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RBM38 is involved in TGF- β -induced epithelial-to-mesenchymal transition by stabilising zonula occludens-1 mRNA in breast cancer

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Background: The transforming growth factor- β (TGF- β) pathway plays a vital role in driving cancer cell epithelial–mesenchymal transition (EMT). Zonula occludens-1 (ZO-1), which is downregulated in response to TGF- β , is able to control endothelial cell–cell tension, cell migration, and barrier formation. However, the molecular mechanism of how TGF- β regulates ZO-1 expression remains unclear.

Methods: Breast cancer cells were treated with TGF- β to induce an EMT progress. Chromatin immunoprecipitation and dual-luciferase reporter assay were performed to investigate direct relationship between Snail and RNA binding motif protein 38 (RBM38). The RNA immunoprecipitation combined with RNA electrophoretic mobility shift assay and dual-luciferase reporter assay were conducted to testify direct relationship between RBM38 and ZO-1. The ZO-1 siRNA was transfected to breast cancer cells that overexpress RBM38 and the control, followed by transwell and Matrigel invasion assays to examine cell migratory and invasive ability.

Results: Transforming growth factor- β induced a remarkable downregulation of RBM38 in breast cancer that was directly regulated by transcription repressor Snail targeting the E-box elements in promoter region of RBM38 gene. Additionally, RBM38 positively regulated ZO-1 transcript via directly binding to AU/U-rich elements in its mRNA 3'-UTR. Moreover, by magnifying RBM38 expression, cell migration and invasion mediated by knockdown of ZO-1 in breast cancer were reversed.

Conclusions: All the results clarified a linear regulation relationship among Snail, RBM38, and ZO-1, implicating RBM38 as a pivotal mediator in TGF- β -induced EMT in breast cancer.

Breast cancer is the most common malignancy in females worldwide (Chen *et al*, 2016; Siegel *et al*, 2016), and the metastasis accounts for the majority of deaths from breast cancer. One potential mechanism for invasion and metastasis in breast cancer is

due to the occurring of epithelial–mesenchymal transition (EMT) that allows the polarised epithelial cells to assume elevated migratory capacity, invasiveness, enhanced resistance to apoptosis, and stem cell properties (Kalluri and Weinberg, 2009; Thiery *et al*,

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2009). Though recent studies suggest that EMT may be dispensable for the carcinoma metastatic cascade (Fischer *et al*, 2015; Zheng *et al*, 2015), it is still not ruled out as a driver of cancer progression for its pivotal role in helping epithelial cancer cells to invade (Li and Kang, 2016; Nieto *et al*, 2016).

The first steps of EMT are the disassembly of epithelial cell–cell contacts. Epithelial cells associate with each other through laterally located, specialised cell–cell contact structures including tight junctions (TJ), adherens junctions (AJ), desmosomes, and gap junctions (Xu *et al*, 2009; Lamouille *et al*, 2014). Zonula occludens-1 (ZO-1, also known as tight junction protein 1) is a critical regulator of TJ assembly that regulates AJ function by coordinating the assembly or dynamics of the cortical cytoskeleton (Fanning and Anderson, 2009).

The EMT is triggered by multiple extracellular signals, including components of the extracellular matrix like collagen and hyaluronic acid, soluble growth factors such as transforming growth factor- β (TGF- β), fibroblast growth factor (FGF), and epidermal growth factor (EGF) (Thiery and Sleeman, 2006). Transforming growth factor- β is one of the best known inducer of EMT. Elevated levels of TGF- β in malignant mammary cells enhance breast cancer invasion, migration, and evasion of immunity. Each of these functions plays a vital role in tumour progression and eventually takes part in the metastatic process (Padua and Massague, 2009). In response to TGF- β , the type II TGF- β receptor phosphorylate the type-I TGF- β receptor, which then initiates downstream signalling through either Smad-mediated canonical signalling or Smad-independent noncanonical signalling (Shi and Massague, 2003). Via interaction with Smad complex, multiple transcription factors are initiated so as to induce EMT. It is widely testified that TGF- β induced a phenotypic change with decreased expression of epithelial markers E-cadherin and ZO-1 (Miettinen *et al*, 1994; Tian and Phillips, 2002) that belong to AJ and TJ, respectively. Unlike the well-illustrated mechanism of E-cadherin regulated by TGF- β (Takano *et al*, 2007; Fransvea *et al*, 2008; Yu *et al*, 2015), it still needs much effort to uncover how TGF- β affects the expression of ZO-1.

RNA binding proteins (RBPs) regulate mRNA splicing, export, stability, or translation mainly by the interaction with a particular sequence of target gene mRNA (Yan *et al*, 2012), and multiple mRNAs are co-mediated by one or more sequence-specific RBPs (Keene, 2007). Previous studies showed that ZO-1 mRNA 3'-untranslated regions (3'-UTRs) readily associated with cytoplasmic RBPs including auxin upregulated F-box protein 1 (AUF1) (Chen *et al*, 2008), human antigen R (HuR) (Yu *et al*, 2011), and TIA-1-related protein (TIAR) (Xiao and Wang, 2014; Yang *et al*, 2014). Previously, our group found that the RNA binding motif protein 38 (RBM38, also called RNPC1), a tumour suppressor in breast cancer, participated in the EMT process (Xue *et al*, 2014). Meanwhile, it regulated expression of various target genes through binding to AU/U-rich elements (AREs) in their mRNA 3'-UTR (Cho *et al*, 2012; Xu *et al*, 2013; Shi *et al*, 2015).

In breast cancer, reduction of ZO-1 level is correlated with decreased tumour differentiation and poor prognosis (Itoh and Bissell, 2003; Martin *et al*, 2004). The ZO-1 possesses several AREs in its mRNA 3'-UTR, presenting as a potential target of RBM38. Furthermore, the promoter region of RBM38 gene contains several E-box DNA sequences (5'-CANNTG-3') that can be recognised by Snail (Batlle *et al*, 2000; Park *et al*, 2012), a prominent mediator of TGF- β -induced EMT that displays a broad spectrum of biological functions in tumour progression, including cancer metastasis, drug resistance, and tumour recurrence (Wang *et al*, 2013). Snail, together with Slug and less explored Smuc, pertains to the Snail family. Although the three members all encode transcription factors of the zinc-finger type and bind to the E-box elements, Snail displays a higher affinity with the target sequences (Nieto, 2002; Bolos *et al*, 2003). Here we put forward the hypothesis that TGF- β

promoted Snail expression that then acts as a transcriptional repressor on RBM38 expression. Additionally, decreased RBM38 level mediated ZO-1 post-transcriptional regulation, thus forming a new pathway that delivered the signals from TGF- β to ZO-1 and eventually modulated EMT progression.

MATERIALS AND METHODS

Cell culture. The human breast cancer cell lines, MCF7, BT474, and MDA-MB-231, were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). For TGF- β treatment, MCF7 and BT474 cells were exposed to 10 ng ml⁻¹ of TGF- β 1 (PeproTech, Rocky Hill, NJ, USA) over different time points (1–5 days). Cells were then harvested for protein or gene expression analysis. For experiments using inhibitor, MCF7 and BT474 cells were pretreated with 10 μ M of SB431542 (Selleckchem, Houston, TX, USA) (Taipaleenmaki *et al*, 2012), a specific inhibitor of TGF- β type I receptor, for 30 min and then stimulated with TGF- β 1 (10 ng ml⁻¹) for 72 h.

