

A83-01 inhibits TGF- β -induced upregulation of Wnt3 and epithelial to mesenchymal transition in HER2-overexpressing breast cancer cells

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Abstract

Purpose The aim of this study is to investigate the mechanisms of interactions between TGF- β and Wnt/ β -catenin pathways that induce and regulate EMT and promote breast cancer cells to become resistant to treatment. **Methods** The effect of TGF- β on Wnt/ β -catenin signaling pathway was examined by using a human Wnt/ β -catenin-regulated cDNA plate array and western blot analysis. The interaction of Twist at promoter of Wnt3 was examined by chromatin immunoprecipitation (ChIP) assay. Secreted Wnt3 level was determined by ELISA assay. **Results** HER2-overexpressing breast cancer cells treated with TGF- β have a reduced response to trastuzumab and exhibited EMT-like phenotype. The TGF- β -induced EMT in HER2-cells was concordant with upregulation of Wnt3 and β -catenin pathways. The TGF- β -induced induction of Wnt3 during EMT was found to be Smad3-dependent. ChIP analysis identified occupancy of Twist at promoter region of Wnt3. Knock-down of Twist by shRNA confirmed the significance of Twist in response to TGF- β regulating Wnt3 during EMT. Subsequently, TGF- β -induced matrix metalloproteinases, MMP1, MMP7, MMP9,

MMP26, Vascular endothelial growth factors (VEGF), and activation of Wnt/ β -catenin signaling were repressed by the shRNA treatment. TGF- β RI ALK5 kinase inhibitor, A83-01 can effectively prevent the TGF- β -induced Twist and Wnt3. Co-treating A83-01 and trastuzumab inhibited TGF- β -induced cell invasion significantly in both trastuzumab responsive and resistant cells.

Conclusions Our data demonstrated an important interdependence between TGF- β and Wnt/ β -catenin pathways inducing EMT in HER2-overexpressing breast cancer cells. Twist served as a linkage between the two pathways during TGF- β -induced EMT. A83-01 could inhibit the TGF- β -initiated pathway interactions and enhance HER2-cells response to trastuzumab treatment.

Keywords TGF- β · Smad3 · Wnt3 · Twist · EMT

Introduction

TGF- β could act as a tumor suppressor by inhibiting cell proliferation in early carcinogenesis, however, at late stage, TGF- β pathway could interact with other oncogenic pathways and promote cancer progression [1–6]. As a tumor promoter, TGF- β has been shown to induce core transcription factors, Snail, Slug, and Twist, and promote EMT and cell invasiveness in different cell types [7–9].

The signal transduction by TGF- β is through its two transmembrane serine/threonine kinase receptors, type II receptor (TGF β RII) and type I receptor (TGF β RI). The TGF β RII activates TGF β RI, which leads to activation of the cellular response to TGF- β [1, 10]. TGF- β -induced EMT could be mediated through both Smad-dependent and Smad-independent pathways [11]. In Smad-dependent pathways, TGF- β induces activation of the receptor

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complex and activates Smad2 and Smad3. The phosphorylated Smad2 and Smad3 recruit Smad4, and translocate it into the cell nucleus, where they associate and/or cooperate with DNA-binding transcription factors to influence the targeted gene transcription [11, 12].

Wnt/ β -catenin signaling pathway also plays a critical role in EMT and cell invasion in cancer cells including breast cancer cells [13–15]. The Wnt signaling pathway can be initiated by binding Wnt ligands to transmembrane receptors of the Frizzled family. This leads to the destruction of APC complex, cell–cell adhesion, and E-cadherin. As a result, β -catenin accumulates in the nucleus, forming the β -catenin/TCF/LEF transcriptional complex, and drives transactivation of the Wnt signaling-targeted genes which promote EMT and cell invasion [16, 17]. We recently demonstrated that in trastuzumab-resistant HER2-overexpressing breast cancer cells, Wnt3 was significantly upregulated [18]. The upregulation of Wnt3 activates β -catenin pathway that leads to the transactivation of EGFR and promotes EMT-like transition. Considering that tumor microenvironments are often enriched in secreted TGF- β [19], hence it is possible that TGF- β signaling pathway could provide a favorable environment for the dysregulation of the Wnt3/ β -catenin pathway in the HER2 cells. There may be pathway interactions or network sustaining that leads to EMT and then activation of EGFR pathway, eventually leading to resistance. In this study we further investigated the interactions between TGF- β and Wnt/ β -catenin pathways in the regulation of EMT and promoting breast cancer cells' invasion. Data from our study demonstrated that TGF- β -induced EMT in HER2 cells was coordinated with the activation of Wnt3/ β -catenin pathways. In response to TGF- β , Twist was able to regulate Wnt3 during EMT and then activated β -catenin pathway. Targeting TGF β R1 ALK5 kinase by A83-01 prevented TGF- β -induced induction of Twist and Wnt3, and enhanced the anti-tumor activity of trastuzumab. The data suggested modulation of TGF- β /Smad pathway by small molecule inhibitors, such as A83-01, may provide a novel approach to therapeutic intervention of TGF- β -initiated pathway interaction and EMT in breast cancer, especially in HER2-overexpressing breast cancer. Furthermore, therapeutic targeting of Twist could inhibit TGF- β -induced upregulation of Wnt3 and enhance the sensitivity of chemo and target therapies, and subsequently could prevent tumor metastases caused by TGF- β .

Methods

Cell lines and culture

The human breast cancer cell lines SKBR3 (HTB-30) and BT474 (HTB-20) were obtained from American Type

Culture Collection. Monolayer cultures of the cells were maintained in DMEM/F12 medium with 10% fetal bovine serum. The cell lines overexpressed the HER2/c-erb-2 (HER2) gene product. The JIMT-1 cell line that was established from a breast cancer patient who clinically demonstrated resistance to trastuzumab was purchased from German Collection of Microorganisms and Cell Cultures. The JIMT-1 cell line also showed HER2 overexpression [20]. Both SKBR3 and JIMT-1 lacked expressions of estrogen and progesterone receptors (ER/PR) and BT474 expresses ER in addition to HER2 receptor.

Treatment with TGF- β , Wnt3, or inhibitors

The cells were serum starved overnight and then incubated with serum-free medium containing 5% Bovine serum albumin (Sigma) and 10 ng/ml of human TGF- β 1 (100-B-001, R&D) at different time points. The A83-01 (SML0788 Sigma) was used at 1 μ M and prior to TGF- β treatment for 2–16 h. The trastuzumab for this study was donated by Genentech. Human recombinant Wnt3 protein was purchased from Abnova and 50 ng/mL of Wnt3 protein was used to induce Smad pathway. The LGK974 (s7143), was obtained from Selleck, USA and 1 nM was used for treatment.

Human Wnt/ β -catenin-regulated cDNA plate array

The Wnt/ β -catenin-regulated cDNA plate array (AP-0171) was purchased from Signosis Sunnyvale, CA and consisted of 22 Wnt/ β -catenin pathway-regulated genes [18]. After treatment with TGF- β for 4 days, total RNA was isolated from cells utilizing RNeasy micro kit (#74004, QIAGEN). The quality of RNA was determined by separation of the RNA via capillary electrophoresis using the Agilent 2100 Bioanalyzer. cDNA was synthesized and plate array was created as per manufacturer's instructions.

