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# Autocrine TGF-β protects breast cancer cells from apoptosis through reduction of BH3-only protein, Bim

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**Cancer cells undergo multi-step processes in obtaining the ability to metastasize, and are constantly exposed to signals that induce apoptosis. Acquisition of anti-apoptotic properties by cancer cells is important for metastasis, and recent studies suggest that transforming growth factor (TGF)-β promotes the survival of certain types of cancer cells. Here, we found that in highly metastatic breast cancer cells, JygMC(A), JygMC(B) and 4T1, TGF-β ligands were produced in autocrine fashion. Pharmacological inhibition of endogenous TGF-β signalling by a TGF-β type I receptor kinase inhibitor in serum-free conditions increased the expression of BH3-only protein, Bim (also known as Bcl2-like 11) in JygMC(A) and JygMC(B) cells, and caused apoptotic cell death. We also found that induction of Bim by TGF-β was not observed in Foxc1 knocked-down cancer cells. These findings suggest that TGF-β plays a crucial role in the regulation of survival of certain types of cancer cells through the TGF-β-Foxc1-Bim pathway, and that specific inhibitors of TGF-β signalling might be useful as apoptosis inducers in breast cancer cells.**

*Keywords*: TGF-β/apoptosis/breast cancer/Bim/Foxc1.

*Abbreviations*: ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CHX, cycloheximide; EMT, epithelial-mesenchymal transition; Foxc1, forkhead box c1; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Hprt1, hypoxanthine phosphoribosyltransferase 1; Id1, inhibitor of DNA binding 1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAP kinase-Erk kinase; PCR, polymerase chain reaction; PI3K, phosphoinositide 3-kinase; RT, reverse transcription; Serpine1, serine peptidase inhibitor, clade E, member 1; siRNA, small interfering RNA; TGF-β, transforming growth factor-β; TβR, transforming growth factor-β receptor, TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

Metastasis is the final stage in the progression of cancer. Cancer cells undergo multi-step processes in metastasis that include liberation from extracellular matrix, intravasation, transport through the circulatory system, extravasation and formation of tumour in a secondary organ (*1*). However, during these multi-step processes, cancer cells are persistently exposed to various stresses such as detachment from microenvironment, mechanical stress in blood vessels, depletion of nutrients and hypoxia. Since they are also exposed to various signals that induce apoptosis, acquisition of anti-apoptotic properties by cancer cells is an important event in metastasis.

Transforming growth factor (TGF)-β, which includes three isoforms (TGF-β1, TGF-β2 and TGF-β3), regulates a wide range of cellular responses, including cell proliferation, differentiation, adhesion, migration and apoptosis (*2*). TGF-β transduces signals through two types of transmembrane serine/threonine kinase receptors, termed type I (TβRI) and type II (TβRII). After TGF-β binding, these receptors form heterotetrameric complexes, and the TβRII kinase phosphorylates the juxtamembrane part of the cytoplasmic domain of TβRI. Phosphorylated TβRI transmits intracellular signalling through phosphorylation of Smads, which are the central mediators of TGF-β signalling (*3*, *4*). Smads are subdivided into three types: receptor-activated Smads (R-Smads), common-mediator Smad (Co-Smad) and inhibitory Smads (I-Smads) (*5*). Smad2 and 3 are R-Smads that transmit TGF-β signalling. Smad2 and 3 are phosphorylated by the TβRI kinase at their carboxy-termini, and form heteromeric complexes with Smad4 (Co-Smad). The Smad complexes then translocate into the nucleus and act as transcriptional regulators of target genes through interaction with other transcription factors and transcriptional regulators. Smad7 (I-Smad), which lacks the typical MH1 domain, interferes with the activation of the R-Smads by interacting with TβRI, and competitively prevents R-Smads from being phosphorylated by TβRI. TGF-β signalling induces expression of Smad7, which provides a TGF-β-induced negative-feedback loop (*6*). Furthermore, TGF-β utilizes a multitude of intracellular signalling pathways in addition to Smads to regulate a wide array of cellular functions. These non-Smad pathways, including various branches of mitogen-activated protein kinase (MAPK) pathways, the Rho-like GTPase pathway and phosphoinositide 3-kinase (PI3K)/Akt pathway, are activated by TGF-β receptors through either phosphorylation or direct interaction (*7*).

During the progression of cancer, TGF-β acts as both an anti-oncogenic and pro-oncogenic factor (*2*, *8*, *9*). In the early stages of carcinogenesis, TGF-β signalling suppresses tumour cell growth and induces apoptosis. On the other hand, in advanced stages of cancer, cancer cells acquire resistance to the growth-inhibitory effects of TGF-β, and TGF-β induces epithelial-mesenchymal transition (EMT) and enhances tumour cell motility and the invasiveness of certain types of cancer cells, such as breast cancer cells and melanoma cells (*10*). TGF-β may thus be a useful molecular target in the treatment of advanced cancer (*11–14*).

We have previously reported that systemic gene transfer of Smad7 inhibits the metastasis of JygMC(A) cells through inhibition of EMT and suppression of cell migration (*15*). We also showed that Dec1, one of the target genes of TGF-β, contributed to the survival of JygMC(A) cells and promoted metastasis of them (*16*). However, the molecular mechanisms of TGF-β-induced cancer cell survival have not been fully elucidated, and there may be other TGF-β target genes involved in the survival of breast cancer cells. In the present study, we investigated the roles of endogenous TGF-β signalling in the apoptosis of breast cancer cells, and identified novel key molecules involved in cancer cell survival.

## Materials and Methods

### Cell culture and reagents

Mouse breast cancer cells (JygMC(A), JygMC(B) and 4T1) were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a 5% $CO_2$ atmosphere at 37°C. Recombinant TGF-β3, bone morphogenetic protein (BMP)-4, and Noggin were purchased from R&D Systems. The TβRI kinase inhibitors SB431542, A-44-03 and LY364947 were used as described previously (*16*).