Lentivirus transfection. Lentivirus constructs of RBM38 over-expression and knockdown were obtained from GenePharma (Shanghai, China) and generated as in our previous study (Shi *et al*, 2015). Briefly, cells were plated in 6-well dishes at 30–40% confluence and infected with the retroviruses. Stable pooled populations of breast cancer cells were generated by selection using puromycin (3 μ g ml⁻¹) for 14 days.

Small interfering RNA (siRNA) and plasmid transfection. The expression vectors encoding wild-type ZO-1 were constructed as described previously (Bryse *et al*, 2012). Plasmids and siRNAs (GenePharma) and were transfected into breast cancer cells using Lipofectamine 3000 (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were cultured in a 6-well plate for 24 to 48 h and the expression level was detected by western blot and qRT-PCR to determine the interference efficiency.

For TGF- β treatment, the medium was supplemented with TGF- β 1 (10 ng ml⁻¹) for another 72 h before siRNA transfection.

Western blot analysis. Cells were lysed and analysed by western blotting as described previously (Shi *et al*, 2015). The primary antibodies were used as follows: anti-rabbit RBM38 (Santa Cruz Biotechnology, Dallas, TX, USA), ZO-1, Snail (Cell Signaling Technology, Danvers, MA, USA), anti-mouse β -actin (Cell Signaling Technology). The anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling Technology. The antibodies were diluted according to the manufacturer's instructions.

Chromatin immunoprecipitation assay (ChIP). The ChIP assay was performed by chromatin immunoprecipitation kit (17-371, EZ-ChIP, Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, MDA-MB-231 and MCF7 cells (with or without TGF- β stimulation) were plated in 15 mm culture dishes, the proteins were crosslinked with DNA by 37% formaldehyde, and then stopped by adding 10 \times glycine solution. Sonication of cell lysate was performed with the condition of 200 cycles/burst, 5.0 duty factor at 70.0 peak power (Covaris S220, Woburn, MA, USA) to shear the chromatin to an average size of 500 bp. The supernatant fractions were diluted and incubated with protein G agarose for 1 h at 4 $^{\circ}$ C. As Input, 10 μ l (1%) of the supernatants was preserved. Then, protein G agarose was pelleted and supernatants were reincubated with 5 μ g primary antibody: anti-Snail (Santa Cruz Biotechnology) or normal mouse IgG, at 4 $^{\circ}$ C overnight with rotation. After this, the supernatants were reincubated with protein G agarose for 1 h at 4 $^{\circ}$ C. After washing the beads, the protein/DNA complexes were eluted twice. Crosslink was reversed by adding 5 M NaCl and incubation at 65 $^{\circ}$ C

overnight. The immunoprecipitated DNAs were purified by treatment with RNase A and proteinase K as well as the Input. Purified DNA was evaluated and analysed by PCR with specific primers listed in the Supplementary Table 1.

Luciferase reporter assay. Luciferase reporter assay was performed with the Dual-Luciferase Reporter Assay System (E1910, Promega, Madison, WI, USA). Briefly, 5 ng of *Renilla* luciferase vector (pRL-TK; Promega), an internal control, and 200 ng of a pGL3 reporter that contained various target regions were co-transfected into breast cancer cells. At 48 h after transfection, cells were harvested to measure the luciferase activity.

RNA isolation, reverse transcription, and qRT-PCR. Total RNA was isolated using Trizol reagent (TaKaRa, Kusatsu, Japan), and cDNA synthesis was performed using Primescript RT Reagent (TaKaRa) according to the manufacturer's instructions. The qRT-PCR was performed using FastStart Universal SYBR Green Master (Roche, Reinach, Switzerland) and then conducted using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), and the abundance of target mRNAs was normalised based on β -actin. The primers used here are listed in Supplementary Table 2 of Supplementary Material.

Immunohistochemical (IHC) staining and analysis. The breast cancer sample tissue microarrays (BC08118) for IHC analysis were purchased from Biomax (Rockville, MD, USA). The same tissue samples were stained with RBM38 and ZO-1 antibody respectively. The RBM38 antibody (LifeSpan Biosciences, Seattle, WA, USA) was used at the dilution of 1:300. The ZO-1 antibody (Abcam, Cambridge, MA, USA) was used with a concentration of $10 \mu\text{g ml}^{-1}$. The IHC staining was conducted and analysed as previously described (Shi *et al*, 2015).

Immunofluorescence (IF). Immunofluorescence assay was performed as described previously (Lou *et al*, 2017). Primary antibodies were used as follows: RBM38 (Santa Cruz Biotechnology) and ZO-1 (Abcam). The FITC-conjugated secondary anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) and Alexa 594-conjugated secondary anti-goat antibody (Jackson ImmunoResearch) were applied. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

RNA immunoprecipitation (RIP). RNA immunoprecipitation was carried out as previously described (Shi *et al*, 2015). The cytosolic extract from MCF7 cells was prepared with RNA immunoprecipitation lysis buffer (Millipore), and then incubated with $5 \mu\text{g}$ of anti-RBM38 (Santa Cruz Biotechnology) or non-immunised rabbit IgG at 4°C overnight. The mRNA-protein immunocomplexes were brought down by protein A/G magnetic beads, followed by RT-PCR and qRT-PCR analysis.

Recombinant protein purification and RNA probes. The recombinant RBM38 proteins were expressed and purified as described previously (Lou *et al*, 2017). To generate RNA electrophoretic mobility shift assay (REMSA) probes, various regions (A–D) in ZO-1 mRNA 3'-UTR were amplified by PCR with T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3'). The primers for probes A–D are listed in Supplementary Material Table 3. Biolabelled RNA probes were made from *in vitro* transcription with a MEGA shortscript Kit (Ambion, Waltham, MA, USA) in the presence of biotin-16-UTP (Roche) according to the manufacturer's instructions.

RNA electrophoretic mobility shift assay. The REMSA was performed with a LightShift Chemiluminescent RNA EMSA Kit (Thermo, Waltham, MA, USA) following the manufacturer's instruction. Briefly, 4 mg ml^{-1} RBM38 and 2 nM biotin-labelled RNA probe were mixed in a REMSA binding buffer (10 mM HEPES (pH 7.3), 20 mM KCl, 1 mM MgCl_2 , 1 mM dithiothreitol)

and incubated for 30 min at room temperature. To prevent nonspecific binding, 10 mg ml^{-1} of tRNA was added in the reaction system. The RNA/protein complexes were then electrophoretically by 4% native polyacrylamide gel and transferred to nylon membrane (Thermo). The RNA was crosslinked with a UV lamp at a distance of 0.5 cm from the membrane for 3 min. The membrane was blocked in blocking buffer for 15 min and then replaced for the blocking buffer with conjugate/blocking buffer (stabilised streptavidin-horseradish peroxidase conjugate 1:300 dilution). After washing with $1 \times$ wash buffer for 3 times, the membrane was incubated in substrate equilibration buffer for 5 min. Then, the membrane was incubated in substrate working solution for 5 min and exposed.

