

# Aspirin inhibits colon cancer cell line migration through regulating epithelial-mesenchymal transition via Wnt signaling

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**Abstract.** The mechanism responsible for the initiation of tumor metastasis and epithelial-mesenchymal transition (EMT) is not well understood. During EMT, epithelial cells lose their polarity and adhesion to surrounding cells and migrate, resulting in transition into mesenchymal cells. Canonical Wnt signaling has been implicated in controlling gene transcription and body axis pattern formation during development. However, canonical Wnt signaling has also been indicated to serve a role in carcinogenesis by regulating EMT. In the present study, it was demonstrated that the expression of several positive regulators of EMT and Wnt signaling was repressed by aspirin treatment in SW480 tumor cells, and that this reduction was due to alterations in the localization of zinc finger E-box binding homeobox 1 and Snail family transcriptional repressor 2. It was also demonstrated that aspirin may be an effective inhibitor of EMT, reducing the viability and migration ability of SW480 tumor cells, including cells induced by TGF- $\beta$ 1.

## Introduction

The source of mesenchymal cells in pathological processes, including tumor invasiveness and metastasis, is still not fully understood; however, increasing evidence suggests that epithelial-mesenchymal transition (EMT) is one of the important processes that lead to the formation of these mesenchymal cells (1). EMT is the process by which a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, undergoes multiple biochemical alterations that enable it to assume a mesenchymal cell phenotype,

including enhanced migratory capacity, invasiveness and elevated resistance to apoptosis (2). Accumulating studies point to a critical series of EMT-like events during tumor progression and malignant transformation, providing the incipient cancer cell with invasive and metastatic properties (3-5). EMT may lead to the metastasis of cancer cells from the primary tumor into surrounding tissue and vasculature (6). Notably, preventing metastasis is the ultimate challenge in overcoming cancer (7).

The hallmark of EMT is the loss of epithelial surface markers, most notably epithelial (E)-cadherin and the acquisition of mesenchymal markers, including Vimentin, and neural (N)-cadherin. Downregulation of E-cadherin during EMT can be mediated by its transcriptional repression through the binding of EMT transcription factors including Snail, Snail family transcriptional repressor 2 (Slug) and twist family bHLH transcription factor 1 (Twist) to E-boxes present in the E-cadherin promoter (8). **Slug is a Snail family transcription factor**, which has been demonstrated to be involved in the control of apoptosis (9). Over expression of Slug has been identified in a number of different cancer types and is correlated with increases in tumor cell proliferation and lymph node metastasis (10).

Wnt signaling is an evolutionarily conserved pathway in animals involved with development but is also implicated in tumor formation. Wnt signaling serves a role in specifying cell fate, body axis patterning, proliferation and migration, which are necessary processes in the formation of vital tissues during embryonic development (11). Wnt mutations have been demonstrated to cause multiple diseases, including breast and prostate cancer, glioblastoma and diabetes. Typically, a Wnt protein binds to the domain of a member of the Frizzled family of receptors. These transmembrane G-protein coupled receptors send a signal to the Disheveled phosphoproteins located in the cytoplasm through direct interactions (12).

Dysregulation of canonical Wnt signaling processes can easily lead to cellular over-proliferation and tumor formation. Common dysregulations leading to tumor formation include mutations in components of the  $\beta$ -catenin destruction complex, overexpression of Wnt ligands, mutations in  $\beta$ -catenin and loss of other Wnt inhibitors and regulators (13). A previous study has demonstrated that repression of Wnt/ $\beta$ -catenin signaling reduces EMT and suppresses metastasis of breast cancer to the lungs (14).

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In this study, two important inducers of EMT: Slug and zinc finger E-box binding homeobox 1 (ZEB1) were focused on. The two proteins bind to E-box consensus sequences on the E-cadherin promoter (15). These proteins facilitate EMT by directly repressing transcription of E-cadherin and other junctional proteins, including claudins and desmosomes (16). Notably, Wnt signaling induces Slug and the mesenchymal marker, Vimentin. Expression of Slug and ZEB1 are induced by the presence of transforming growth factor (TGF)- $\beta$  during embryogenesis to regulate the formation of different organs; however, certain types of metastatic cancer have been demonstrated to activate TGF- $\beta$  signaling (17).

Aspirin is one of the oldest known and most widely used medicines. It is known to reduce inflammation by irreversibly inhibiting cyclooxygenase (COX)-1 and COX-2 (18), inhibiting the formation of inflammatory prostaglandins (PGs) and eicosanoids by disrupting COX's formation of their precursors from arachidonic acid (17). Aspirin inhibits the activation of the Wnt signaling pathway by modulating Wnt/PGE<sub>2</sub> crosstalk and by promoting the degradation of  $\beta$ -catenin (19). Recently, there has been an increased focus on the role of aspirin as a chemopreventative drug. In the preset study it was demonstrated that in addition to its anti-inflammatory and cardioprotective characteristics, aspirin serves a role in inhibiting EMT (20,21).