Measurement of Wnt3 in conditioned medium utilizing ELISA test

The cells were treated with TGF- β , TGF- β + anti-TGF- β antibody, TGF- β + anti-Wnt3 antibody, or antibodies alone for 24 h and then collected the medium to measure Wnt3 by ELISA using Human wingless-type MMTV integration site family, member 3 ELISA Kit (MyBioSource #MBS919345). The attached cells were collected and counted. The Wnt3 level was calculated and normalized as ng/ml, per 1 million cells/ml according to manufacturer's instructions.

Knock down of Twist1 gene

shRNA construct for human Twist1 (TF308556) was purchased from OriGene, Rockville, MD. One set of shRNA

constructs contains four Twist1-specific shRNA sequences (A, B, C, D) expressed in pRFP-C-RS vector. Lipofectamine™ 2000 transfection reagent (11668-019, Invitrogen) was used for transfection following the manufacturer's instructions, and Puromycin (20 mg/ml) was used for clonal selection. The negative sequence for Twist1 (purchased from the same company) was also transfected in the cell lines.

Scratch wound healing assay

The cells were seeded in a 6-well plate until the adherent monolayer reached 70–80% confluence and the scratches were created. After washing with PBS some of the cells were placed in fresh medium containing trastuzumab and/or A83-01 and others were placed in fresh medium without Trastuzumab and/or A83-01. Each condition was duplicated. The images were taken after 48 h and three measurements of wound width were made at random points.

Boyden chamber invasion assay

The invasive assay was done in 24-well cell culture chambers using inserts with 8 µm pore membranes pre-coated with Matrigel (Sigma). Cell suspensions were placed in the upper wells without serum and medium containing serum or MDA-MB231 cells were filled in the lower wells. After 24 h, the cells were fixed by 0.5% glutaraldehyde and stained with 0.5% toluidine blue. The numbers of invaded cells were counted utilizing 20× objective microscope from three files per membrane and then normalized with total number of cells. Each experiment was performed twice and each conditioning was duplicated.

Chromatin immunoprecipitation real-time PCR assay (ChIP-qPCR)

The cells were treated with or without TGF-β for 48 h. The chromatin protein complex from the cells was prepared by immunoprecipitation of the chromatin with Twist1 antibody (ab50887, abcam) using Magna ChIP assay kit (17-10085, Millipore) following the manufacturer's instruction and then qPCR with Wnt3 promoter primers was performed. The primer sequences were designed to cover the Twist1-binding region of the promoter of Wnt3 (640–660): Lt: 5'-AAGGAGCCACCCGTTTCAT-3' and Rt: 5'-CCAGCAAACCTTGGGCAAAA-3'. Fold enrichment in the Twist1-precipitated samples was calculated relative to the mock samples (precipitated with normal IgG).

Immunoblotting analysis

The NE-PER nuclear and cytoplasmic extraction reagents (78833, Thermo-Scientific) were used to extract the nuclear

and cytoplasmic protein following the manufacturer's instructions. The antibodies used for Immunoblotting were β-catenin (cell signaling); TGF-βRI, TGF-βRII, phospho-Smad3 at ser423/425 and E-cadherin (Santa Cruz Biotechnology); Twist1 and Wnt3 (abcam). β-actin (sigma), α-Tubulin (Santa Cruz Biotechnology), and Histone 3 (H3) (cell signaling) were used for loading control or for confirmation of cytoplasmic and nuclear protein extraction.

Immunofluorescence (IF)

IF was carried out using antibodies for phospho-Smad3, Twist, and β-catenin according to manufacturer's recommended dilutions and incubated overnight followed by incubation with fluorescence-conjugated secondary antibodies for 30 min and then mounted with Vectashield, mounting medium. Cell nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI) (D9542; Sigma-Aldrich). Image was taken with a microscopy digital camera (Olympus BioScapes) under the light microscope reviewed by pathologist.

Statistical analyses

Data were analyzed using one-way Anova or the *t* test with SPSS 13.0 software. *p* < 0.05 indicates a statistically significant difference.

Results

TGF-β induces mesenchymal phenotype in HER2 cells and concordantly upregulates Wnt3 and β-catenin pathway signaling

We first treated HER2-overexpressing breast cancer cells with TGF-β over several days. Morphologic changes in SKBR3 cells were observed after two days of TGF-β treatment, as shown in Fig. 1a(left), the cells started losing cell–cell contact and displayed spindle shape. However, the cell growth was inhibited during the first three days of TGF-β-treatment and then the cell growth increased again after 4 days of treatment (Fig. 1a(right)). Cadherin switch, decreasing E-cadherin and increasing N-cadherin, and upregulation of zinc finger transcription factors, Snail, Slug, and Twist (Twist1), were detected after 24 h treatment with TGF-β (Fig. 1b). For the same period of time the cell invasiveness was found to have been increased by 35% (Fig. 1c). Data in Fig. 1d show the changes in the protein levels of Twist, N-cadherin, and E-cadherin by TGF-β. As shown in Fig. 1d the protein levels of Matrix Metalloproteinase-7 (MMP7) were also increased upon TGF-β induction.