### Apoptosis assays

For detection of cytosolic DNA ladder formation, both floating and adherent cells were collected and lysed with a lysis buffer [20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% Triton-X100]. Cell extracts were incubated with 0.1 mg/ml RNase A and 0.2 mg/ml Proteinase K at 42°C for 1 h. DNA was purified by standard phenol-chloroform extraction and ethanol precipitation. Dry DNA pellets were then resuspended in TE containing 0.2 mg/ml RNase A, and samples were electrophoretically separated on 2% agarose gel containing 0.01% ethidium bromide. For terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assays, cells were fixed in 3.7% formaldehyde. After permeabilization in PBS containing 0.1% Triton X-100, the *In situ* Cell Death Detection Kit TMR red (Roche Diagnostics) was used. The nuclei were stained by SYTOX Green Nucleic Acid Stain (Invitrogen). Fluorescence was examined using an Axiovert 200M microscope (Carl Zeiss) and measured with the LSM Image Browser (Carl Zeiss).

### Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed essentially as previously described (*17*). Total RNAs were extracted using Isogen reagent (Nippon Gene). First-strand cDNAs were synthesized using the PrimeScript II First Strand cDNA Synthesis (Takara) with oligo(dT) primers. Semiquantitative RT-PCR analysis was performed using the primers listed in Supplementary Fig. S1A. *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) was used to normalize the total amount of cDNA in each sample. PCR conditions were 30 cycles of 95°C (20 s), 58°C (30 s) and 72°C (1 min). PCR products were separated by electrophoresis in 1% agarose gel or in 2.5% agarose gel and then visualized with ethidium bromide. As a negative control, a PCR reaction for each set of primers was run against $H_2O$. Quantitative real-time RT-PCR analysis was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and SYBR Green reagent (Applied Biosciences). The primer sequences used are listed in Supplementary Fig. S1B. All samples were run in duplicate for each experiment. Values were normalized to *Hprt1* (hypoxanthine phosphoribosyltransferase 1).

### Immunoblotting

Immunoblotting was performed as described previously (*17*). Anti-phospho-Smad2 (Ser465/467) antibody, anti-phospho-Smad1/5 antibody (Ser463/465), anti-Smad1 antibody and anti-Bim antibody were purchased from Cell Signaling Technology. Anti-Smad2/3 antibody and anti-Foxc1 antibody were purchased from BD Transduction and Abcam, respectively. Anti-α-tubulin antibody was from Sigma. Detection of immunoblotted proteins was performed by enhanced chemiluminescence.

### RNA interference and oligonucleotides

Stealth small interfering RNA (siRNA) duplex oligoribonucleotides against mouse Bim or Foxc1 were synthesized by Invitrogen. JygMC(A) cells were transfected in the presence of 60 nM of either siRNA or control siRNA in a 500-μl volume with 20-μl Lipofectamine 2000 reagent (Invitrogen) per well of a 6-well plate according to the manufacturer's protocols. To confirm knockdown efficiency, cells were harvested 36 h after siRNA transfection and subjected to quantitative real-time RT-PCR analysis.

## Results

### Activation of TGF-β signals in highly metastatic breast cancer cells

In the present study, we examined the role of endogenous TGF-β family signalling in breast cancer cells, i.e. JygMC(A), JygMC(B) and 4T1. We first assessed the phosphorylation of Smads in these cells by immunoblot analysis (Fig. 1A). Smad2 and Smad1/5 were phosphorylated in response to TGF-β and BMP-4, respectively, in these cells. Phosphorylation of Smad2 was detected even in the absence of exogenous TGF-β, and was eliminated by treatment with the TβRI kinase inhibitor SB431542. Quantitative real-time RT-PCR analysis revealed that exogenous TGF-β induced the expression of *Serpine1* (serine peptidase inhibitor, clade E, member 1), and that BMP-4 induced that of *Id1* (inhibitor of DNA binding 1) in these cells (Fig. 1B). Treatment with SB431542 attenuated the expression of *Serpine1* gene even in the absence of exogenous ligands. Consistent with these findings, mRNAs for *Tgfb1* and *Tgfb3* were detected in these cells by semi-quantitative RT-PCR analysis (Fig. 1C). Taken together, these findings suggest that TGF-β is produced in breast cancer cells in autocrine fashion, and that endogenous TGF-β signalling is transduced in these cancer cells.

### Endogenous TGF-β protects breast cancer cells from apoptosis

To explore the roles of endogenous TGF-β signalling in the survival of breast cancer cells, we examined whether TGF-β promotes survival of JygMC(A) cells under serum-free conditions. Although JygMC(A) cells were able to survive even in the absence of serum, they died when treated with SB431542, as reported previously (Fig. 2A) (*16*). Fragmentation of DNA was assessed by TUNEL assay under serum-free conditions, and anti-apoptotic effects of TGF-β were