## Materials and methods

**Cell line and reagents.** The human colorectal adenocarcinoma SW480 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 with 10% fetal bovine serum (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA). All associated antibodies [E-cadherin (cat. no. ab15148; 1:1,000)], N-cadherin (cat. no. ab18203; 1:1,000), Vimentin (cat. no. ab8978; 1:3,000), Slug (cat. no. ab27568; 1:1,000), Snail (cat. no. ab53519; 1:1,000), Twist (cat. no. ab50581; 1:2,000), ZEB1 (cat. no. ab124512; 1:1,000), ZEB2 (cat. no. ab138222; 1:1,000) and GAPDH (cat. no. ab8245; 1:5,000) were purchased from Abcam (Cambridge, UK). Aspirin and TGF- $\beta$ 1 were available from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

**Cell migration assay.** Cells were plated at  $1 \times 10^5$  cells/well in 200  $\mu$ l of culture medium in the upper chamber of Transwell permeable supports (Corning Inc., Corning, NY, USA; with an 8.0- $\mu$ m pore size, polycarbonate membrane, 6.5-mm diameter and a 24-well plate format) to track the migration of SW480 cells. Migrated cells incubated in DMEM+10% FBS on the lower surface of the membranes were fixed with 4% paraformaldehyde for 10 min at 37°C and stained with hexamethylparosaniline chloride for 20 min at 37°C. The number of cells was counted in three randomly selected fields of the wells under a light microscope.

**Plasmid construction and cell transfection.** The E-cadherin promoter region was cloned by polymerase chain reaction (PCR) and inserted into PGL-3 basic plasmid (cat. no. VT1554; YouBio, Hunan, China) which expressed the luciferase-reporting gene. The recombinant plasmid (5  $\mu$ g) was transferred into competent cells and positive clones were identified by PCR and restriction enzyme digestion prior

to being sequenced. Following the amplification of recombinant plasmid using a QIAGEN Plasmid Maxi kit (12162; Qiagen, Inc., Valencia, CA, USA), the E-cadherin luciferase report plasmid was used for cell transfection. For the ZEB-RFP and Slug-RFP constructs, cDNA fragments of ZEB and Slug were amplified by PCR and inserted into PCDNA3.1-RFP vector (cat. no. VPY0003; Yrgene, Changsha, China). Briefly, a large number of ZEB-RFP and Slug-RFP plasmids were obtained according to the above step. The siRNAs targeting *ZEB1* gene were designed and synthesized by the Shanghai GenePharma Co., Ltd., (Shanghai, China). Small interfering (si)RNA sequences for three sites of the *ZEB1* gene are listed in Table I. Transfections (10  $\mu$ mol siRNA) were performed using the Lipofectamine® 2000 kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

**Cell proliferation assay.** SW480 cells were seeded in 96-well plates. Cell proliferation was assessed using a Cell counting Kit-8 (CCK-8; cat. no. 1166/1; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The number of cells was measured using a microplate reader at a wavelength of 450 nm.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized by adding purified RNA and oligo(dT) primers to SuperScript II at 4°C (Invitrogen; Thermo Fisher Scientific, Inc.). QPCR was performed using SYBR-Green (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (primers are listed in Table II). The reactions were performed as follows: 95°C for 15 min, 95°C for 10 sec, 60°C for 32 sec, 72°C for 32 sec and 85°C for 6 sec for 40 cycles; the melting curve analysis ranging from 60 to 95°C, gradually increased at a speed of 0.5 kC every 10 sec. The relative expressions levels were normalized to GAPDH and expressed using the  $2^{-\Delta\Delta C_t}$  method (22).

**Western blotting.** Cells were lysed in ice-cold radioimmunoprecipitation buffer containing a protease inhibitor cocktail (RocheDiagnostics, Basel, Switzerland). The concentration of protein was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Proteins (60  $\mu$ g) were separated by 8% SDS-PAGE and were transferred onto nitrocellulose membranes. The membranes were blocked in PBS containing 5% skimmed milk for 1 h at 37°C and incubated overnight at 4°C with the primary antibodies against the protein of interest. Goat anti-mouse immunoglobulin (Ig)G H&L horseradish peroxidase (HRP; cat. no. ab6789; 1:5,000; Abcam) and goat anti-rabbit IgG H&L (HRP; cat. no. ab6721; 1:5,000; Abcam) were then added to each membrane and incubated for 2 h at 37°C. The presence of target proteins was detected using enhanced chemiluminescence system (cat. no. K820-50; BioVision, Inc., Milpitas, CA, USA). Each protein band was analyzed by densitometry using Image-Pro Plus 6.0 software (National Institutes of Health, Bethesda, MD, USA) and normalized to expression of the internal control GAPDH.

Table I. siRNA sequences for zinc finger E-box binding homeobox1 gene silencing.

Site	Sense	Antisense
siRNA-313	CCAUGAUCCUAAUGUUGAAGA	UUCAACAUUAGGAUCAUGGUU
siRNA-1227	GGCAAGUGUUGGAGAAUAUC	UUAUUCUCCAACACUUGCCUU
siRNA-1963	GGACAGCACAGUAAAUCUACA	UAGAUUUGCUGUGCUGUCCUG

Si, small interfering.