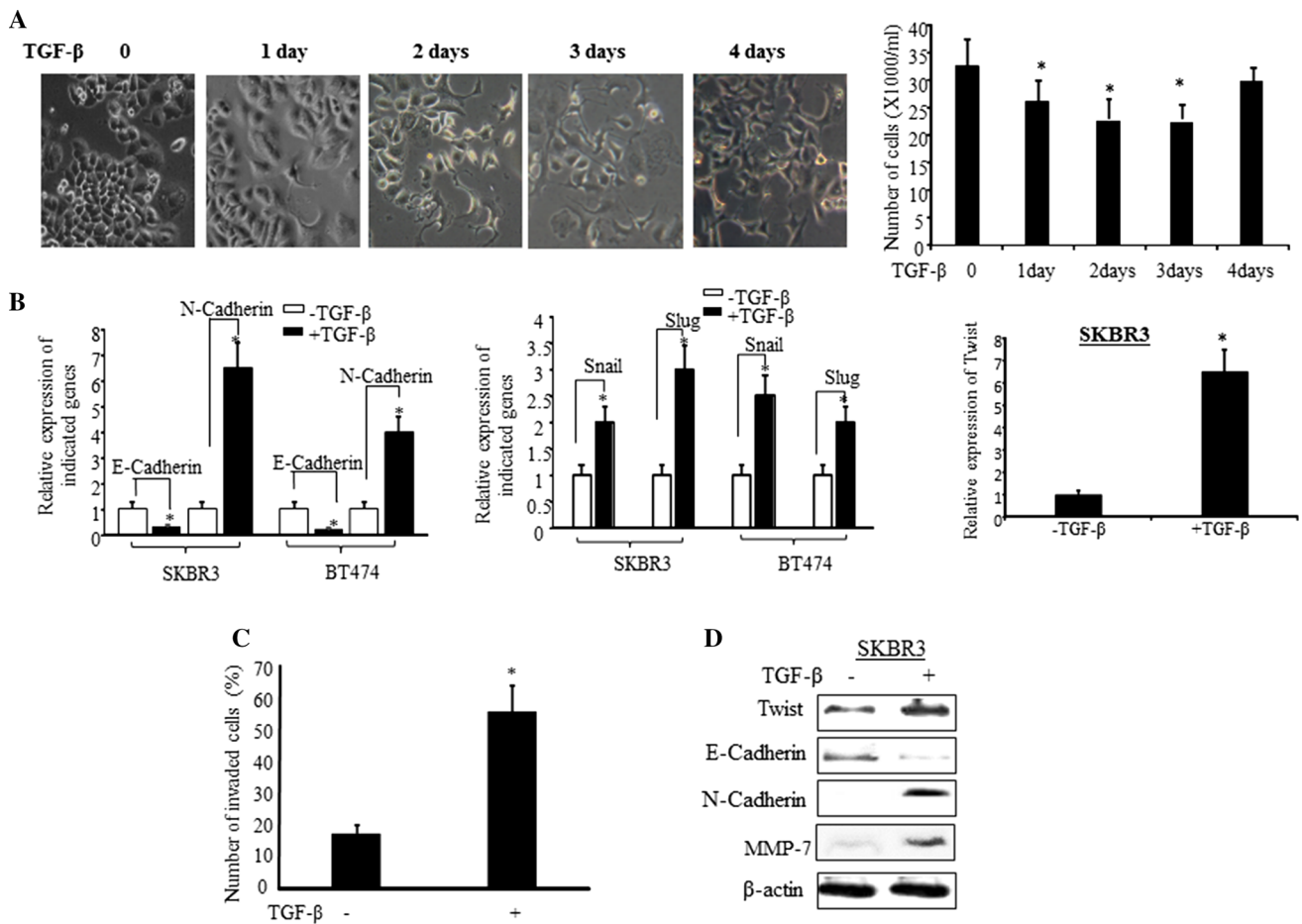


Fig. 1 TGF- β induces EMT phenotype enhances cell invasion of HER2-overexpressing breast cancer cells. **a** SKBR3 cells were treated with TGF- β for 4 days and changes in cell morphology were observed at the indicated days (left panel). The bar graph in right is quantified data of the left panel. The bars indicated mean number of total cells plus standard deviation counted from five different areas, $*p < 0.05$ compared to untreated cells. **b** SKBR3 and BT474 cells were treated with or without TGF- β for 24 h and RT-PCR was performed with the indicated genes' primers. The bars indicate relative expression of the

indicated genes (mean \pm SD from three determinations) adjusted with 18S, $*p < 0.05$ compared to untreated cells. **c** SKBR3 cells were treated with or without TGF- β for 24 h and cell invasiveness was measured by Boyden Chamber Invasion assay as described in "Methods" section. The invasive cells (mean \pm SD from five different areas) were counted, $*p < 0.05$ compared to untreated cells. **d** SKBR3 cells were treated with or without TGF- β and the indicated protein expressions were analyzed by Western blot analysis. The β -actin was used as loading control

Previously we have identified that upregulation of Wnt3 promoting EMT in HER2-overexpressing breast cancer cells is an important mechanism leading to trastuzumab resistance [18]. The Wnt3 expression was increased more than 3-fold in JIMT-1 and more than 2-fold in SKBR3/100-8 (a Trastuzumab-resistant cell line generated from SKBR3 [18]) compared to SKBR3 and BT474 (Supplement-Fig. 1A). The secreted Wnt3 in conditioned medium from JIMT1 cells was also higher than that in the conditioned medium from SKBR3 cells, and the upregulated mRNA levels of Wnt3 and Twist in JIMT1 were confirmed again (Supplement-Fig. 1B). The protein levels of TGF- β Rs (TGF- β RII and TGF- β RI) and phosphorylated Smad3 were also higher in Trastuzumab-resistant cell, JIMT1, compared with the sensitive cells, SKBR3 and BT474 (Supplement-Fig. 1C). The data suggested that TGF- β and

Wnt3 pathways are constitutively upregulated in the Trastuzumab-resistant cells. Hence, it is important to understand that if the two pathways sustain each other to affect HER2 cell response to treatment.

Next, we examined the effect of TGF- β on Wnt/ β -catenin signaling pathway using a human Wnt/ β -catenin-regulated cDNA plate array. The data showed that 64% of the genes in the array were upregulated more than 2 fold upon TGF- β induction. Among those upregulated genes, c-Jun, c-Myc, NOS2, PPAR δ were upregulated by 5 fold, while Nanog and Vascular endothelial growth factor (VEGF) were upregulated approximately by 16 fold (Fig. 2a). Furthermore, all four matrix metalloproteinases, MMP1, MMP7, MMP9, and MMP26 were significantly upregulated. To determine if the TGF- β -induced activation of Wnt/ β -catenin pathway signaling is ligand dependent,