Cell migration and invasion assay. Cell invasiveness was conducted using 24-well transwell inserts (Millicell Hanging Cell Culture Insert, PET $8 \mu\text{m}$, Billerica, MA, USA) coated with $50 \mu\text{l}$ of Matrigel (1 mg ml^{-1} ; BD Biosciences, Franklin Lakes, NJ, USA). Inserts were seeded with 5×10^4 cells in $200 \mu\text{l}$ DMEM supplemented with 0.1% FBS, and $500 \mu\text{l}$ of medium with 10% FBS was added to the lower chamber. Cell migration assay was carried out without the Matrigel. Cells were incubated at 37°C for 36 h, after which noninvading cells were wiped from the upper side of the membrane. The number of invading or migrating cells was counted under the microscope in five independent fields and the average number of cells per field was represented in the graphs.

Statistical analysis. All experiments were repeated in triplicate, unless otherwise specified. The data were analysed using the SPSS 20.0 software (Chicago, IL, USA). The χ^2 test was used to assess the correlation between RBM38 and the clinicopathological parameters. For all the continuous variables, Student's *t*-test was used to analyse the statistical significance of the differences between groups, and $P < 0.05$ was considered to indicate a statistical significance.

RESULTS

Repression of RBM38 expression during TGF- β -induced EMT in breast cancer. To explore the alteration of RBM38 expression during TGF- β -induced EMT, MCF7 and BT474 cells were cultured in the absence or presence of TGF- β over 5 days and monitored for a phenotypic switch (Figure 1A). The EMT induction was confirmed by the activation of Smad3, Snail, Slug, as well as the reduction of ZO-1 in protein level (Figure 1B). Notably, RBM38 expression showed a significant decrease in response to TGF- β (Figure 1B). The reduction of RBM38 level was also observed in BT474 cells when treated with TGF- β to induce EMT (Figure 1C). The expression of an activated version of the TGF- β type I receptor could be inhibited via the potent and selective chemical inhibitor (i.e., SB431542) that blocks EMT and promotes an enhanced epithelial phenotype in cell culture (Xu *et al*, 2009). We blocked TGF- β signalling by SB431542 that evidently inhibited down-regulation of RBM38 protein levels (Figure 1D and E) by TGF- β . These data demonstrated that RBM38 expression was markedly reduced in breast cancer cells undergoing EMT and functioned as a mediator in TGF- β signalling pathway.

Snail caused reduction of RBM38 levels in breast cancer. We transfected MDA-MB-231, a cell line that relatively expressed Snail highly (Dong *et al*, 2012), with small interfering RNAs (siRNAs) targeting Snail. It was found that RBM38 expression was evidently increased by knockdown of Snail at protein and mRNA levels ($P < 0.05$; Figure 2A). The reduction of RBM38 in MCF7 cells that were treated with TGF- β was also restrained with Snail knockdown ($P < 0.05$; Figure 2B), whereas the phenomenon was not observed in MCF7 cells with Slug knockdown (Supplementary Figure S1A and 1B).

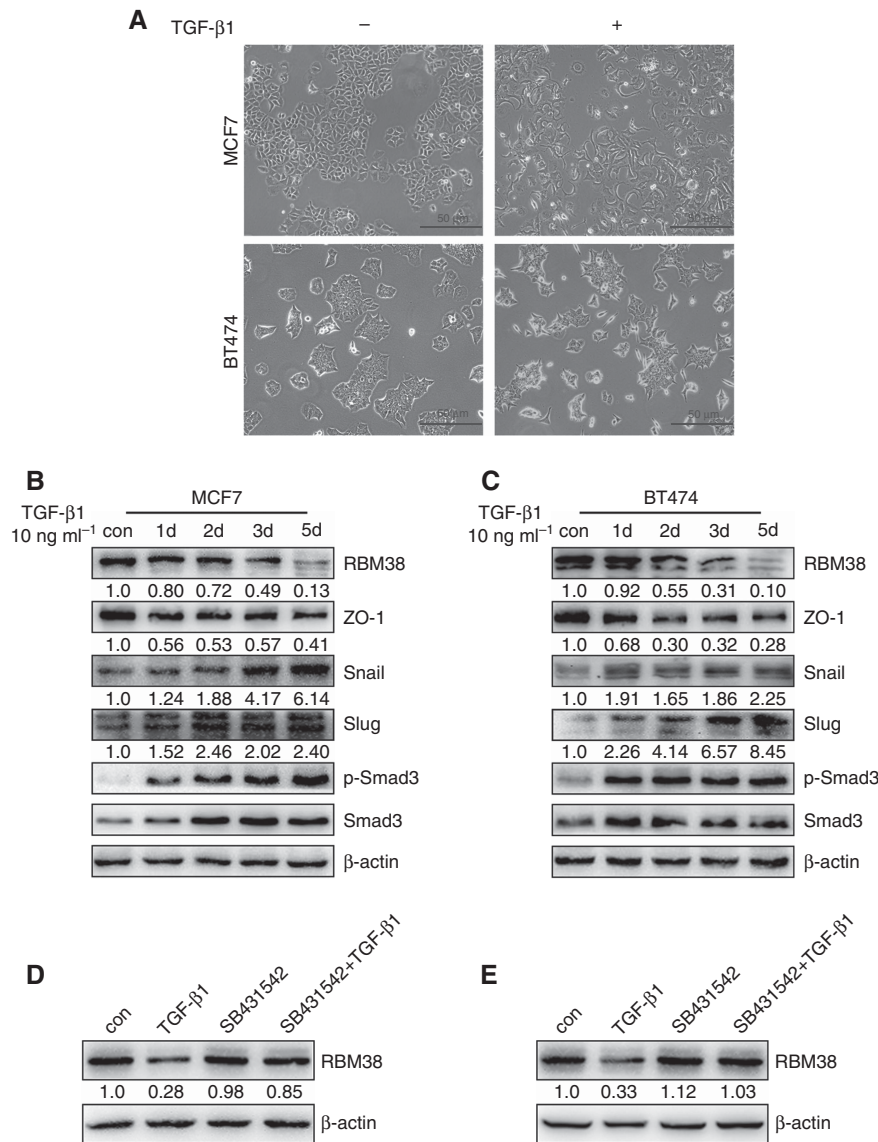


Figure 1. The RBM38 expression decreased during TGF- β -induced EMT. **(A)** Morphological contrast photos of MCF7 and BT474 cells treated without or with TGF- β 1 (10 ng ml⁻¹) for 5 days. Cells showed a more mesenchymal morphology than that in the absence of TGF- β . Scale bars indicated 50 μ m. **(B)** The MCF7 cells and **(C)** BT474 cells were exposed to TGF- β 1 (10 ng ml⁻¹) for 0, 1, 2, 3, and 5 days. The EMT progress was confirmed by monitoring ZO-1, Snail, Slug, and p-Smad3 expression. Meanwhile, expression of RBM38 was remarkably reduced. **(D)** The MCF7 and **(E)** BT474 cells were cultured without (con) or with TGF- β 1 (10 ng ml⁻¹) followed by the addition of selective inhibitor SB431542 (10 μ M). After 72 h of treatment, the reduction of RBM38 expression induced by TGF- β was restored by the addition of SB431542.