Table II. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward sequence	Reverse sequence
E-cadherin	5'-CTGAGAACGAGGCTAACG-3'	5'-TTCACATCCAGCACATCC-3'
N-cadherin	5'-ATCCTACTGGACGGTTCG-3'	5'-TTGGCTAATGGCACTTGA-3'
Vimentin	5'-CCAGGCAAAGCAGGAGTC-3'	5'-CGAAGGTGACGAGCCATT-3'
Slug	5'-AGGAATCTGGCTGCTGTG-3'	5'-ATGCCTTTGGACTTTATTTGTC-3'
Snail	5'-ACAAGCACCAAGAGTCCG-3'	5'-CCCTCCCTCCACAGAAAT-3'
Twist	5'-CGGGAGTCCGCAGTCTTA-3'	5'-CACGCCCTGTTTCTTTGA-3'
ZEB1	5'-AAGTGCGCGTAGATGGTA-3'	5'-TGTTGTATGGGTGAAGCA-3'
ZEB2	5'-TATTCCTGGGCTACGACC-3'	5'-TCTCCCTGAAATCCTTGT-3'

E, epithelial; N, neural; ZEB, zinc finger E-box binding homeobox; Snail, snail family transcriptional repressor 1; slug, snail family transcriptional repressor 2; twist, twist family bHLH transcription factor 1.

**Immunofluorescence.** SW480 cells transfected with ZEB-RFP and Slug-RFP plasmids were seeded on cell culture slides in 12-well plates at  $5 \times 10^5$  cells/well and grown until 40% confluence. Following treatment with TGF- $\beta$ 1 and TGF- $\beta$ 1+aspirin for 24 h, cells were fixed with 4% paraformaldehyde for 30 min at 37°C and washed three times with PBS (5 min each time). Next, cells were incubated with 1% Triton X-100 for 10 min. After washing with PBS, 3% H<sub>2</sub>O<sub>2</sub> was added in order to neutralize endogenous peroxidase. The glass slide containing cells was blocked using 5% bovine serum albumin (cat. no. A1933, Sigma-Aldrich; Merck KGaA) for 30 min and the samples were incubated with primary antibodies against ZEB-1 (1:100) and Slug (1:100) overnight at 4°C. The next day, secondary antibody goat-anti-rabbit-596 nm (1:400) was added to the cells and incubated for 2 h at room temperature. DAPI staining (10 min) was used to label cell nuclei at 37°C. Subsequently, slides were mounted using mounting medium and signals were observed using a fluorescence microscope.

**Luciferase reporter assay.** SW480 colon cancer cells were seeded at a density of  $1 \times 10^5$  cells/well in 48-well plates. The firefly luciferase reporter gene assay kit (Shanghai Kangnian Biotechnology Co., Ltd., Shanghai, China) was performed in uninduced and TGF- $\beta$ 1-induced SW480 cells and in aspirin-treated and untreated (10 ng/ml) SW480 cells. Samples (10  $\mu$ l) were assayed for *Gaussia* luciferase activity using a microplate luminometer in the *Renilla* luciferase assay buffer. A simple *Gaussia* luciferase assay buffer (PBS containing 1.43  $\mu$ M coelenterazine) was also tested and yielded similar results. Luciferase activity was measured with a luminometer

(Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

**Statistical analysis.** The data were analyzed by one-way analysis of variance with a Tukey's post-hoc test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The data were expressed as the mean  $\pm$  standard deviation.  $P < 0.05$  was considered to indicate a statistically significant difference. Each experiment was repeated three times.

## Results

**Aspirin inhibits EMT due to its anti-inflammatory and Wnt inhibitor effects.** This hypothesis was tested by treating SW480 cells and measuring their viability and migration ability via the Transwell assay. A line of Dukes' type B, human colorectal adenocarcinoma SW480 cells was obtained from the ATCC. These cells have a mutation in codon 12 of the *ras* proto-oncogene and express *C-myc*, *K-ras*, *H-ras*, *N-ras*, *Myb*, *Sis* and *Fos* oncogenes. This line also has a G to A mutation in *p53* and forms tumors at 100% frequency following 21 days in mice.

**Aspirin inhibits the viability and migration ability of colon cancer SW480 cells.** SW480 cells were cultured in the presence of 0.5-10 mM aspirin for 2 days and the results were quantified using a CCK-8 kit. A decreased level of viable cells was observed as the concentration of aspirin was increased. To determine the effect of aspirin on SW480 cell migration, SW480 cells were grown on a membrane and treated with 0.5-10 mM aspirin for 2 days. A CCK-8 was used to quantify