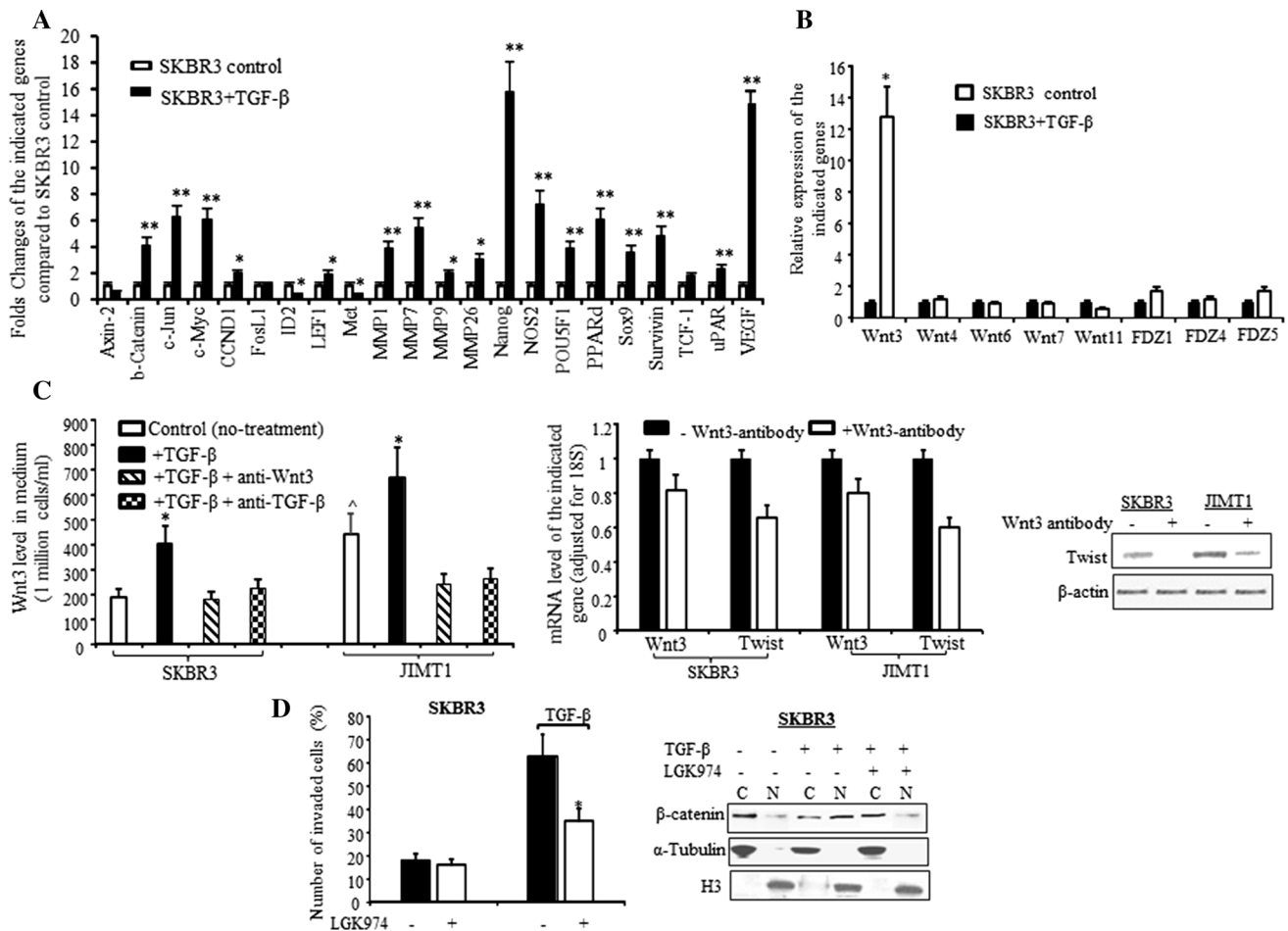


Fig. 2 TGF- β upregulates Wnt3 and activates β -catenin pathway. **a** Bar graph of regulated Wnt/ β -catenin pathway signaling in SKBR3 treated with TGF- β (dark bars) compared to untreated SKBR3 (open bars) analyzed by specific Wnt/ β -catenin mRNA array as described in “Methods” section. The relative level of each indicated gene was adjusted with GAPDH and each bar indicates mean fold change and SD from four determinations, * $p < 0.05$ and ** $p < 0.01$ compared to untreated cells. **b** SKBR3 cells were treated with TGF- β for 24 h and RNA was extracted. RT-qPCR was performed with the primers for Wnt ligands (left) and FZD receptors (right). The bars indicate relative expression of the indicated genes adjusted to 18S (mean \pm SD from three determinations), $^{\wedge}p < 0.05$ compared to the untreated SKBR3 cells, * $p < 0.05$ compared to untreated cells. **c** The cells were treated with TGF- β \pm anti-TGF- β antibody for 24 h or TGF- β \pm anti-Wnt3 antibody. The medium was collected and the attached cells were used for extraction of RNA or protein. **c** (left) The Wnt3 level in the conditioned medium was measured by ELISA and calculated as per one million cells/ml as described in “Methods” section. The bars indicate mean from two experiments (each

experiment had duplicated measurements) plus standard deviation, * $p < 0.05$ compared to untreated cells, $^{\wedge}p < 0.05$ compared to untreated SKBR3 cells (control). **c** (middle) RT-qPCR was performed with Wnt3 and Twist primers. The bars indicate relative expression of Wnt3 or Twist (mean \pm SD from three determinations) and adjusted for 18S. The dark and open bars indicate RNA from the cells without or with incubation with Wnt3 antibody respectively. The fold changes were compared to cells without incubation with Wnt3-antibody. **c** (right) Western blot was performed with antibody specific against Twist and β -actin was used for loading control. **d** (left), SKBR3 was pre-treated with LGK974 for 16 h and then treated with TGF- β for additional 24 h. Cell invasiveness was measured by Boyden Chamber Invasion assay as described in “Methods” section. The invasive cells (mean \pm SD from five different areas) were counted. * $p < 0.05$ compared to untreated cells. **d** (right) The nuclear or cytoplasm expressions of β -catenin were determined by Western blot analysis upon treatment with TGF- β \pm LGK974. The α -Tubulin and H3 were used for loading control of cytoplasm and nuclear protein respectively

especially if it is Wnt3 dependent, we examined several Wnt ligands and Fizzled receptors upon TGF- β induction. Among the different Wnt ligands TGF- β significantly upregulated Wnt3 and slightly upregulated FZD1 and FZD5 in SKBR3 cells (Fig. 2b). The baseline level of Wnt3 in conditioned medium from JIMT1 cells was higher than that in conditioned medium from SKBR3 cells and the

secretion of Wnt3 was further increased significantly followed by TGF- β induction in both SKBR3 and JIMT1 cells (Fig. 2c(left)). The TGF- β -induced Wnt3 secretion was repressed by co-treating the cells with TGF- β and TGF- β antibody or TGF- β with Wnt3 antibody (Fig. 2c(left)). The immunodepletion of Wnt3 not only repressed Wnt3 secretion and expression, but also downregulated mRNA

and protein level of Twist in both SKBR3 and JIMT1 cells (Fig. 2c(middle and right)). LGK974 is an inhibitor of PORCN and blocks secretion of Wnts. Pre-treated SKBR3 cells with LGK974 repressed TGF- β -induced cell invasion (Fig. 2d(left)). The TGF- β -induced nuclear accumulation of β -catenin was also blocked by LGK794 (Fig. 2d(right)). Those data further confirmed that the TGF- β -induced cell invasion is Wnt dependent.

TGF- β -induced EMT and Wnt3 are Smad3 dependent

Next, we examined the role of Smad pathway in TGF- β -induced EMT and upregulation of Wnt3 in HER2-over-expressing cells. SKBR3 cells were treated with TGF- β from 15 min to 24 h, and the phosphorylation of TGF- β receptors was examined. As shown in Fig. 3a, the

phosphorylation of TGF β RII was observed at 15 min, peaked at 30 min, and then decreased after 30 min of induction, while the phosphorylation of TGF β R I remained for 1 h. The phosphorylation of Smad2/3 (pSmad2/3) was also increased by TGF- β , however, the pSmad3 (lower band) increase was more conspicuous (Supplement-Fig. 2). The increased pSmad2/3 was completely blocked by the A83-01 (Supplement-Fig. 2). Then we examined the effect of TGF- β in the upregulation of pSmad3. The TGF- β -induced pSmad3 protein at serine 423/425 was observed at 1 h and remained up to 24 h (Fig. 3a). While the protein level of Twist was upregulated upon 4 h of TGF- β induction and the E-cadherin was downregulated at the same time (Fig. 3a). Figure 3a also shows that Wnt3 protein was upregulated after TGF- β induction for 6 h and significantly increased after TGF- β treatment for 24 h. Similar observation was made with JIMT1 and BT474