Snail repressed RBM38 expression via directly targeting E-box elements in the promoter of RBM38 gene. Then, we noticed that the RBM38 promoter region contained six Snail-binding E-boxes (5'-CANNTG-3'). A ChIP assay was performed by using three sets of primers each containing two E-box elements (Figure 2C). Snail showed a stronger ability to associate with E-box-1 and E-box-2 in the promoter region of RBM38 gene in MCF7 cells that had undergone TGF- β -mediated EMT (Figure 2D). Additionally, endogenous Snail bound to the same region of RBM38 promoter in MDA-MB-231 cells (Figure 2E). We next cloned two RBM38 promoter-luciferase constructs, I (-1880 to -1672) and II (-1221 to -1028), based on the location of E-box-1 and E-box-2 (Figure 2F). With Snail knockdown in TGF- β -treated MCF7 cells, RBM38 promoter activity significantly increased ($P < 0.05$; Figure 2G). Meanwhile, knockdown of Snail in MDA-MB-231 cells raised RBM38 promoter luciferase as well ($P < 0.05$; Figure 2H). These evidences indicated that Snail directly repressed RBM38 expression by binding to E-box elements in its promoter region.

RBM38 overexpression counteracted cell migration and invasion induced by TGF- β in breast cancer cells. To further investigate whether RBM38 took part in TGF- β -mediated cell migration and invasion, RBM38-overexpressed MCF7 cells and the control were exposed or not to TGF- β . We found that cell migration and invasion process induced by TGF- β were distinctly attenuated via the overexpression of RBM38 (Figure 3A and B). Quantified data were the fold change of cell number with the exposure of TGF- β divided by that with the control, indicating that TGF- β -mediated migration was dropped from 1.66 to 1.29 with RBM38 overexpression and the invasion was decreased from 2.10 to 1.61 with RBM38 overexpression.

RBM38 regulated the expression of ZO-1 in breast cancer. In TGF- β -treated MCF7 cells, overexpression of RBM38 blocked the reduction of ZO-1 induced by TGF- β (Figure 3C). Similar result was obtained in BT474 cells (Figure 3D). Next, MCF7, BT474, and MDA-MB-231 cells were transfected with lentivirus to stably

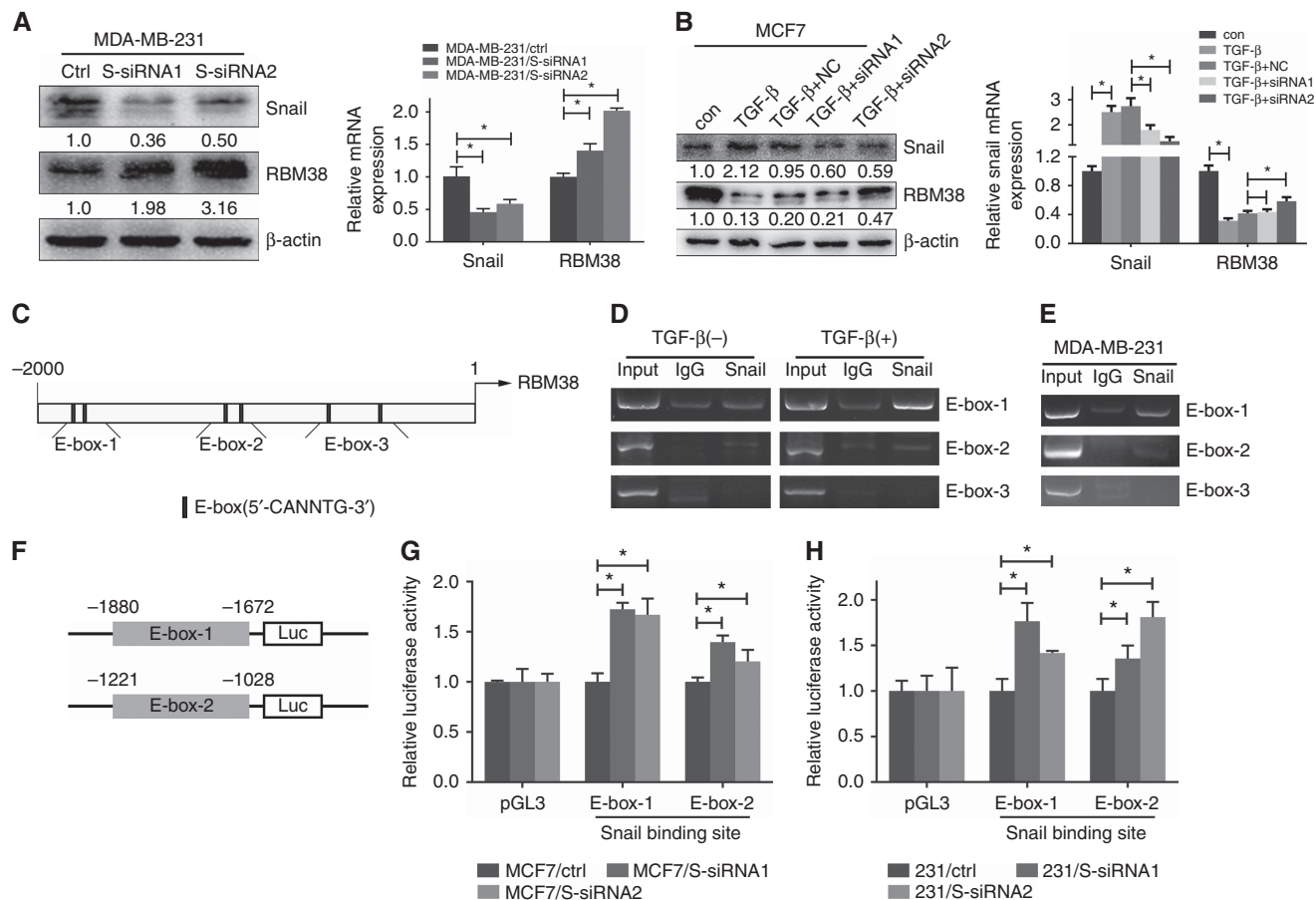


Figure 2. Repression of Snail restored RBM38 levels via binding to E-box in promoter region of RBM38 gene. **(A)** Expression of RBM38 was significantly increased after transfecting with siRNAs (S-siRNA1, S-siRNA2) to knock down the expression of Snail in MDA-MB-231 cells. **(B)** Interference of Snail reversed TGF- β -stimulated downregulation of RBM38 expression in MCF7 cells. Western blot and qRT-PCR were applied to detect the expression of Snail and RBM38. The relative quantification was calculated by the $\Delta\Delta C_t$ method and normalised based on β -actin. $*P < 0.05$. **(C)** Schematic diagram exhibiting six potential Snail-binding E-box elements in promoter region of RBM38 gene. **(D)** The ChIP analysis of regions E-box-1, -2, and -3 was performed after treatment with or without TGF- β 1 (10 ng ml $^{-1}$) in MCF7 cells. With RBM38 promoter fragments, E-box-1 and E-box-2, Snail was detected brighter in TGF- β -stimulated cells than that without TGF- β . No difference was observed in E-box-3. **(E)** ChIP analysis was performed in MDA-MB-231 cells. A relatively brighter band was detected in Snail compared with the control IgG with E-box-1 and -2. No band was observed with E-box-3. **(F)** Schematic representation of the luciferase plasmid with E-box-1 and E-box-2 in promoter region of RBM38 gene. **(G)** The luciferase activity for the reporter carrying RBM38 E-box-1 and -2 was increased in TGF- β -treated MCF7 cells by knockdown of Snail. **(H)** The luciferase activity for the reporter carrying RBM38 E-box-1 and -2 was increased by interfering with the level of Snail in MDA-MB-231 cells (231). Data represented mean \pm s.d. of three independent experiments. $*P < 0.05$.

overexpress or knock down RBM38. The ZO-1 expression was increased in MCF7 cells alongside upregulation of RBM38 at both protein and mRNA levels ($P < 0.05$; Figure 3A). Additionally, there was a significant decrease in ZO-1 protein and transcript expression in RBM38-knockdown MCF7 cells ($P < 0.05$; Figure 3C). It indicated that RBM38 positively affected ZO-1 expression in breast cancer cells. Similar results were observed in BT474 (Figure 3B and D) and MDA-MB-231 cells (Supplementary Figure S2A–D). These data suggested that RBM38 positively regulated ZO-1 expression in breast cancer cells.

