMGB3 expression using a HMGB3 siRNA reversed the EMT phenotype in 5FUR CRC cells and results in reduced 5FU resistance in CRC. These results pointed to HMGB3 as a target protein to enhance the sensitivity of CRC to 5FU. The determination of HMGB3 may have potential to be a useful marker in screening patients that respond positively to 5FU-based therapy. Moreover, our further experiments found that downregulation of HMGB3 expression inhibited Wnt/ $\beta$ -catenin signaling, suggesting that HMGB3 could regulate 5FU-induced activation of the Wnt/ $\beta$ -catenin pathway. The regulatory effects of HMGB3 on the EMT were also validated by a study of Zhang et al. [16]. Multiple signaling pathways are involved in the activation of the EMT, including NF- $\kappa$ B, Notch, and Wnt/ $\beta$ -catenin pathways. Wnt/ $\beta$ -catenin signaling could regulate the proliferation and growth of colon cancer cells by promoting EMT [37]. Another study demonstrated that Destruxin B inhibited the proliferation of hepatocellular carcinoma cells by regulating the EMT via Wnt/ $\beta$ -catenin signaling. Similarly, we identified that EGb 761 treatment significantly inhibited 5FU-induced Wnt/ $\beta$ -catenin activation. Collectively, the above data suggested that HMGB3 controlled Wnt/ $\beta$ -catenin signaling was involved in the EGb 761-sensitization of CRC cells to 5FU.

In a broader point of view, several other mechanisms have been proposed to regulate tumor cell response to 5FU in CRC. Once EMT occurs, several molecular-based signaling pathways are activated, resulting in the activation of transcriptional regulators such as TWIST1. Suppression of TWIST in CRC cells also sensitizes the cells to 5FU [38]. Therefore, the other factors controlling 5FU-induced EMT need to be elucidated in CRC cells. Further investigation is

needed to evaluate other ways by which EGb 761 chemosensitizes 5FU cells.

In summary, to our knowledge this is the first report showing that EGb 761 chemosensitizes CRC cells to chemotherapeutic agent 5FU by reversing the HMGB3-mediated EMT through the Wnt/ $\beta$ -catenin signaling pathway.

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#### Disclosure of conflict of interest

None.

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