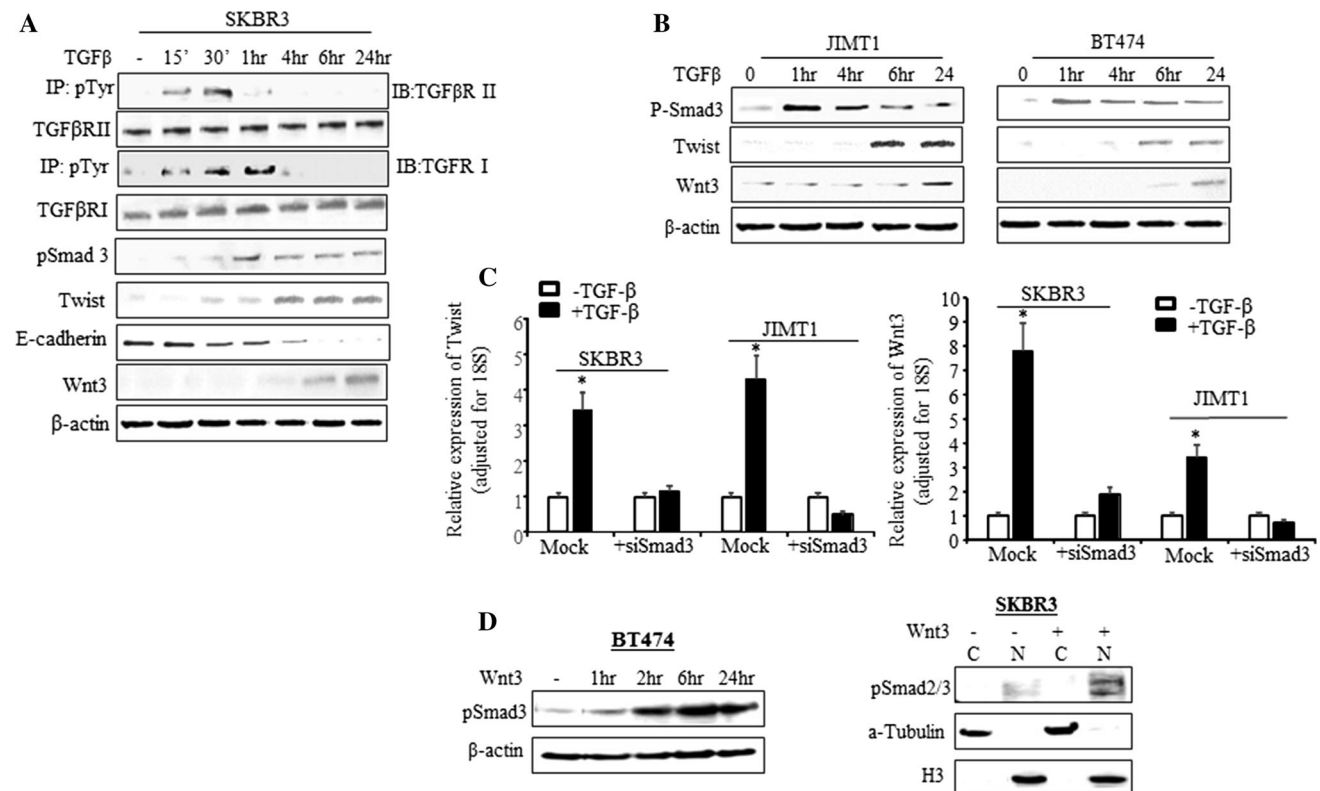


Fig. 3 TGF- β -induced EMT and Wnt3 are Smad3 dependent. **a** SKBR3 cells were treated with TGF- β for 15 min to 24 h. Total protein was extracted at the indicated time points. To measure the phosphorylated TGF- β R I and TGF- β R II, immunoprecipitation was performed by incubating protein lysis with phosphor-Tyrosine antibody first followed by Western blot analysis with antibodies specific to TGF β R I and TGF β R II. The phospho-Smad3 (pSmad3), Twist, E-cadherin, and Wnt3 protein levels were also determined by Western blot analysis. The β -actin was used as loading control. **b** BT474 and JIMT1 cells were treated with TGF- β from 1 to 24 h and Western blot analysis was performed with the indicated antibodies. **c** Cells were treated with siSmad3 or negative sequence (Mock) for 48 h. TGF- β was added into the media containing

siSmad3 or negative sequence after 24 h siSmad3 treatment. RT-qPCR was performed with Twist (*left*) and Wnt3 (*right*) primers. The bars indicate relative expression of the indicated genes adjusted to 18S (mean \pm SD from three determinations), * p < 0.01 compared to untreated cells. **d** In the *left* panel, BT474 cells were treated with recombinant human Wnt3 protein at the indicated time and total protein was extracted. The pSmad3 was determined by Western blot analysis. The β -actin was used as loading control. In *right* panel, SKBR3 cells were treated with or without recombinant human Wnt3 protein for 24 h and then cytoplasmic and nuclear fragment was extracted. The pSmad2/3 was determined by Western blot analysis. The α -Tubulin and H3 were used for loading control of cytoplasm and nuclear protein respectively

(Fig. 3b). Knock down of Smad3 by siRNA inhibited TGF- β -induced upregulation of Twist and Wnt3 in both SKBR3 and JIMT1 cells, suggesting the Smad3-dependent regulation of Twist and Wnt3 by TGF- β in HER2 cells (Fig. 3c). Next we questioned if the TGF- β -mediated Wnt signaling could interact with the Smad pathway. We exogenously treated SKBR3 and BT474 cells with recombinant human Wnt3 protein for 24 h. The data showed that pSmad3 protein was significantly upregulated in BT474 cells which were treated with Wnt3 for 2 h and the upregulated pSmad3 was sustained after 24 h (Fig. 3d(left)). The Fig. 3d(right) demonstrates that Wnt3 was able to induce nuclear accumulation of both pSmad2 and pSmad3 in SKBR3 cells after 24 h treatment. The data suggested that there is a feedback loop between TGF- β and Wnt/ β -catenin pathways inducing EMT in HER2-overexpressing breast cancer cells.

Twist binding to E-box at the Wnt3 promoter to regulate Wnt3 during TGF- β -induced EMT

Twist, a basic helix-loop-helix (bHLH) transcription factor, is characterized by a basic DNA-binding domain that targets the consensus E-box sequence 5'-CANNTG-3' [21]. Using MatInspector on the Genomatix server (www.genomatix.de) [22], an E-box sequence at the promoter of Wnt3 gene located at -640 to -660 relative to the transcription start site +1 was identified. Following TGF- β treatment, chromatin immunoprecipitation confirmed the Twist binding to E-box at the Wnt3 promoter to regulate Wnt3 during the TGF- β -induced EMT. Data in Fig. 4a showed that the occupancy of Wnt3 promoter region by Twist was approximately 5-fold enriched in SKBR3 cells and 4-fold enriched in JIMT1 cells compared with IgG control. TGF- β further increased the occupancy of Twist at Wnt3 promoter region by 2-fold in SKBR3 and 4-fold in JIMT1 compared with the cells without treating with TGF- β , respectively. Knock down of Twist by shRNA decreased Wnt3 protein expression (Fig. 4b(right)). Western blot data in Fig. 4c showed that nuclear accumulation of β -catenin by TGF- β was also inhibited by the shRNA knocking down Twist. Subsequently, TGF- β -induced MMPs, VEGF, and activation of Wnt/ β -catenin signaling were repressed by the shRNA treatment (Fig. 4d). These findings indicate the critical role of Twist in the interactions between the TGF- β and Wnt/ β -catenin pathways.

Inhibition of Smad3-dependent TGF- β pathway by A83-01 prevented TGF- β -induced Twist and Wnt3, enhanced HER2 cells' response to trastuzumab treatment

The A83-01, has been shown to inhibit TGF β R1 ALK5 kinase activity, prevent phosphorylation of Smad2, and

subsequently inhibit TGF- β -induced EMT [23]. In our study A83-01 inhibited TGF- β -induced pSmad3 at serine 423/425 and prevented nuclear accumulation of pSmad3. As shown in Fig. 5A, SKBR3 cells pre-treated with A83-01 inhibited TGF- β -induced pSmad3 and Twist. The nuclear translocation of pSmad3 by TGF- β was inhibited (Fig. 5B(a–f)) and Twist expression in cell nucleus was also repressed (Fig. 5B(g–l)). TGF- β -induced nuclear expression of β -catenin was also inhibited by A83-01 (Fig. 5C). Besides, A83-01 prevented the TGF- β -induced upregulation of Wnt3 protein (Fig. 5D).