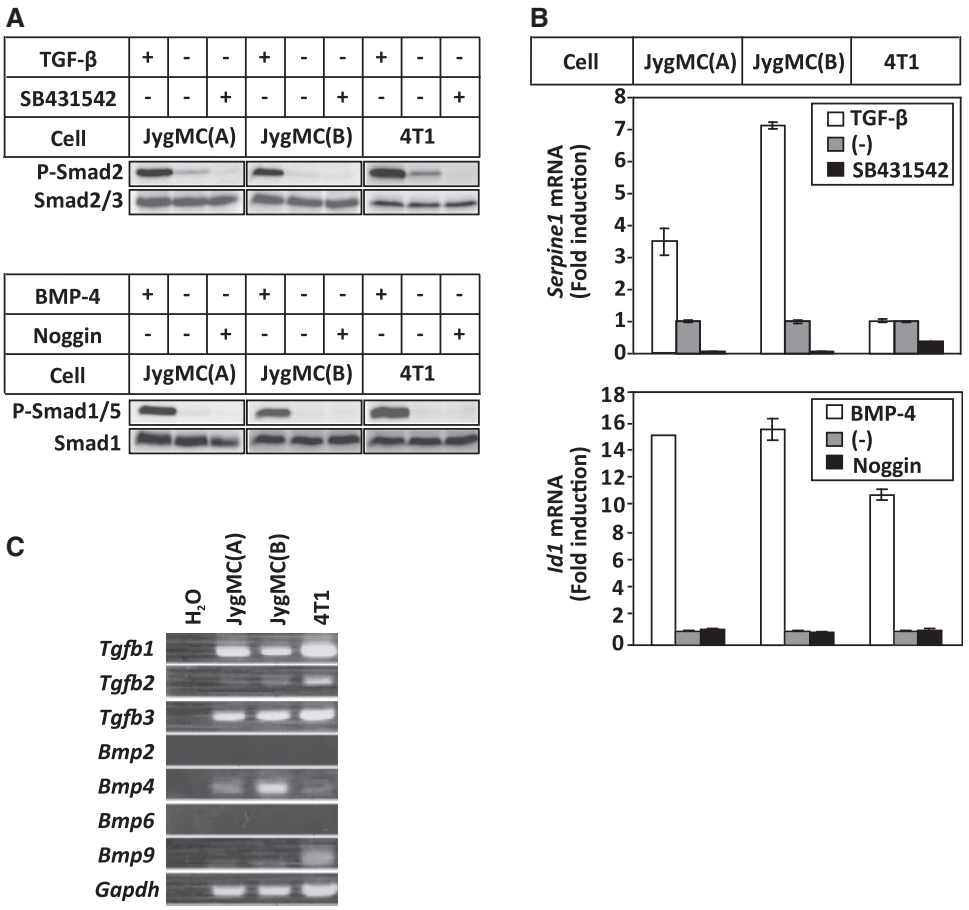



**Fig. 1 TGF-β family signals are activated in highly metastatic breast cancer cells in autocrine fashion.** (A) JygMC(A) cells, JygMC(B) cells and 4T1 cells were treated with TGF-β3 (1 ng/ml), BMP-4 (30 ng/ml), SB431542 (10 μM) or Noggin (30 ng/ml) in serum-free conditions for 1 h. Cells lysates were immunoblotted with anti-phospho-Smad2 (P-Smad2) antibody, anti-phospho-Smad1/5 (P-Smad1/5) antibody, anti-Smad2/3 antibody and anti-Smad1 antibody. (B) JygMC(A) cells, JygMC(B) cells and 4T1 cells were treated with TGF-β3 (1 ng/ml), BMP-4 (30 ng/ml), SB431542 (10 μM) or Noggin (30 ng/ml) in serum-free conditions for the indicated times. Levels of expression of *Serpine1* and *Id1* mRNA were examined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of *Hprt1*. Columns, means of duplicate determinations; bars, SDs. (C) Production of *Tgfb1*, *Tgfb2*, *Tgfb3*, *Bmp2*, *Bmp4*, *Bmp6* and *Bmp9* by JygMC(A) cells, JygMC(B) cells and 4T1 cells was examined using semi-quantitative RT-PCR analysis.

observed in all three cell lines tested (Fig. 2B and C). Cytosolic DNA ladder formation assay also revealed that apoptosis of these breast cancer cells was enhanced by SB431542 (Fig. 2D). These findings suggest that endogenous TGF-β serves as a pro-survival factor of cancer cells against apoptotic stimuli.

### *Profiling of pro-apoptotic genes regulated by TGF-β signalling in JygMC(A) cells*

To examine the alterations caused by the inhibition of TGF-β signalling in breast cancer cells, we made use of comprehensive gene expression data sets for JygMC(A) cells previously obtained using oligonucleotide microarray analysis (*16*). In that study, Dec1 was identified as an anti-apoptotic transcription factor, expression of which was sustained by endogenous TGF-β in JygMC(A) cells. However, well-known regulators of apoptosis, which were regulated by endogenous TGF-β in breast cancer cells, still remain unidentified. We therefore hypothesized that endogenous TGF-β suppressed the expression of pro-apoptotic genes, and attempted to find novel candidate genes which were involved in the induction of apoptosis and regulated by TGF-β signalling.

As shown in Fig. 3A, we purified 18 genes down-regulated by TGF-β. mRNA for Bcl2-like 11 (*Bcl2l11*, also known as Bim) was included among them. Bim was identified as a BH3-only protein, and originally reported to inactivate pro-survival proteins on the mitochondrial membrane to induce apoptosis (*18–20*). Recent reports suggested that gene deletion or epigenetic silencing of Bim expression may play a critical role in the pathogenesis of Burkitt lymphoma, and loss of Bim expression has been observed in melanoma and renal cell carcinoma (*21–23*). Moreover, metastasis of renal cell carcinoma was more strongly induced in patients who exhibited reduced expression of Bim than in those who expressed Bim well (*23*). We therefore focused on the expression of Bim in apoptosis of breast cancer cells.