IHC staining of RBM38 and ZO-1 in human breast cancer tissues. To identify correlation of location and expression between RBM38 and ZO-1 in breast cancer tissues, IHC analysis was performed in 90 breast cancer tissues. Both RBM38 and ZO-1 were mainly expressed in the cytoplasm (Supplementary Figure S3A). The ZO-1 staining was obviously stronger in RBM38 high-expressed breast cancers compared with RBM38 low-expressed breast cancers ($P < 0.05$; Supplementary Figure S3B). The correlation between RBM38 expression and clinicopathological features was analysed as shown in Supplementary Table 4. The RBM38 and

ZO-1 cellular localisation in breast cancer cells was confirmed using immunofluorescence (Supplementary Figure S4).

RBM38 enhanced ZO-1 mRNA stability in breast cancer. To further explore whether RBM38 promotes ZO-1 expression by regulating its mRNA stability, RBM38-overexpressed MCF7 and the control were treated with 5 μ g ml $^{-1}$ actinomycin-D (ActD), a transcription inhibitor, for various times. We found that overexpression of RBM38 prolonged the half-life of ZO-1 transcript from 2.1 to 4 h (Figure 4A). Knockdown of RBM38 shortened the half-life of ZO-1 transcript from 3.3 to 2 h (Figure 4C). Similar results were also found in BT474 cells (Figure 4B and D). These data indicated that RBM38 could increase ZO-1 mRNA stability.

RBM38 directly bound to the AREs in the ZO-1 mRNA 3'-UTR. To determine whether RBM38 physically binds to ZO-1 transcript, RNA immunoprecipitation assay was employed. The RBM38 antibody and control IgG were applied to immunoprecipitate RBM38-RNA complex in extracts from MCF7 cells. As Input, 10% of cell extracts were utilised. The ZO-1 was detected in RBM38 rather than in the control IgG by RT-PCR (Figure 4E) and qRT-PCR (Figure 4F). Both HuR and p21 were detected as positive

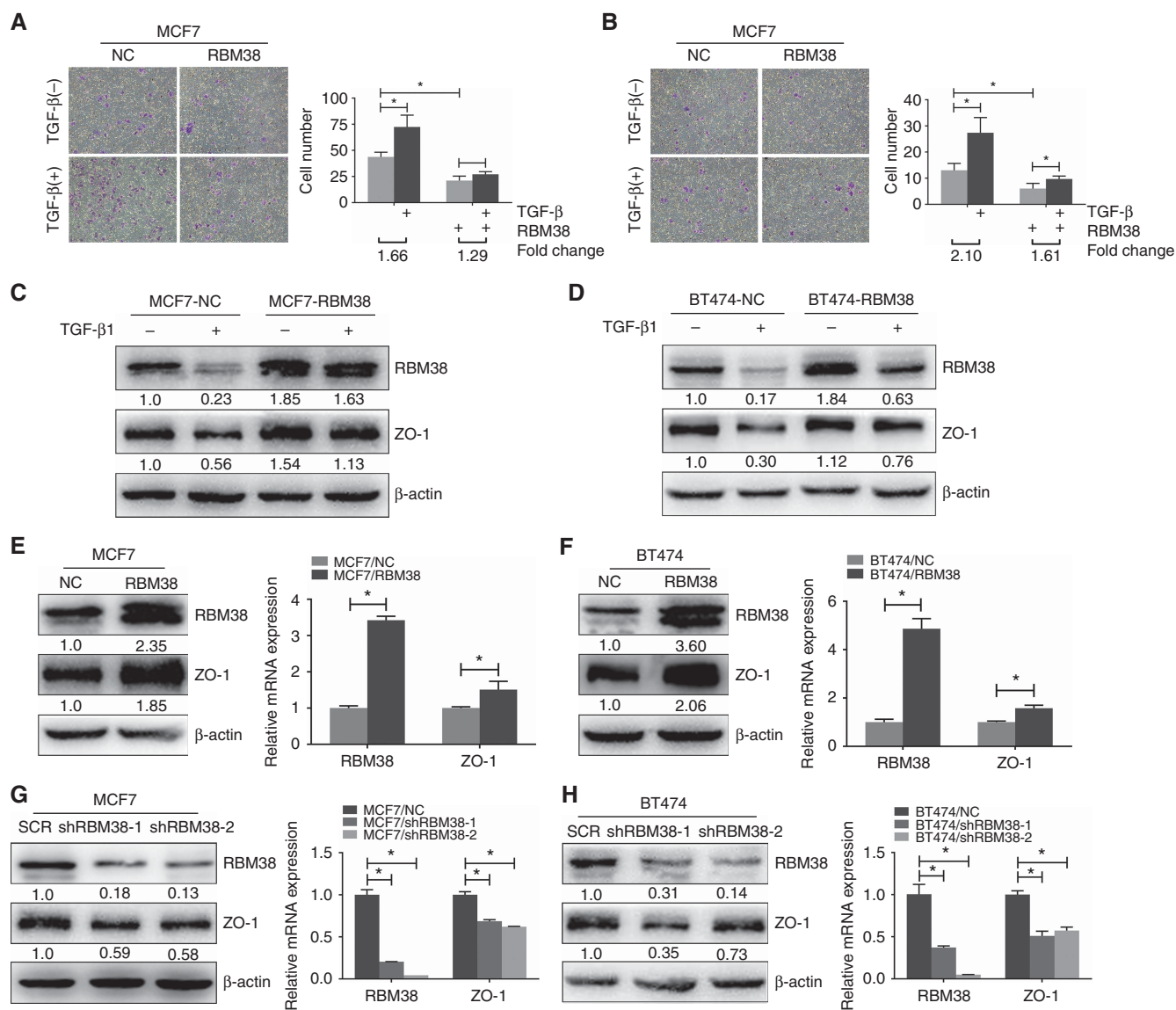


Figure 3. The RBM38 reinstated reduction of ZO-1 expression induced by TGF- β and regulated ZO-1 gene and protein expression in breast cancer. The RBM38 counteracted the promotion of cell migration and invasion induced by TGF- β . **(A and B)** MCF7 cells that overexpress RBM38 and the control were exposed to TGF- β or not. **(A)** Cell migration and **(B)** invasion assay were examined and representative images of migrated or invaded cells are shown. Right panel represents the migrating or invading cell numbers corresponding to the left panel. The assay was done in triplicate and presented as mean \pm s.d. * P <0.05. **(C)** MCF7 and **(D)** BT474 cells overexpressing RBM38 and the control were treated with or without TGF- β 1 (10 ng ml⁻¹). Reduction of ZO-1 expression induced by TGF- β was blocked by upregulating RBM38. **(E)** The MCF7 and **(F)** BT474 cells were transfected with lentivirus to overexpress RBM38 (RBM38) and the control (NC). The ZO-1 expression was obviously increased after upregulating RBM38 at both protein and mRNA levels. **(G)** The MCF7 and **(H)** BT474 cells were transfected with the control (SCR) and RBM38 knockdown lentivirus (shRBM38-1, shRBM38-2). The ZO-1 level was distinctly decreased after downregulating RBM38 at both protein and mRNA levels. Western blot and qRT-PCR were applied to detect the expression of RBM38 and ZO-1. The relative quantification was calculated by the $\Delta\Delta$ Ct method and normalised based on β -actin. Data were means of three separate experiments and presented as mean \pm s.d. * P <0.05.

controls, in accordance with the previous reports (Shu *et al*, 2006; Cho *et al*, 2010). No obvious β -actin band presented in either RBM38 or control IgG. Similarly, in BT474 cells, ZO-1, HuR, and p21 transcripts existed in RBM38 rather than in the control IgG (Figure 4G and H). These results indicated that RBM38 could physically bind to ZO-1 mRNA.