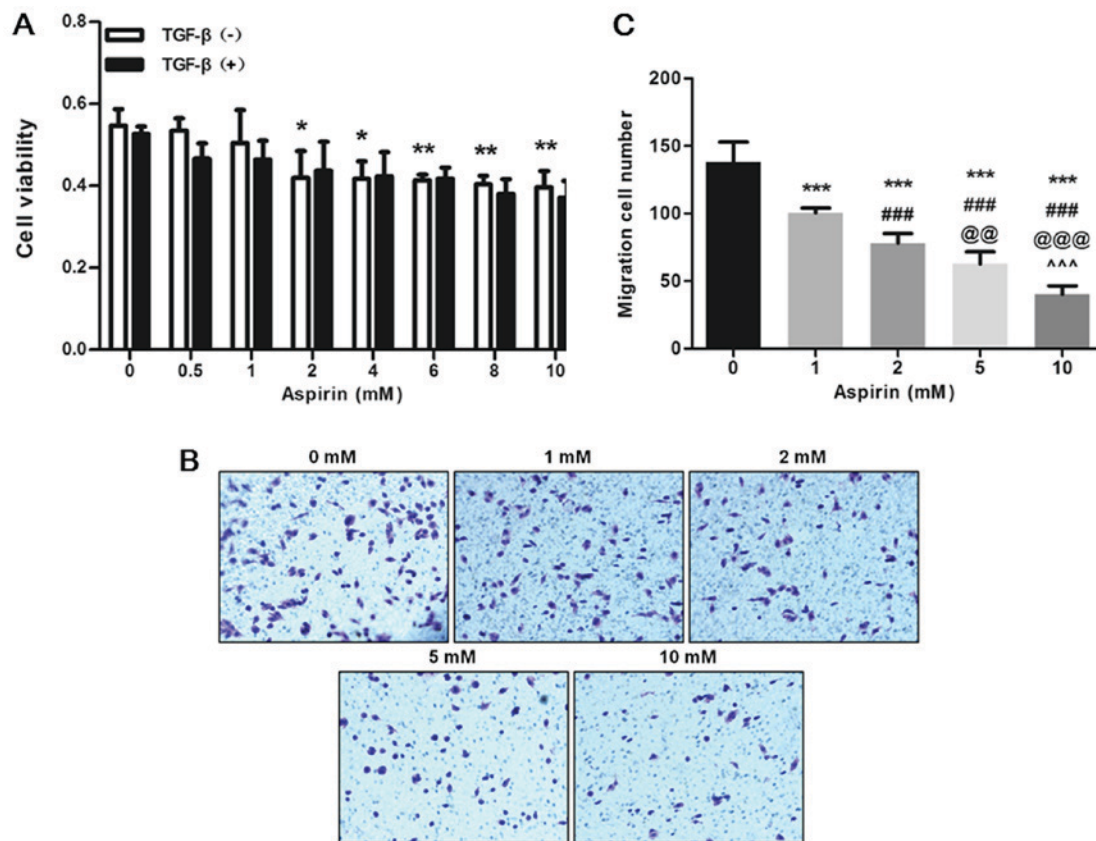


Figure 1. Aspirin reduces the proliferation and migration ability of SW480 cells. (A) The effect of aspirin on cell viability was determined. TGF- $\beta$ -induced and uninduced cells were treated with 0.5–10 mM aspirin for 2 days and cell proliferation was determined using a Cell Counting Kit-8 kit. \* $P < 0.05$  TGF- $\beta$  (-) and TGF- $\beta$  (+) group. (B) The inhibitory effect of aspirin on SW480 cell migration ability was assessed using a Transwell assay. Cells were stained with hexamethylparosaniline chloride and observed under a light microscope. Magnification,  $\times 100$ . (C) Relative quantitation of the number of migrated cells observed in panel B was performed. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. non-treated group; ### $P < 0.001$  vs. 1 mM aspirin; @@ $P < 0.01$  and @@@ $P < 0.001$  vs. 2 mM aspirin; ^^^ $P < 0.001$  vs. 5 mM aspirin. TGF, transforming growth factor.

the number of cells that had migrated from the membrane. The results indicated that aspirin reduced the migratory ability of SW480 cells (Fig. 1).

**Aspirin reduces TGF- $\beta$ -induced EMT in colon cancer SW480 cells.** In order to determine the effects of aspirin on the SW480 cancer cells that had been induced by TGF- $\beta$ 1, SW480 cells were cultured and treated for 24 h with 5 ng/ml TGF- $\beta$ 1 in order to promote their differentiation into a mesenchymal cell type. Cells were subsequently treated with 10 mM aspirin. The transdifferentiated cells were cultured, their viability was determined and another Transwell assay was performed to assess the effect of aspirin on the ability of cells to migrate. It was observed that the application of aspirin reduced the migration ability and viability of TGF- $\beta$ 1-induced cells (Fig. 2A). mRNA was extracted from treated cells and RT-qPCR was used to determine the relative expression of several EMT-associated genes: *E-cadherin*, *N-cadherin*, *Vimentin*, *Slug*, *Snail*, *Twist*, *ZEB1* and *ZEB2*. The application of aspirin increased the relative expression of epithelial cell marker *E-cadherin* but decreased the relative expression levels of mesenchymal marker *N-cadherin*. As indicated in Fig. 2B, *N-cadherin*, *Vimentin*, *Slug*, *Snail*, *Twist*, *ZEB1* and *ZEB2*, which are all inducers of Wnt signaling and positive regulators of EMT, were decreased in aspirin-treated samples. In order to determine if the changes to relative gene expression levels

observed caused a change to the protein levels, a western blot was performed on TGF- $\beta$ 1-exposed samples with or without aspirin treatment. Notably, similar effects with regards to the protein expression levels of the corresponding assayed genes were observed (Fig. 2C).