### *Autocrine TGF-β protects JygMC(A) and JygMC(B) cells from apoptosis through reduction of Bim*

Since SB431542 is reported to inhibit kinases other than TβRI kinase (*24*), we first exclude the possibility

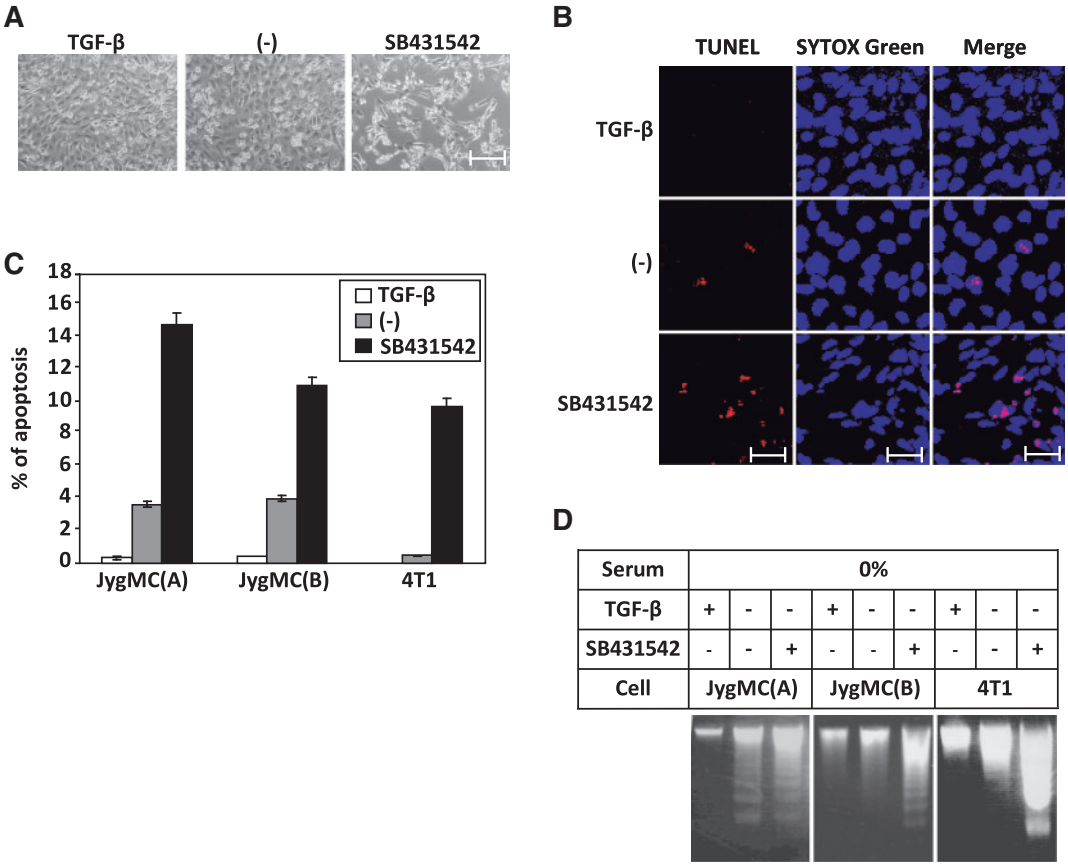



**Fig. 2 TGF-β protects breast cancer cells from apoptosis.** (A) JygMC(A) cells were treated with TGF-β3 (1 ng/ml) or SB431542 (10 μM) in serum-free conditions for 48 h. Cells were observed by phase-contrast microscopy. Scale bar, 100 μm. (B) Cells in (A) were fixed and subjected to TUNEL staining. Cell nuclei were counterstained with SYTOX Green Nucleic Acid Stain. Red, TUNEL; Blue, SYTOX Green; Scale bar, 50 μm. (C) Percentages of TUNEL-positive cells among SYTOX Green-positive cells in (B) were determined. Columns, means of ten determinations; bars, SDs. (D) JygMC(A) cells, JygMC(B) cells and 4T1 cells were treated in the same fashion as in (A), and apoptotic cell death was assessed by DNA-fragmentation assay. Characteristic DNA ladders were observed after ethidium bromide staining.

the involvement of other kinases in the regulation of the Bim expression (Fig. 3B). Quantitative real-time RT-PCR analysis revealed that two other types of TβRI kinase inhibitors, A-44-03 and LY364947, up-regulated the expression of Bim in JygMC(A) cells equally well to SB431542. We also determined the effects of TβRII/Fc chimera which inhibits TGF-β signalling (Supplementary Fig. S2). Although low molecular compounds were more potent to block the autocrine TGF-β signalling within the cells than TβRII/Fc chimera, TβRII/Fc chimera weakly up-regulated the expression of Bim in the cells. These results suggest that the effect of SB43152 was based on the inhibition of TGF-β signalling in the cells.

We next examined whether Bim is one of the down-stream targets of TGF-β in breast cancer cells, using quantitative real-time RT-PCR analysis (Fig. 3C). Expression of *Bcl2l11* mRNA was down-regulated in response to TGF-β in JygMC(A) cells and JygMC(B) cells, whereas SB431542 induced the transcription of *Bcl2l11* mRNA under serum-free conditions in these cells. In 4T1 cells, we failed to detect a correlation between TGF-β signalling and expression of *Bcl2l11* mRNA. Consistent with the regulation of *Bcl2l11* mRNA by TGF-β, downregulation of the Bim protein in JygMC(A) and JygMC(B) cells was also confirmed by immunoblot analysis (Fig. 3D).

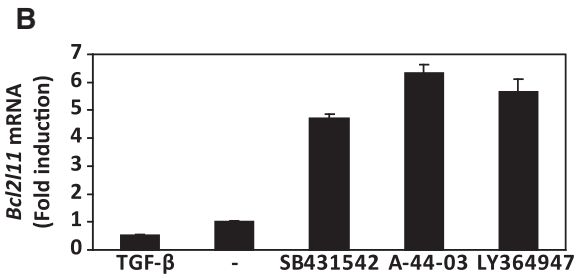
Next, to determine whether Bim plays a critical role in apoptosis of JygMC(A) cells, we knocked-down the expression of Bim in JygMC(A) cells using the siRNA method. Expression of Bim was efficiently silenced by transfection of siRNA targeting *Bcl2l11* (siRNA/Bim) (Fig. 4A), and the reduction of *Bcl2l11* mRNA resulted in decrease in the number of apoptotic cells even in the absence of TGF-β under serum-free conditions (Fig. 4B and C). These findings suggest that suppression of the pro-survival effect of TGF-β by SB431542 in breast cancer cells is dependent on the regulation of Bim expression in these cells.