Furthermore, targeting TGF β R1-ALK5 kinase by A83-01 resulted in reducing TGF- β -induced cell invasion/migration and enhancing HER2 cells' sensitivity to trastuzumab treatment. Data from this study showed that TGF- β reduced the growth rate in the first 3 days, and then the cell growth rate was increased after 4 days (Fig. 6a). Consistent with the previous report [23], A83-01 blocked the growth-inhibitory effect of TGF- β (Fig. 6a). However, the cell invasion was significantly increased after 24 h of TGF- β treatment (Fig. 6b). The TGF- β -induced cell invasion was inhibited 24% by trastuzumab, and 35% by A83-01, respectively, compared to the cells treated with TGF- β alone. The trastuzumab in combination with A83-01 was able to completely block the TGF- β -induced cell invasion (Fig. 6b). The TGF- β -induced cell migration in JIMT1 cells was also reduced greatly by the combination treatment (Fig. 6c). The Fig. 6c(top) demonstrated the migration of JIMT1 cells upon different treatment and Fig. 6c(bottom) showed the quantified migrating level (wound width compared to Control cells) of JIMT1 cells at each treatment. As shown in Fig. 6c(bottom) that A83-01 alone reduced approximately 17% cell migration of JIMT1 and trastuzumab alone inhibited 20% cell migration, while in combination of A83-01 and trastuzumab synergistically inhibited JIMT1 cell migration up to 90%. The inhibitory effects of A83-01 suggest the therapeutic potential of inhibiting ALK5 kinase for treating TGF- β -induced HER2-positive tumor.

Discussion

TGF- β -induced EMT plays an important role in cancer cell resistance to chemotherapy and progression to metastases [24–26]. Wnt/ β -catenin signaling pathway is also implicated in playing a critical role in EMT and appears to be associated with resistance to therapeutic treatment in aggressive types of cancer [16, 17, 27–29]. Data from our previous study suggested that activation of Wnt3/ β -catenin signaling pathway in HER2-overexpressing breast cancer cells leads to transactivation of EGFR and trastuzumab resistance [18]. Although both TGF- β and Wnt/ β -catenin

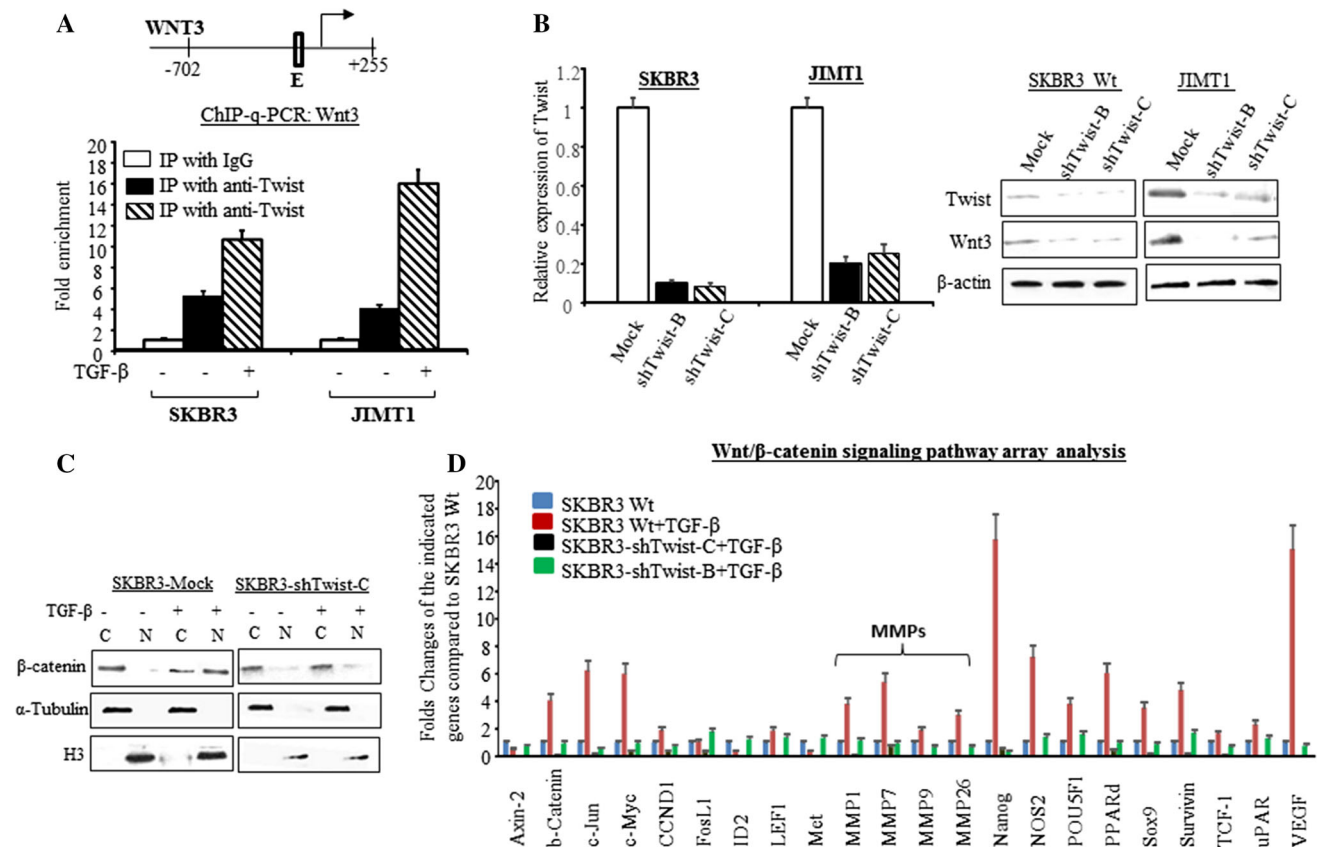


Fig. 4 TGF- β induces Twist transcriptionally upregulating Wnt3 and leads to activate Wnt/ β -catenin pathway. **a** *top* E-box located in promoter of Wnt3 where Twist binding allowed; *bottom* Cells were treated with or without TGF- β and ChIP-qPCR assay was performed as described in “Methods” section. The bars indicate the fold enrichment of Twist binding to the promoter of Wnt3 in the indicated cells with or without TGF- β induction and compared to Mock cells (immunoprecipitated with IgG). **b** The cells were treated with shRNA Twist or negative sequence for 48 h. RT-qPCR was conducted with Twist primer and the bars in left indicated relative expression of Twist (mean \pm SD from three determinations) adjusted with 18S, * p < 0.05 compared to untreated cells. In the right, Western blot was performed with antibodies specific for Twist and Wnt3. The β -actin

was used for loading control. **c** The cells were treated with Twist shRNA or negative control sequence and then induced with or without TGF- β . The nuclear or cytoplasm expressions of β -catenin were determined by Western blot analysis. The α -Tubulin and H3 were used for loading control of cytoplasm and nuclear protein respectively. **d** The bar graph indicates the regulation of Wnt/ β -catenin pathway signaling in SKBR3 wild type (blue bars), wild-type cells treated with TGF- β (red bars) and shRNA Twist knocking down cells (black and green bars) treated with TGF- β compared to untreated SKBR3 (blue bars) analyzed by specific Wnt/ β -catenin mRNA array. The relative level of each indicated gene was adjusted with GAPDH and each bar indicates mean fold change and SD from four determinations

signaling pathways play a dominant role in the initiation of EMT, the two pathways often cooperate in regulating gene expression and the EMT process [30]. In our current study we continually investigated the mechanism of TGF- β and Wnt pathways cooperatively regulating EMT.