To further confirm the effects of aspirin on TGF- $\beta$ 1-induced SW480 cells, a luciferase-based reporter system was used. A construct was generated by fusing a luciferase reporter gene with the promoter of *E-cadherin*. SW480 cells were transformed with this reporter construct and treated as above. Cells treated with aspirin exhibited significantly increased luciferase activity in the TGF- $\beta$ 1-induced and uninduced cells when compared with untreated cells ( $P < 0.001$ ; Fig. 3).

**Aspirin inhibits the transnuclear behavior of Slug and ZEB1 in colon cancer SW480 cells.** It was hypothesized that the increase in *E-cadherin*: Luciferase reporter activity in aspirin-treated cells was caused by a change to the localization of a negative regulator of *E-cadherin* expression. A previous study has revealed that ZEB1 and ZEB2 can directly bind the *E-cadherin* promoter to inhibit *E-cadherin* expression (23). Therefore, the nuclear protein fraction from aspirin-treated and untreated TGF- $\beta$ 1-induced cells was isolated. A western blot was performed to determine the expression levels of ZEB1, ZEB2 and Slug in the nuclear and cytoplasmic fractions. Results indicated that the expression levels of ZEB1,



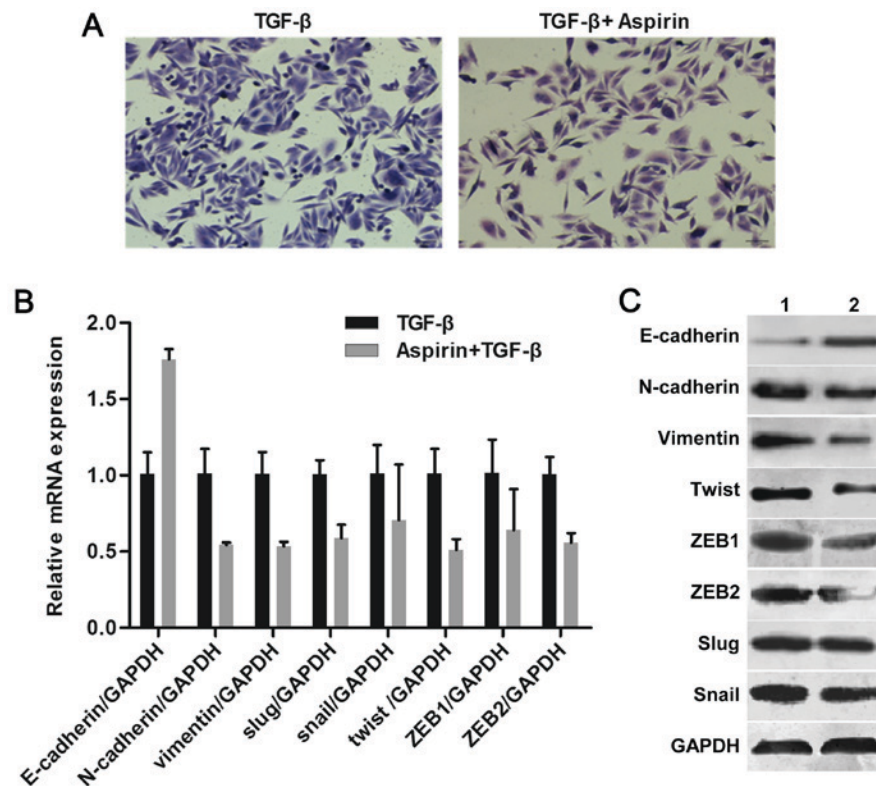


Figure 2. Aspirin reduces TGF- $\beta$ -induced epithelial-mesenchymal transition. (A) Inhibition of aspirin on EMT in SW480 was determined. Cellular migration was measured via a Transwell assay. TGF- $\beta$  (5 ng/ml for 24 h) was added to cells to induce epithelial mesenchymal transformation. Magnification, x100. (B) Transcriptional levels of EMT-associated genes were detected by reverse transcription-quantitative polymerase chain reaction. (C) Protein expression levels of EMT-associated genes were detected by western blotting. Notably, aspirin influenced the levels of E-cadherin, N-cadherin and vimentin in the process of EMT, resulting in the alterations of cell morphology. EMT, epithelial mesenchymal transition; TGF, transforming growth factor; e, epithelial; N, neural; ZEB, zinc finger E-box binding homeobox; Snail, snail family transcriptional repressor 1; slug, snail family transcriptional repressor 2; twist, twist family bHLH transcription factor 1.