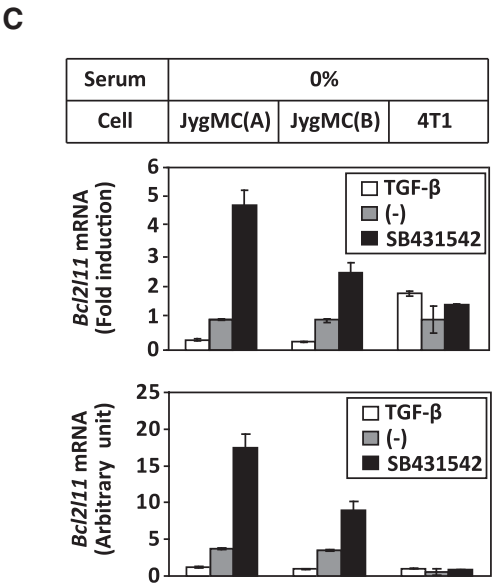
### *Erk pathway is dispensable for the transcription of Bim in JygMC(A) cells*

To determine the involvement of non-Smad signal pathways by TGF-β, JygMC(A) cells were treated with several types of MAPK inhibitors, i.e. U0126 for MAP kinase-Erk kinase (MEK)-1/2, SB203580 for p38, SP600125 for c-Jun N-terminal kinase (JNK) and LY294002 for PI3 kinase (Supplementary Fig. S3A). Among them, only phosphorylation of Erk1/2 was dependent on TGF-β signalling in these

**A**

| Probe Set | Accession | Gene | | Fold change | | | |
|---|---|---|---|---|---|---|---|
| | | Symbol | Name | LacZ/cont | Smad7/cont | Smad7/LacZ | Tb24/cont |
| 1 1415996_at | AF173681 | Txnip | thioredoxin interacting protein | 0.821 | 1.278 | 1.556 | 0.739 |
| 2 1417500_a_at | BC016492 | Tgm2 | transglutaminase 2, C polypeptide | 1.004 | 1.889 | 1.881 | 0.722 |
| 3 1419735_at | NM_007786 | Csn3 | casein kappa | 0.901 | 6.674 | 7.405 | 0.560 |
| 4 1421153_at | NM_053083 | Loxl4 | lysyl oxidase-like 4 | 0.995 | 1.691 | 1.699 | 0.652 |
| 5 1422510_at | NM_133710 | Ctdspl | CTD small phosphatase-like | 0.987 | 1.638 | 1.659 | 0.776 |
| 6 1424921_at | BC008532 | Bst2 | bone marrow stromal cell antigen 2 | 1.010 | 1.838 | 1.821 | 0.659 |
| 7 1427447_a_at | BC003984 | Triobp | TRIO and F-actin binding protein | 1.014 | 1.795 | 1.770 | 0.736 |
| 8 1433428_x_at | AW321975 | Tgm2 | transglutaminase 2, C polypeptide | 0.993 | 1.517 | 1.529 | 0.665 |
| 9 1435448_at | BM120925 | Bcl2l11 | BCL2-like 11 (apoptosis facilitator) | 0.872 | 1.799 | 2.063 | 0.787 |
| 10 1437277_x_at | BB550124 | Tgm2 | transglutaminase 2, C polypeptide | 0.940 | 1.428 | 1.520 | 0.793 |
| 11 1448830_at | NM_013642 | Dusp1 | dual specificity phosphatase 1 | 0.964 | 1.723 | 1.787 | 0.548 |
| 12 1448991_a_at | BC018383 | --- | --- | 1.038 | 1.877 | 1.809 | 0.634 |
| 13 1450264_a_at | NM_013490 | Chka | choline kinase alpha | 1.172 | 2.440 | 2.081 | 0.684 |
| 14 1455900_x_at | BB041811 | Tgm2 | transglutaminase 2, C polypeptide | 1.079 | 1.764 | 1.635 | 0.765 |
| 15 1460287_at | M93954 | Timp2 | tissue inhibitor of metalloproteinase 2 | 0.890 | 1.541 | 1.731 | 0.694 |
| 16 1429863_at | AK016522 | Lonrf3 | ring finger protein 127 | 1.301 | 2.998 | 2.305 | 1.048 |
| 17 1436200_at | BE956940 | A830039N02Rik | RIKEN cDNA A830039N02 gene | 1.039 | 2.360 | 2.272 | 0.756 |
| 18 1443832_s_at | AV064339 | Sdpr | serum deprivation response | 1.270 | 2.824 | 2.223 | 0.795 |