Our data demonstrated that TGF- β -induced EMT in HER2 cells was coordinated with the upregulation of Wnt/ β -catenin pathway. There was a Smad3-dependent feedback loop between TGF- β and Wnt/ β -catenin pathways inducing EMT in the HER2 cells. The TGF- β -induced Wnt3 acts as a secreted factor and further drives not only β -catenin signaling, but also Smad pathway. The small molecular inhibitor, LGK974 is a potent and specific PORCN inhibitor, and has been tested blocking Wnt1, -2, -3, -3A, -6, -7A, and -9As [31]. It has also been shown to

have strong efficacy in animal tumor models, and to be well tolerated [31]. In this study TGF- β -induced cell invasion was repressed by LGK974, suggesting that the Wnt-dependent invasion was induced by TGF- β . The siRNA knocking down Smad3 confirmed Smad3-dependent interaction between TGF- β and canonical Wnt pathway. Smads, especially Smad2 and Smad3, have been recognized as important intracellular mediators of TGF- β signaling and controlling transcriptional effects of TGF- β signaling during EMT [11, 32]. The nuclear localization of Smad3 was shown to enhance Twist, leading to EMT by repressing ID2 interaction with transcription factor 3 (ATF3) in epithelial cells from different organs including mammary epithelial cells, MCF-10A [33]. Our data in this study showed that nuclear accumulation of Smad3 by TGF-

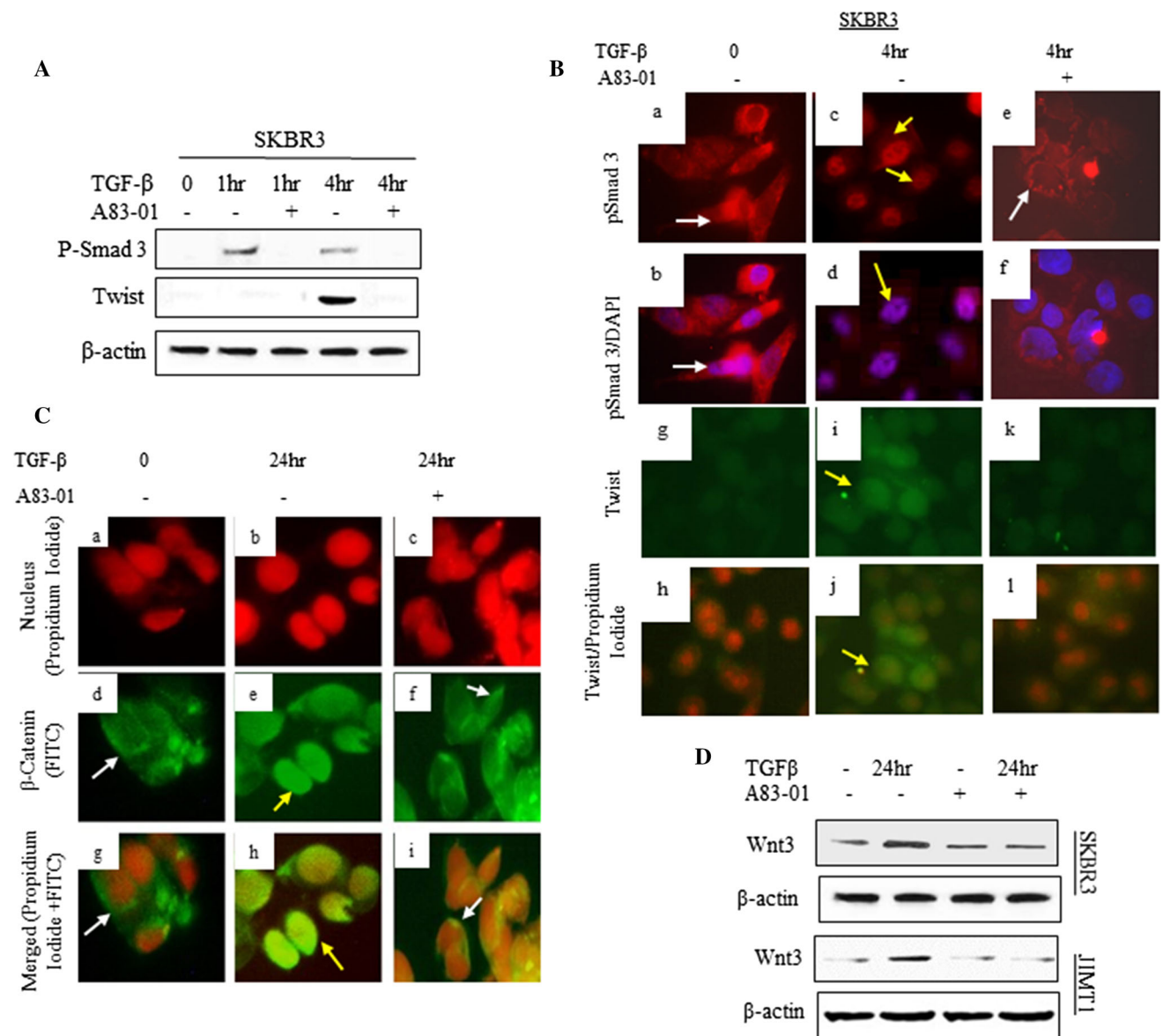


Fig. 5 Targeting TGF-βRI ALK5 kinase by A83-01 inhibited TGF-β-induced Twist and Wnt3. The cells were incubated with A83-01 for 16 h and then treated with TGF-β at indicated time. **A** total protein levels of pSmad3 and Twist were determined by Western blot analysis. **B** Localization of pSmad3 (*a, c, e* Texas red) and Twist (*g, i, k* FITC green) protein was determined by Immunofluorescence analysis (IF) as described in the “Methods” section. White arrows indicate cytoplasmic expression and yellow arrows indicate nuclear expression. *b, d, f* Merged pSmad3-Texas red and cell nuclei labeled

with DAPI blue, and *h, j, l* merged Twist-FITC (green) and cell nuclei labeled with propidium iodide (PI) (red). **C** Localization of β-catenin was determined by IF. *a–c* show cell nuclei labeled with PI (red), and *d–f*, show β-catenin labeled with FITC (green). *g–i* Merged β-catenin-FITC and cell nuclei labeled with PI. White arrows indicate cytoplasmic expression and yellow arrows indicate nuclear expression. **D** Total protein level of Wnt3 was determined by Western blot analysis

β led to the upregulation of Twist protein in HER2-over-expressing epithelial cells.

The A83-01 was reported to be able to block ALK4/5/7 kinase, such that it inhibits not only TGF-β but also activin/nodal signaling [23]. It has been shown that A83-01 inhibits the transcriptional activity induced by constitutively active ALK5 and has twice the potency compared to other small molecular inhibitor for ALK5 [23]. Therefore A83-

01 might be useful for treating cancer progression by blocking TGF-β-induced EMT. We show in this study that targeting TGFβRI ALK5 kinase by A83-01 inhibited TGF-β-induced phosphorylation of Smad3, and subsequently repressed Twist and prevented TGF-β-induced induction of Wnt3 and EMT in HER2 cells.