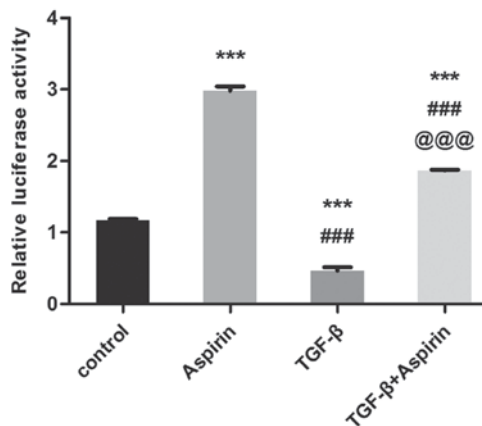


Figure 3. Confirmation of the effects of aspirin on TGF- $\beta$ 1-induced EMT using the luciferase reporter assay. Relative luciferase activity was measured in cells treated with TGF- $\beta$ 1, aspirin and TGF- $\beta$ 1+aspirin. Non-aspirin-treated and non-TGF- $\beta$ -treated cells were used as negative controls. \*\*\*P<0.001 vs. the control; ###P<0.001 vs. the aspirin only; @@@P<0.001 vs. the TGF- $\beta$  only group. TGF, transforming growth factor.

ZEB2 and Slug were increased in the cytoplasm and decreased in the nucleus of aspirin-treated cells (Fig. 4A). To confirm the localization of ZEB1 and Slug, an immunofluorometric assay was used. A construct was generated using ZEB1 and Slug fusion with RFP and the SW480 cells were transformed with the constructs. DAPI was used to stain the nuclei of the cells.

Following aspirin treatment and immunostaining, ZEB1-RFP and Slug-RFP signals were localized in the cytoplasm in aspirin-treated cells, whereas in untreated cells ZEB1-RFP and Slug-RFP signals were localized in the nucleus (Fig. 4B). These data led to the conclusion that aspirin inhibits that transport of Slug and ZEB1 to the nucleus in TGF- $\beta$ 1-induced, aspirin-treated SW480 cells.

*Aspirin inhibits EMT in ZEB1 knockdown of SW480 cells.* After observing that aspirin could impact the nuclear transport of Slug and ZEB1, it was hypothesized that a similar inhibitory effect on EMT could be achieved by knocking down ZEB1. In order to achieve this, different siRNA constructs were generated and three were selected that could successfully knock down ZEB1: siRNA-313, siRNA-1227 and siRNA-1963. siRNA-NC, a non-complementary siRNA to ZEB1, was used as a control. The relative amount of ZEB1 mRNA was quantified using RT-qPCR following isolation of the total mRNA from transformed SW480 cells (Fig. 5A). Successful knockdown of the expression level of ZEB1 mRNA was observed and a western blot was then conducted to confirm that this knockdown could successfully reduce the expression of ZEB1 protein (Fig. 5B). The western blot confirmed that the knockdown siRNA constructs were working effectively. siRNA ZEB1- and siRNA NC-transformed cells were then induced with TGF- $\beta$ 1 as indicated above and treated with aspirin (Fig. 5C). A western blot was performed to assess the expression level of