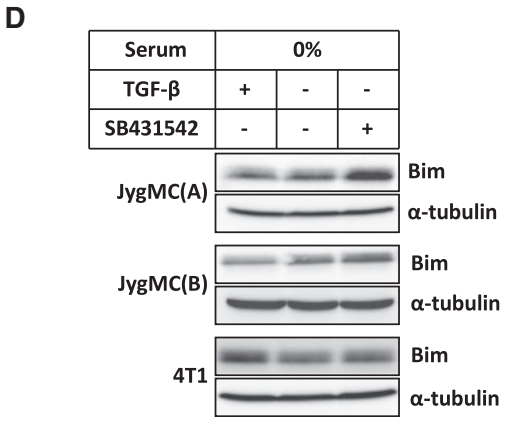




**Fig. 3 Bim is negatively regulated by TGF-β in breast cancer cells.** (A) Total RNAs were extracted from JygMC(A) cells treated as follows: untreated (cont), treated with TGF-β3 (1 ng/ml) for 24 h (Tb24), transduced with *LacZ* gene (LacZ) and transduced with *Smad7* gene (Smad7). Total RNAs were used to prepare cDNA and subjected to oligonucleotide microarray analysis using GeneChip Mouse Genome 430 2.0 Array (Affymetrix). Three statistical criteria were applied to the 45 102 genes analysed: (i) the signal intensities in control and LacZ were >50. (ii) Signal intensities were decreased by Tb24 by <0.3-fold compared to control or LacZ. (iii) Signal intensities were increased by Smad7 by >0.7-fold compared to control or LacZ. All of the 18 of 45,102 genes that met these criteria are listed. (B) JygMC(A) cells were treated with TGF-β3 (1 ng/ml), SB431542 (10 μM), A-44-03 (1 μM) or LY364947 (1 μM) in serum-free conditions for 24 h. Levels of expression of *Bcl2l11* mRNA were examined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of *Hprt1*. Columns, means of duplicate determinations; bars, SDs. (C) JygMC(A) cells, JygMC(B) cells and 4T1 cells were treated with TGF-β3 (1 ng/ml) or SB431542 (10 μM) in serum-free conditions for 24 h. Expression of *Bcl2l11* mRNA was examined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of *Hprt1*. Columns, means of duplicate determinations; bars, SDs. (D) JygMC(A) cells, JygMC(B) cells and 4T1 cells were treated in the same fashion as in (C). Cell lysates were subjected to 12% SDS-gel electrophoresis, and immunoblotted with anti-Bim antibody.

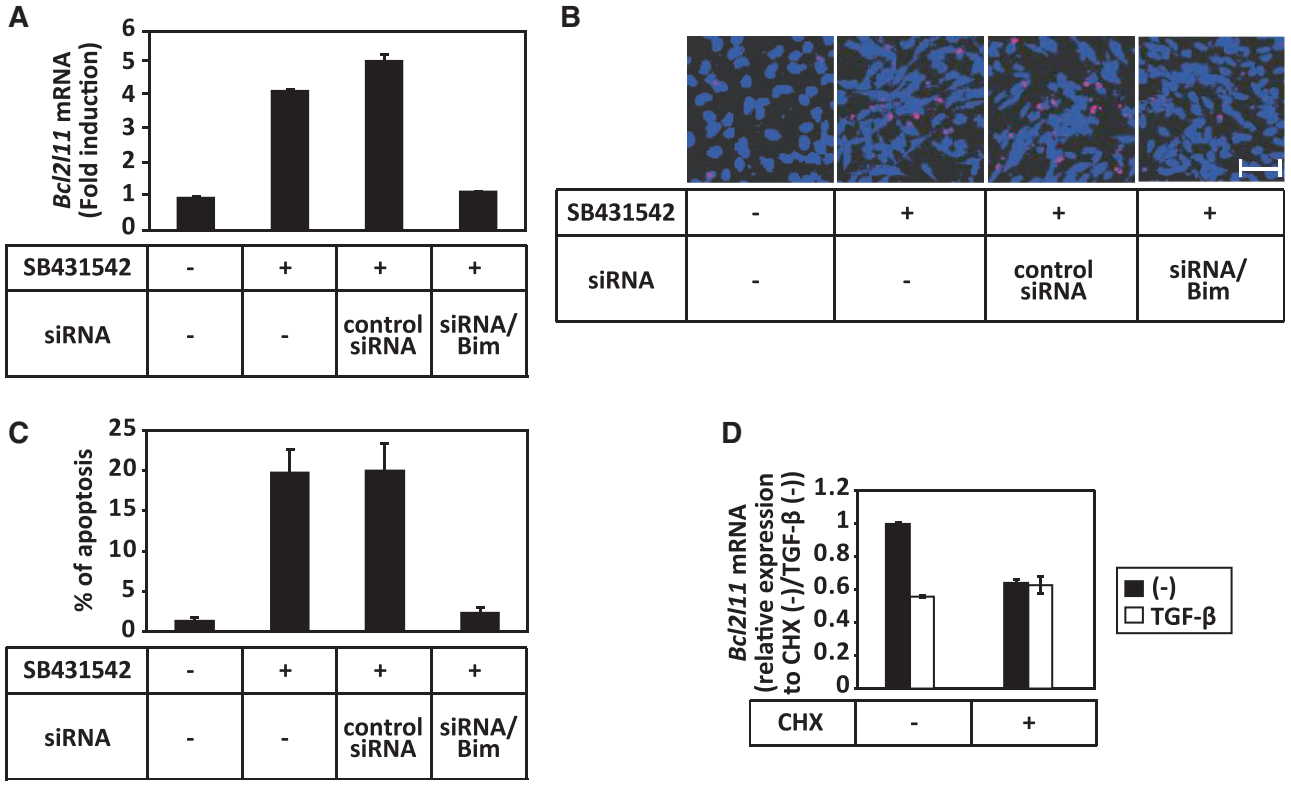



**Fig. 4 Autocrine TGF-β protects breast cancer cells from apoptosis through the reduction of Bim.** (A) JygMC(A) cells were transfected with control siRNA or Bim-targeting siRNA (siRNA/Bim), and levels of expression of *Bcl2l11* mRNA were determined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of *Hprt1*. Columns, means of duplicate determinations; bars, SDs. (B) Twelve hour after transfection, cells were cultured in serum-free medium with SB431542 (10 μM) for 48 h. Cells were then fixed and subjected to TUNEL staining. Cell nuclei were counterstained with SYTOX Green Nucleic Acid Stain. Red, TUNEL; Blue, SYTOX Green; Scale bar, 50 μm. (C) Percentages of TUNEL-positive cells among SYTOX Green-positive cells in (B) were determined. Columns, means of 10 determinations; bars, SDs. (D) JygMC(A) cells were pre-treated with CHX (2 mg/ml) in serum-free conditions for 2 h, and then cells were treated with TGF-β3 (1 ng/ml) for 22 h. Levels of expression of *Bcl2l11* mRNA were examined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of *Hprt1*. Columns, means of duplicate determinations; bars, SDs.

cells. We therefore further examined whether the Erk signal cascade is involved in the regulation of Bim expression. However, quantitative real-time RT-PCR analysis revealed that U0126 had no effect on the transcription of *Bcl2l11* mRNA in JygMC(A) cells (Supplementary Fig. S3B). Thus, Erk pathways downstream of TGF-β did not appear to participate in the regulation of Bim expression in these cells.