Twist has been shown to transcriptionally repress E-cadherin gene expression through E-box elements on its

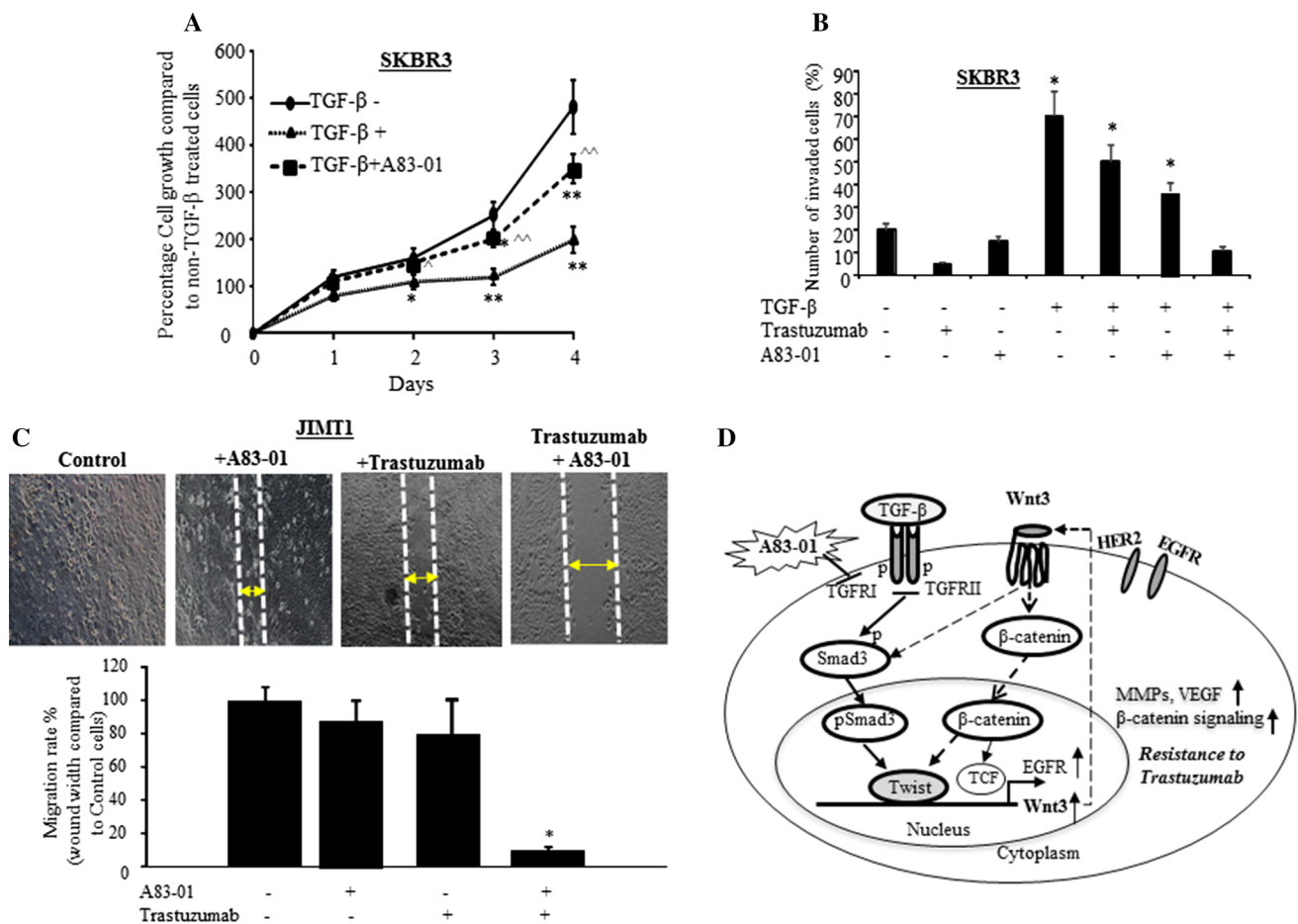


Fig. 6 A83-01 enhanced inhibition activity of trastuzumab on TGF- β -induced cell invasion and migration. **a** SKBR3 cells were treated with TGF- β , TGF- β plus A83-01, or without any treatment for 4 days and MTT assay was performed at each day as described in “Methods” section. Each data point was six measurements and the graph showed mean of six measurements plus standard deviation, * $p < 0.05$ and ** $p < 0.01$ compared to untreated cells, $^{\wedge}p < 0.05$ and $^{\wedge\wedge}p < 0.01$ compared to the TGF- β treatment alone. **b** SKBR3 cells were incubated with A83-01 for 16 h and then the cells were treated with either TGF- β , trastuzumab, or A83-01 alone or as indicated combination treatment for 24 h. Cell invasiveness was measured by Boyden Chamber Invasion assay as described in “Methods” section. The bars indicated mean number of invaded cells plus standard deviation counted from five different areas, * $p < 0.05$ compared to untreated cells. **c** Cell migration was determined by wound healing (Gap Closure) assay. The cells were pre-treated with or without A83-01 for

16 h and then treated with TGF- β \pm Trastuzumab. The photos of cell migration were taken after 48 h treatment (top). The bar graph in bottom is quantification of the wound width. The bar indicates the percentage migration rate (wound width compared to untreated control cells) of each condition, and three views were counted, * $p < 0.05$ compared to the control cells. **d** Proposed model of the feedback loop between TGF- β and Wnt/ β -catenin pathways. TGF- β initiates phosphorylation of Smad3 and nuclear translocation of pSmad3, and then induces Twist binding to E-box at the Wnt3 promoter to regulate Wnt3 during EMT. The upregulated Wnt3 by TGF- β in turn activates Wnt/ β -catenin signaling to further upregulated EGFR [18] and Smad pathway. The interaction between the two pathways sustains each other, induces EMT and promotes HER2 cell resistance to therapy. Targeting TGF- β R1 ALK5 kinase could inhibit the TGF- β -initiated pathway interactions and enhance HER2 cell response to trastuzumab treatment

promoter and by inducing EMT [34]. In this study we demonstrated for the first time that Twist could regulate Wnt3 by directly binding to Wnt3 promoter region through E-box element on its promoter. Therefore, Twist served as a linkage between TGF- β and canonical Wnt pathways during the time TGF- β -inducing EMT.

It was known that Twist is not only critical for EMT process but also necessary for the metastatic process [35–40]. Through an in vivo mouse model in our previous study, we found that Twist expression was associated with

mammary tumors that were more likely to exhibit invasive and metastatic characteristics [41]. Our data in the current study showed that the angiogenesis- and metastases-associated molecules, such as VEGF and MMPs were significantly upregulated upon TGF- β induction. As a result, the cells were more invasive.

It has been reported recently that EMT may not be required for metastases, but more likely to contribute to resistance to chemotherapy [42, 43]. We showed in this study that inhibition of TGF- β -induced EMT by A83-01 in

HER2-overexpressing breast cancer cells enhanced anti-tumor activity of trastuzumab. Co-targeting TGF- β /Smad and HER2 pathways has significantly inhibited cell migration in both SKBR3 and JIMT1 cells.

Therefore, we propose a model in which TGF- β initiated direct interaction between phosphorylation of Smad3 and Twist to regulate Wnt3, consequently, induced canonical Wnt signaling and led to resistance (Fig. 6d). Inhibition of TGF- β /Smad pathway by A83-01 could prevent the TGF- β -initiated pathway interaction and inhibit EMT induced by TGF- β , and subsequently enhance HER2 cell response to trastuzumab treatment. Our study also suggests that targeting Twist could inhibit the interaction between TGF- β and Wnt pathways that could not only prevent breast cancer progressing to EMT and enhancing sensitivity of cancer treatment, but could also inhibit the TGF- β - and Wnt3-induced MMPs and VEGF. Thus, therapeutic targeting of Twist could prevent tumor metastases and improve outcomes of breast cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The experiments in this study comply with the current laws of the country in which they were performed.

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