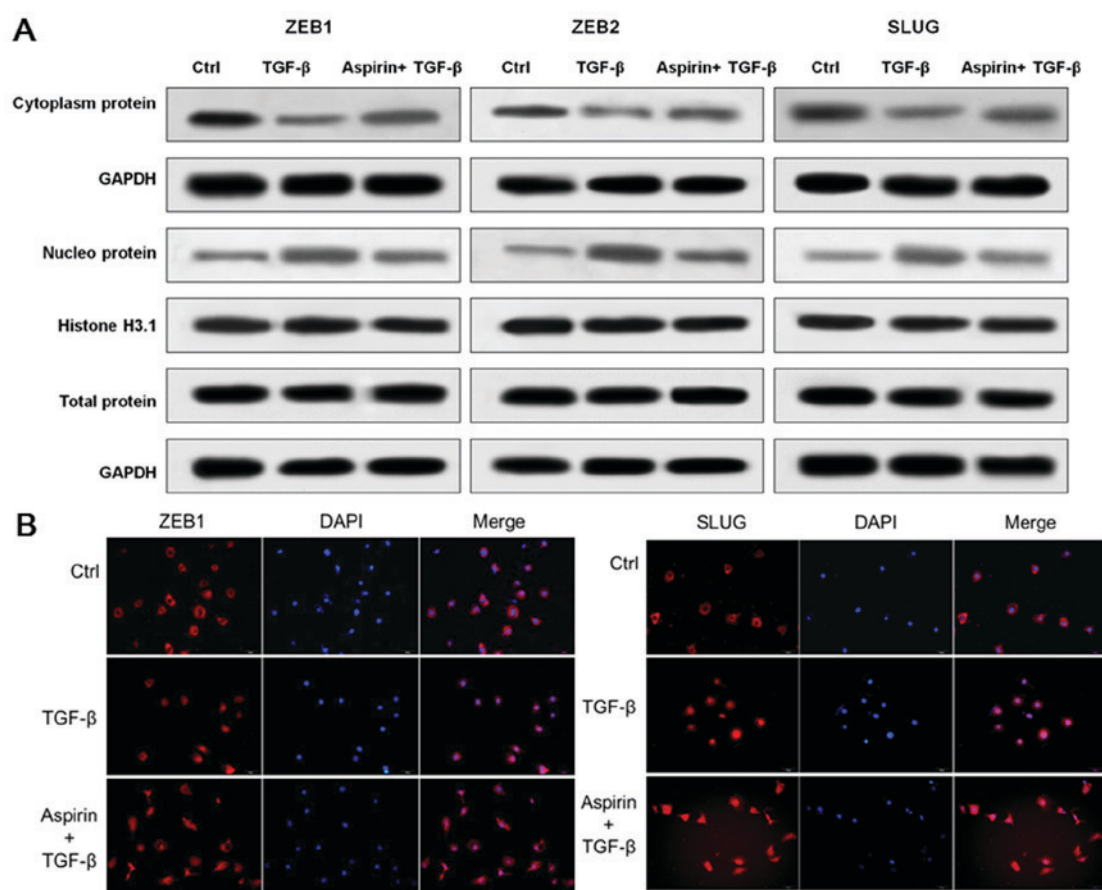


Figure 4. Aspirin inhibits the transnuclear behavior of Slug and ZEB1 in SW480 colon cancer cells. (A) Detection of Slug, ZEB1 and ZEB2 protein expression levels in the nucleus and cytoplasm by western blotting. (B) Slug and ZEB1 localization was indicated using immunofluorometric assay; magnification, 200x. ZEB, zinc finger E-box binding homeobox; slug, snail family transcriptional repressor 2; Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole.

E-cadherin present in these cells. It was observed that siRNA *ZEB1*-transformed cells exhibited an increased expression level of E-cadherin and aspirin treatment further increased the level of E-cadherin present, augmenting the effect of siRNA *ZEB1*. Furthermore, the transformed cells were cultured and a Transwell assay was performed as above following induction with TGF- $\beta$ 1 and aspirin treatment. Cells that received no treatment with aspirin and siRNA NC-transformed cells were used as controls. Notably, a significant reduction in the migratory ability of SW480 cells that had been treated with siRNA *ZEB1* was indicated compared with the NC group ( $P < 0.01$ ; Fig. 5D and E). Furthermore, this reduction was further reduced by aspirin treatment, reflecting the increase in E-cadherin that was observed earlier. As presented in Fig. 5E, relative quantitation of the number of migrated cells demonstrated the same tendency with transwell assay (Fig. 5E).

## Discussion

The findings presented above convincingly suggested that aspirin may be an effective drug for inhibiting the migration of neoplastic cells. The results demonstrated that aspirin caused SW480 cells to retain their epithelial surface markers but reduced the level of mesenchymal markers by limiting the nuclear transport of Slug and ZEB1. The results also suggested that the absence of ZEB1 may lead to transcriptional alterations, affecting Wnt signaling and further limiting EMT.

Previous studies have demonstrated that aspirin treatment causes a reduction in the level of  $\beta$ -catenin available for Wnt signaling and that this reduction is due to increased ubiquitination and increased phosphorylation sites on  $\beta$ -catenin, marking it for degradation (24,25). In addition, a previous study indicated that aspirin can inactivate the PP2A complex and is involved in multiple levels of Wnt signaling via autophosphorylation (25). Given these previous findings, it is not unexpected that aspirin caused the reduction in EMT that was observed.

A total of three possible mechanisms were proposed to be responsible for the effects of aspirin treatment observed in the present study. Firstly, it was proposed that aspirin affects the nuclear transport of Slug. Previous studies have demonstrated that aspirin can downregulate the expression of *Slug* in aggressive *K-ras*-expressing cancer cells (26,27). A previous study has also implicated aberrant nuclear transport of Snail family member transcription factors in tumor formation (28). Taken together, these data create a convincing foundation for a mechanism by which EMT is suppressed in aspirin-treated tumor cells. Secondly, aspirin may repress Wnt signaling. As mentioned above, aspirin can negatively regulate a number of proteins involved in Wnt signaling. Therefore, it is not surprising that a reduction of  $\beta$ -catenin caused by aspirin can reduce EMT. Thirdly, aspirin may alter ZEB1 acetylation, possibly affecting Slug acetylation. In H358 non-small-cell lung cancer cells, reduced acetylation was identified in histones