Using bio-UTP-labelled probes (probes A, B, C, and D), REMSA was performed containing various fragments of ZO-1 mRNA 3'-UTR (Figure 5A) to find out potential RBM38 binding site in ZO-1 mRNA. Bio-UTP-labelled p21 probe was utilised as a positive control. The RBM38 protein was able to form RNA-protein complexes (RPCs) with probe B, but not probes A, C, and

D. Formation of the complex was weakened with the addition of p21 cold probe (Figure 5B).

To validate that AREs in ZO-1 mRNA 3'-UTR were required for RBM38 to promote ZO-1 expression, a dual-luciferase assay was performed using pGL3 control reporter or pGL3 reporters with ZO-1 3'-UTR-A, -B, -C, and -D downstream, whose sequences were identical to probes A, B, C, and D, respectively (Figure 5C). We found that the reporter carrying 3'-UTR-B and -D exhibited a relatively stronger luciferase activity in RBM38-overexpressed MCF7 cells (P <0.05; Figure 5D). Although the luciferase activity for a reporter carrying fragment B or D in ZO-1 3'-UTR was obviously increased by RBM38, the REMSA assay for fragment D was not definite when p21 cold probe

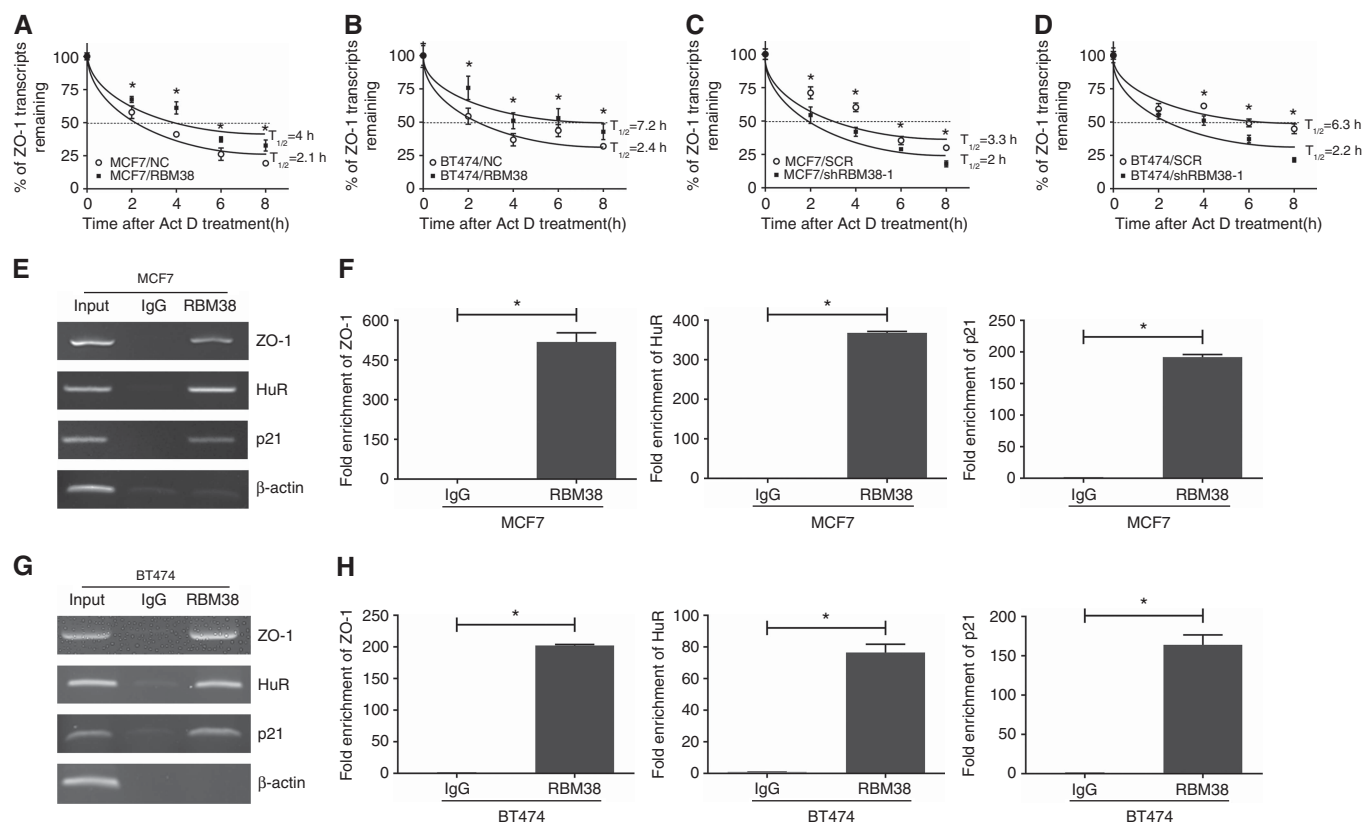


Figure 4. The RBM38 regulated ZO-1 mRNA stability by directly binding to its transcript. **(A and B)** Ectopic expression of RBM38 prolonged the half-life of ZO-1 transcript. **(A)** The MCF7 and **(B)** BT474 cells were transfected with lentivirus to overexpress RBM38. The RBM38 overexpression cells (RBM38) and the control (NC) were treated with $5 \mu\text{g ml}^{-1}$ actinomycin D for 0, 2, 4, 6, and 8 h. **(C and D)** Knockdown of RBM38 shortened the half-life of ZO-1 transcript. **(C)** The MCF7 and **(D)** BT474 cells were transfected with lentivirus to knock down the expression of RBM38 (shRBM38-1) and the control (SCR). The experiment was performed as described in RBM38 overexpression. Relative quantification was calculated by the $\Delta\Delta\text{Ct}$ method and normalised based on β -actin. $*P < 0.05$. **(E–H)** The RBM38 associated with ZO-1 transcript *in vivo*. **(E and F)** MCF-7 and **(G and H)** BT474 cell lysates were collected and immunoprecipitated with RBM38 antibody or control IgG followed by RT-PCR **(E and G)** and qRT-PCR **(F and H)** measuring transcript levels of ZO-1, HuR, and p21 in the control or RBM38-mRNA immunocomplexes. Upon normalisation of the level of β -actin transcript, the data were calculated from three separate experiments and performed as mean \pm s.d. $*P < 0.05$.

was added and not vice versa. Altogether, we concluded that ZO-1 3'-UTR-B was the binding site of RBM38 in ZO-1 mRNA 3'-UTR.