Next, to determine whether Bim is a direct target of TGF-β in JygMC(A) cells, we examined the expression of Bim in the presence of cycloheximide (CHX). Treatment of JygMC(A) cells with CHX resulted in decreased expression of *Bcl2l11* mRNA to the extent similar to that after TGF-β treatment (Fig. 4D). Stimulation with TGF-β in the presence of CHX did not cause further down-regulation of *Bcl2l11* mRNA. Taken together, these findings suggest that TGF-β-induced Bim suppression in JygMC(A) cells is mediated by repression of certain constitutively expressed endogenous protein(s).

### *Foxc1 is a target of TGF-β in breast cancer cells*

To identify the endogenously expressed protein(s) that regulates the transcription of *Bcl2l11* mRNA under the control of TGF-β signalling in breast cancer cells, we again made use of the comprehensive gene expression data sets (*16*). As shown in Fig. 5A, we purified 17 genes down-regulated by TGF-β in the early phase (2 h after TGF-β stimulation). Among them, mRNA for the transcription factor, Forkhead box C1 (*Foxc1*), was included. Foxc1 belongs to the FOX family, and has been identified as an important factor in development, while its role in the apoptosis of cancer cells has yet to be determined (*25*). To determine whether Foxc1 is one of the factors regulating the expression of *Bcl2l11* mRNA downstream of TGF-β signalling in breast cancer cells, quantitative real-time RT-PCR analysis was performed (Fig. 5B). Expression of *Foxc1* mRNA was down-regulated in response to TGF-β in JygMC(A) cells and JygMC(B) cells, whereas SB431542 activated the transcription of *Foxc1* mRNA under serum-free conditions in these cells. Since the expression of *Foxc1* mRNA in 4T1 cells was not suppressed by TGF-β, the TGF-β-Foxc1-Bim axis appears to be important only in certain cell types, e.g. JygMC(A) cells and JygMC(B) cells.

### *Regulation of Bim transcription by Foxc1 in JygMC(A) cells*

Finally, to determine whether Foxc1 regulates the expression of Bim in JygMC(A) cells, we knocked

**A**

| Probe Set | Accession | Gene | | Fold change | |
|---|---|---|---|---|---|
| | | Symbol | Name | Tb2/cont | Tb24/cont |
| 1 1417395_at | BG069413 | Klf4 | Kruppel-like factor 4 (gut) | 0.688 | 0.695 |
| 2 1419486_at | BB759833 | Foxc1 | forkhead box C1 | 0.627 | 0.611 |
| 3 1422782_s_at | NM_126166 | Tlr3 | toll-like receptor 3 | 0.658 | 0.696 |
| 4 1423017_a_at | NM_031167 | Il1rn | interleukin 1 receptor antagonist | 0.608 | 0.627 |
| 5 1423450_a_at | AV226060 | Hs3st1 | heparan sulfate (glucosamine) 3-O-sulfotransferase 1 | 0.526 | 0.371 |
| 6 1423891_at | BC003903 | Gstt3 | glutathione S-transferase, theta 3 | 0.784 | 0.708 |
| 7 1424647_at | BC027245 | Gabrp | gamma-aminobutyric acid (GABA-A) receptor, pi | 0.549 | 0.358 |
| 8 1424647_at | BC027245 | Gabrp | gamma-aminobutyric acid (GABA-A) receptor, pi | 0.583 | 0.421 |
| 9 1425416_s_at | BC008994 | Psrc1 | proline/serine-rich coiled-coil 1 | 0.600 | 0.676 |
| 10 1425675_s_at | M77196 | Ceacam1 | CEA-related cell adhesion molecule 1 | 0.677 | 0.518 |
| 11 1427443_at | BF162286 | --- | --- | 0.691 | 0.532 |
| 12 1448494_at | BB550400 | --- | --- | 0.515 | 0.459 |
| 13 1449146_at | NM_010929 | Notch4 | Notch gene homolog 4 (Drosophila) | 0.601 | 0.442 |
| 14 1450784_at | NM_016678 | --- | --- | 0.658 | 0.511 |
| 15 1433837_at | AV365503 | 8430408G22Rik | RIKEN cDNA 8430408G22 gene | 0.547 | 0.831 |
| 16 1433868_at | AV028445 | Btbd3 | BTB (POZ) domain containing 3 | 0.642 | 0.720 |
| 17 1453448_at | AK010086 | 2310067E19Rik | RIKEN cDNA 2310067E19 gene | 0.715 | 0.661 |

**B**

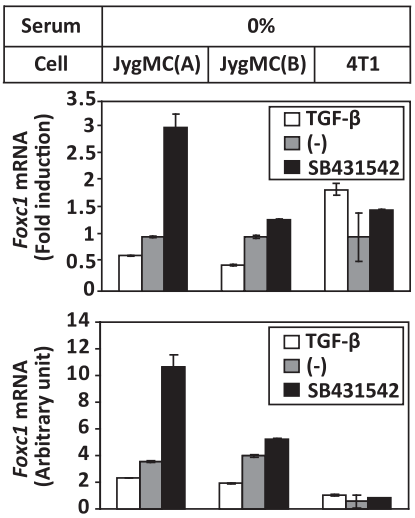



**Fig. 5 Expression of Bim in breast cancer cells is regulated by constitutively expressed endogenous protein.** (A) JygMC(A) cells were treated as follows: untreated (cont), treated with TGF-β3 (1ng/ml) for 2 h (Tb2), and treated with TGF-β3 (1 ng/ml) for 24 h (Tb24). Oligonucleotide microarray analysis was then performed as described in Fig. 3A. Three statistical criteria were applied to the 45,102 genes analysed: (i) the signal intensities in control were >50. (ii) Signal intensities were decreased by Tb2 by <0.3-fold compared to control. (iii) Signal intensities were decreased by Tb24 by <0.3-fold compared to control. The 17 of 45,102 genes that met these criteria are listed. (B) JygMC(A) cells, JygMC(B) cells and 4T1 cells were treated with TGF-β3 (1 ng/ml) or SB431542 (10 μM) in serum-free conditions for 24 h. Levels of expression of *Foxc1* mRNA were examined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of *Hprt1*. Columns, means of duplicate determinations; bars, SDs.