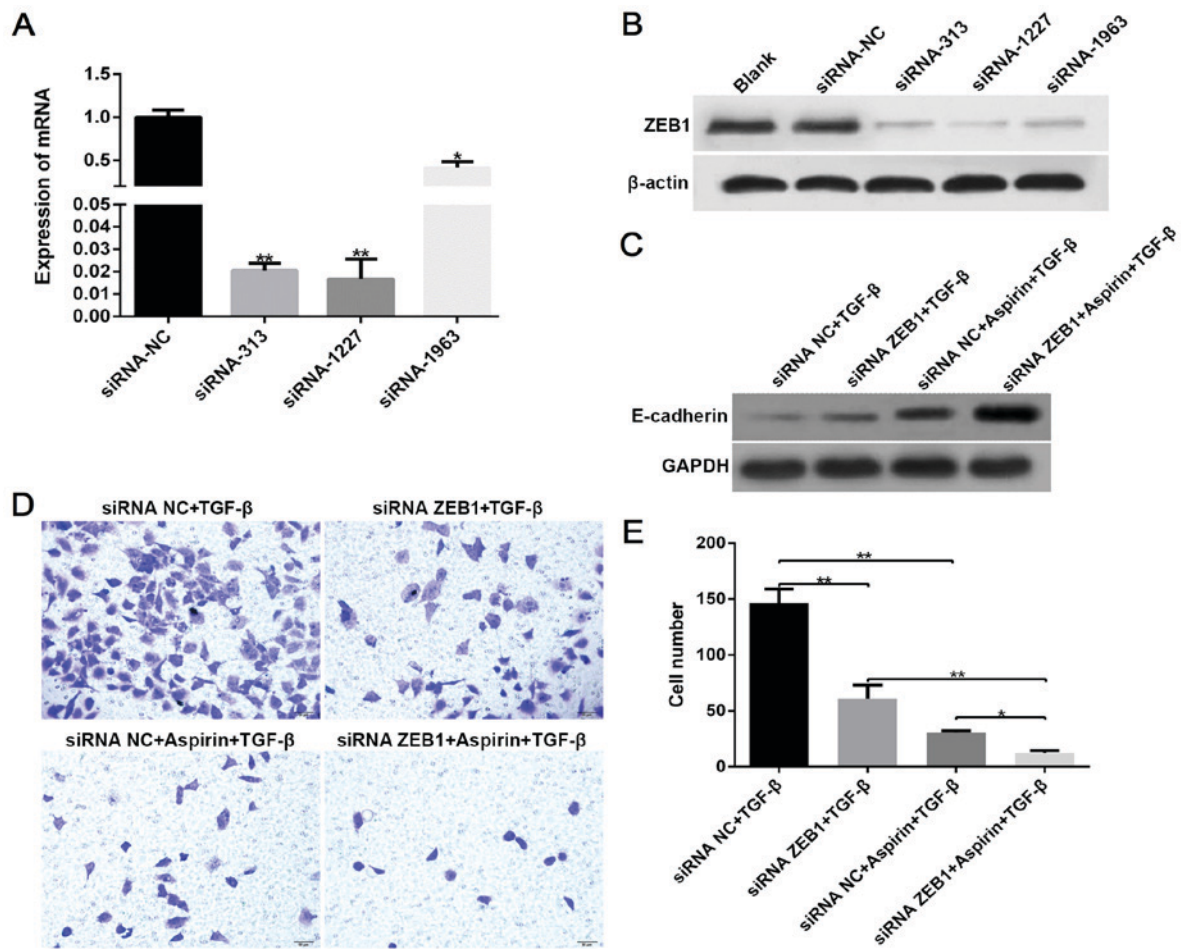


Figure 5. Aspirin inhibits epithelial mesenchymal transition in ZEB1 knockdown of SW480 cells. Identification of *ZEB1* gene silencing efficiency was indicated by (A) reverse transcription-quantitative polymerase and (B) western blotting. siRNA-31, siRNA-1227 and siRNA-1963 were selected to interfere with *ZEB1* expression. (C) Effects of *ZEB1* gene silencing on E-cadherin expression level after aspirin treatment in TGF-β 1-pretreated cells. (D) Cells were stained with hexamethylparosaniline chloride and observed under a light microscope; magnification, 50x. (E) Relative quantitation of the number of positively-stained cells in panel D is indicated. \*P<0.05 and \*\*P<0.01 vs. the siRNA-NC. Si, small interfering; NC, negative control; TGF, transforming growth factor; ZEB, zinc finger E-box binding homeobox; E, epithelial.

surrounding ZEB1 binding sites (29). Epigenetic alterations like these can increase the availability of ZEB1 binding sites in the promoters of endothelial marker genes. Previous research has also demonstrated that the deacetylase SIRT2 can rapidly destabilize Slug in basal-like breast cancer cells (30). These findings suggest aspirin may serve a role in affecting the two acetylation changes, inhibiting EMT.

The novel research in the present study highlights the positive effects aspirin has on inhibiting EMT. Taken together, these data suggest that aspirin can inhibit tumor metastasis by inhibiting tumor cells from acquiring the ability to invade surrounding tissue and circulate to other tissues. The data from the present study reflect results of previous findings, which have demonstrated that aspirin can increase the survival rate and reduce the occurrence of colorectal cancer in individuals that take aspirin on a regular basis (31). Although the present research provides insights into the effects of aspirin treatment on tumor cells, more remains to be discovered regarding the mechanism of action of aspirin. Recent studies suggest that aspirin has beneficial effects on preventing colorectal cancer; however, aspirin seems to have no effect on lowering the number of circulating tumor cells identified in metastatic

breast cancer patients (32-34). In addition, much remains to be developed in the medical field before screening for EMT markers becomes commonplace. The advent of innovative EMT screening strategies in the clinical setting makes for an exciting outlook for future research.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.



## Authors' contributions

SJ performed all the experiments, drafting and revised the manuscript. XW participated in the development of the research concept and experimental design, and proposed revision suggestions. All the authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no conflicts of interest.

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