RBM38 overexpression rescued cell migration and invasion induced by knockdown of ZO-1. ZO-1 is proved to be a tumour suppressor in breast cancer, controlling endothelial cell-cell tension, cell migration, and barrier formation (Martin *et al*, 2004; Tornavaca *et al*, 2015). To examine the role of RBM38 on ZO-1-mediated cell migration and invasion, we silenced ZO-1 via transfecting with siRNAs, termed as Z-siRNA1 and Z-siRNA2, in MCF7 cell lines that expressed ectopic RBM38 and the control (Figure 6A). Then, Z-siRNA1 was selected for further study. Upon silencing of ZO-1 expression by Z-siRNA1, both RBM38-overexpression group and the control had stronger ability of migration and invasion. Moreover, by the overexpression of RBM38, MCF7 cell migration and invasion were evidently alleviated (Figure 6C and D). The fold change is the ratio of cell number with the transfection of Z-siRNA1 divided by that with the control, indicating that interfering of ZO-1-mediated migration was dropped from 1.98 to 1.43 with RBM38 overexpression. Furthermore, invasion was reduced from 2.57 to 1.79 with RBM38 overexpression. We obtained similar events in BT474 cells (Figure 6E and F). Cell migration was decreased from 1.76 to 1.20 with RBM38 overexpression. In addition, cell invasion was lessened from 2.03 to 1.69 with RBM38 overexpression. All the results suggested that overexpression of RBM38 had a stronger

effect on inhibiting migration and invasion that would reverse the same biological process mediated by ZO-1 knockdown. To explore whether overexpression of ZO-1 counterbalanced silencing of RBM38 *in vitro*, we inversely transfected MCF7 and BT474 cells with vector encoding the ZO-1 (Supplementary Figure S5A–5C). However, the effect of ZO-1 on RBM38 mediated cell migration was not that clear (Supplementary Figure S5B–D).

DISCUSSION

In this study, we elucidated a new pathway of RBM38 involved in TGF- β -induced EMT. The RBM38 could stabilise ZO-1 mRNA by directly binding to AREs in ZO-1 mRNA 3'-UTR and therefore inhibited cell migration and invasion. In addition, with active TGF- β signalling, RBM38 expression remarkably reduced that was directly regulated by transcription factor Snail targeting E-box elements in promoter region of RBM38 gene.

Our previous study demonstrated that RBM38 affected EMT process in breast cancer (Xue *et al*, 2014). Here, we identified a distinct reduction of RBM38 in mammary cells undergoing TGF- β -mediated epithelial dedifferentiation. Meanwhile, the blockage of TGF- β signalling by selective inhibitor SB431542 restored RBM38 expression levels, suggesting that RBM38 expression was modulated by TGF- β . Considering the importance of Snail in TGF- β -

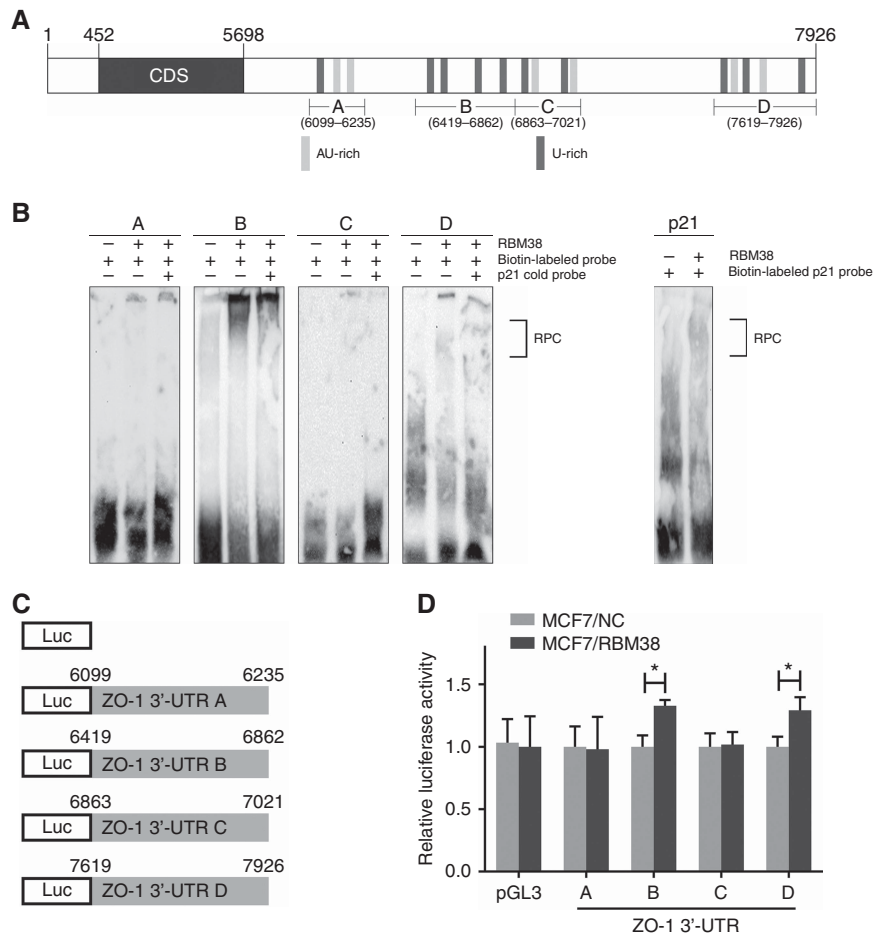


Figure 5. Multiple regions in the ZO-1 mRNA 3'-UTR were bound by RBM38. **(A)** Schematic representation of ZO-1 transcript and the location of probes used for REMSA. **(B)** Probes B and p21, but not probes A, C, and D, associated with RBM38. REMSA was performed by mixing bio-UTP-labelled probes A, B, C, D, or p21 with RBM38 protein, respectively. The bracket indicated RNA-protein complexes (RPCs). For a competition assay, an excess amount of p21 cold probe was added to the reaction mixture. **(C)** Schematic representation of the luciferase plasmid with various regions in ZO-1 mRNA 3'-UTR. **(D)** The luciferase activity for the reporter carrying ZO-1 3'-UTR-B, -D was increased by RBM38. The MCF7 cells with RBM38 overexpression lentivirus (RBM38) and the control (NC) were transfected with pGL3 control reporter or pGL3 reporter carrying various regions in 3'-UTR of ZO-1 mRNA for 48 h, respectively. The fold increase in relative luciferase activity was a product of the luciferase activity induced by RBM38 divided by that induced by NC. Data represented mean \pm s.d. of three independent experiments. * $P < 0.05$.

induced EMT, the work was focussed on the relationship between Snail and RBM38. By interfering the expression of Snail, RBM38 level was accumulated. Furthermore, as six E-box elements, the Snail recognising sequences, exist in RBM38 promoter region, the ChIP assay and dual-luciferase reporter assay were conducted and proved that Snail bound to E-box-1 and E-box-2 in promoter region of RBM38 gene and therefore restrained the RBM38 expression.

With the enhanced expression of Snail induced by TGF- β , TJ proteins, including claudin-1, occludin, and ZO-1, were down-regulated (Ohkubo and Ozawa, 2004; Li *et al.*, 2011). Additionally, Zhang *et al.* (2013b) reported that the Rho/ROCK signalling pathway mediated by TGF- β played a role in the dissolution of TJs during EMT. Here we found that enhanced expression of RBM38 prevented the loss of ZO-1 expression stimulated by TGF- β , indicating that ZO-1 might be the downstream of RBM38. We further investigated a significant correlation between RBM38 and ZO-1 expression in breast cancer cells. Overexpression of RBM38 increased ZO-1 expression, whereas RBM38 knockdown decreased ZO-1 expression. The RBM38 was able to increase ZO-1 mRNA stability by prolonging its half-life, whereas RBM38 knockdown obviously decreased the stability of ZO-1 transcript. We further confirmed that RBM38 could bind to ZO-1 transcript directly by

RIP assay. Previous studies found that RBM38 bound to mRNA 3'-UTR of many target genes and altered the stability of the transcripts that all contain multiple AREs (Zhang *et al.*, 2010, 2013a; Yin *et al.*, 2013; Cho *et al.*, 2015; Li *et al.*, 2017). Consistent with this, ZO-1 mRNA 3'-UTR contains several AREs that can be bound by RBPs (Chen *et al.*, 2008; Nagaoka *et al.*, 2012). Our results indicated that RBM38 directly bound to fragment B in ZO-1 mRNA 3'-UTR. Binding of ZO-1 AREs within its 3'-UTR by RBM38 led to enhanced stability of ZO-1 transcript. Here we uncovered a novel mechanism that RBM38 is a positive post-transcriptional regulator of ZO-1 in breast cancer.

Accordingly, TJs in epithelial cells function in an adhesive manner and prevent cell dissociation, being the first barrier that cancer cells must overcome for metastasis (Martin and Jiang, 2009). As a member of the ZO family, ZO-1 contains multiple domains with numerous binding partners (Fanning and Anderson, 2009) that controls endothelial cell-cell tension, cell migration, angiogenesis, and barrier formation (Tornavaca *et al.*, 2015). Here we demonstrated that the cell migration and invasion progression caused by the loss of ZO-1 was rescued by upregulation of RBM38. However, it is not obvious that ZO-1 counterbalances silencing of RBM38 *in vitro*. Altogether, we proposed that under stimulation of TGF- β , RBM38 was downregulated through transcription

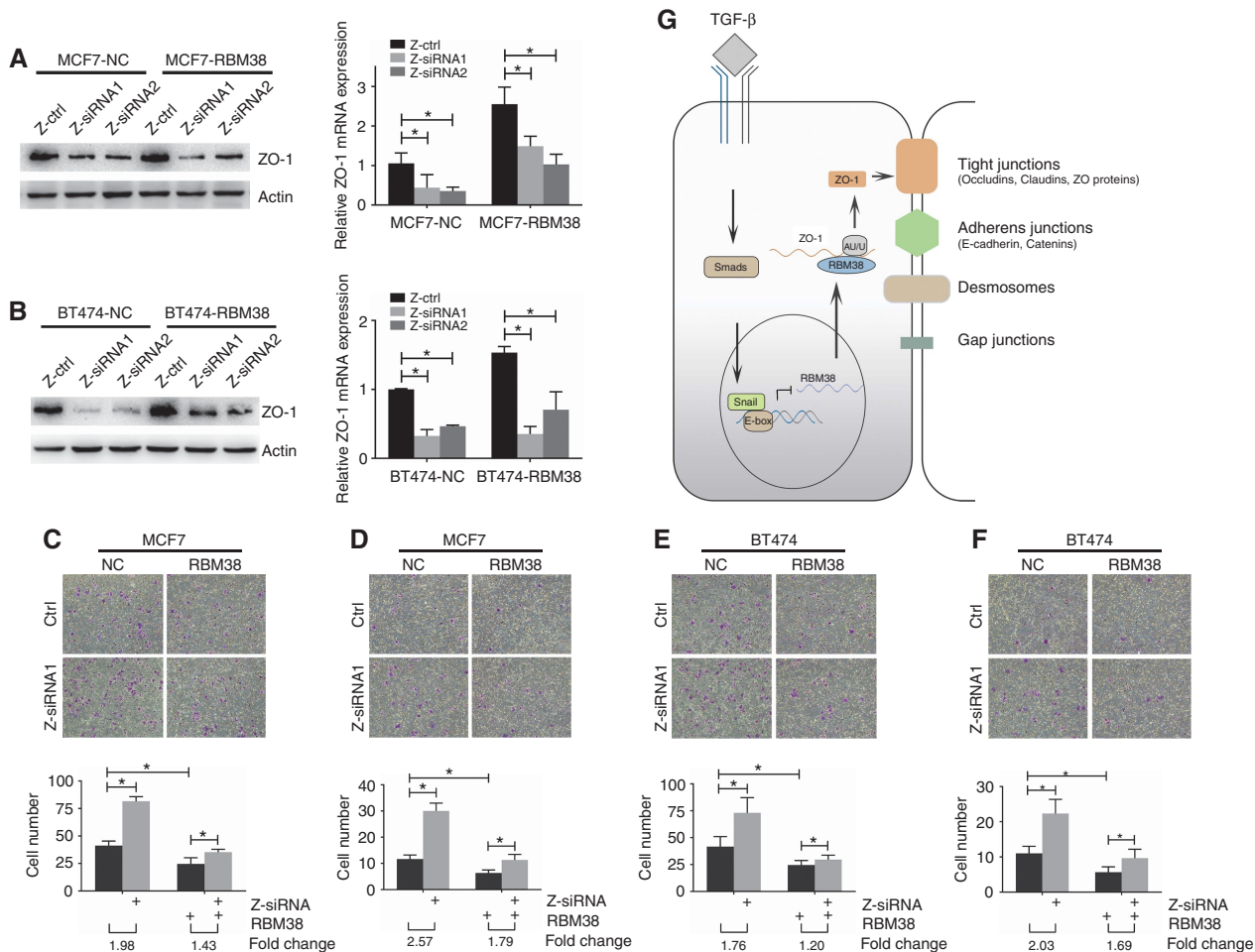


Figure 6. The RBM38 rescued the enhancement of cell migration and invasion induced by knockdown of ZO-1. (A) The MCF7 and (B) BT474 cells were transfected with siRNAs to silence ZO-1 expression in both RBM38-overexpressed cells and the control. Cell lysates were collected and analysed by western blotting and qRT-PCR. (C–F) The RBM38 rescued the promotion of cell migration and invasion induced by knockdown of ZO-1. (C) Cell migration and (D) cell invasion assay were performed using the cells treated as in (A). Lower panel represents the migrating and invading cell numbers corresponding to the upper panel. (E) Cell migration and (F) cell invasion assay were performed in BT474 cells and analysed as in (C and D). The assay was done in triplicate and a representative image is shown. **P*<0.05. (G) A model for the new pathway of RBM38 involved in TGF-β-induced EMT in breast cancer.

suppressor Snail, and low level of RBM38 led to decreased expression of ZO-1, and then contributed to cell invasion and migration (Figure 6G).

Considering the fact that TGF-β activates various signalling pathways, and the interactions among these pathways are quite complex (Gonzalez and Medici, 2014), the mechanism through which TGF-β controls the expression of RBM38 requires further investigation. Furthermore, more researches are required to clarify RBM38 regulation networks so as to explore the utility of RBM38 as a biomarker for breast cancer diagnosis and prognosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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