down endogenous *Foxc1* mRNA by siRNA-targeting Foxc1 (siRNA/Foxc1). As shown in Fig. 6A, we efficiently knocked down the expression of endogenous *Foxc1* mRNA in JygMC(A) cells in the absence of TGF-β under serum-free conditions. Interestingly, induction of *Bcl2l11* mRNA was attenuated in the *Foxc1* knocked-down JygMC(A) cells compared to those treated with control siRNA. Furthermore, reduction of *Foxc1* mRNA resulted in decrease in the number of apoptotic cells in the absence of TGF-β under serum-free conditions (Fig. 6B and C). These findings indicate that the expression of Foxc1 is down-regulated by TGF-β, and that Foxc1 positively regulates the expression of Bim in JygMC(A) cells under serum-free conditions (Fig. 6D).

## Discussion

In the present study, we showed that survival of JygMC(A), JygMC(B) and 4T1 breast cancer cells was sustained by autocrine TGF-β signalling. We also demonstrated that the TβRI kinase inhibitor SB431542 effectively induced apoptosis of breast cancer cells through induction of the pro-apoptotic protein Bim in JygMC(A) cells and JygMC(B) cells.

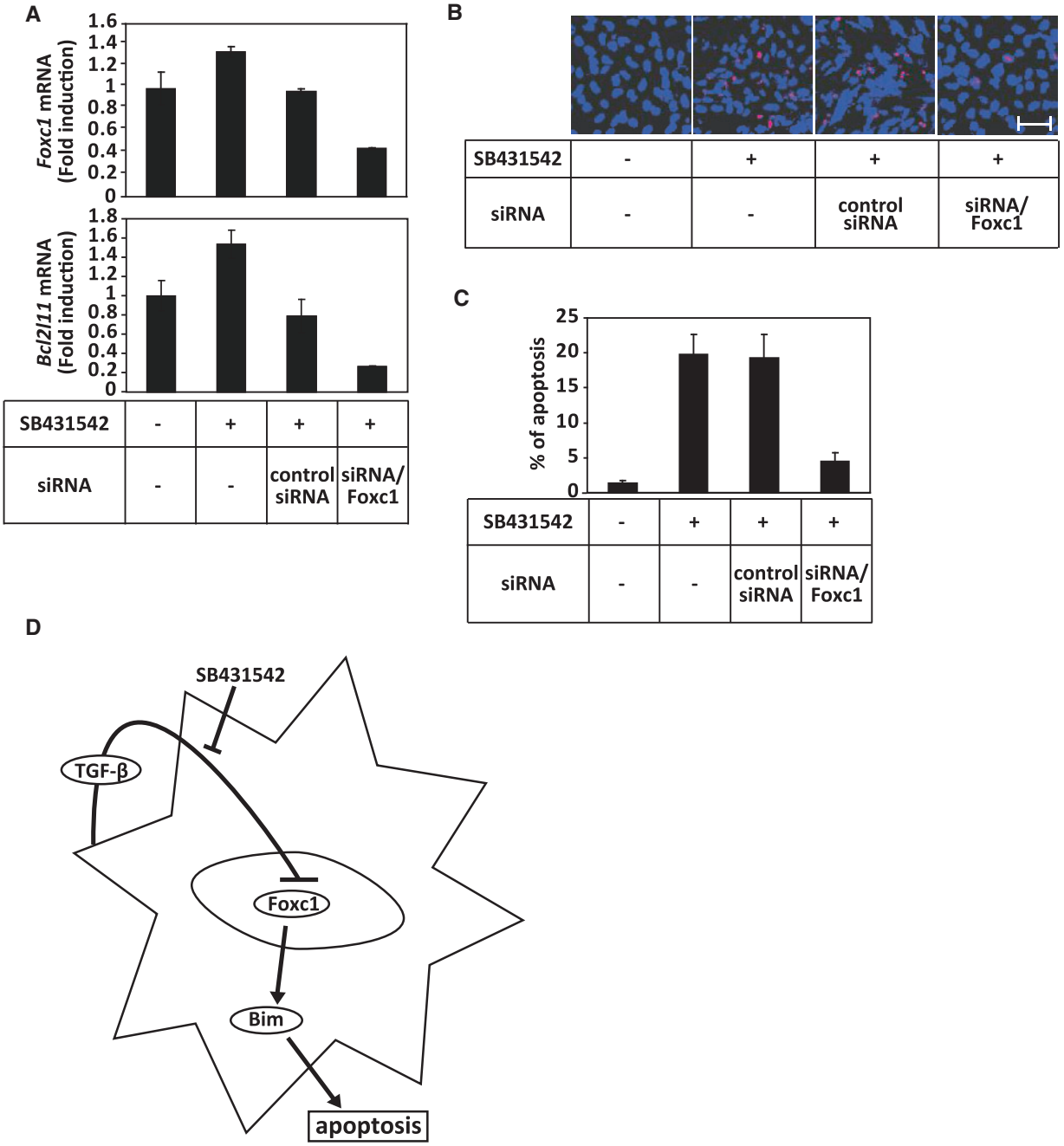



**Fig. 6 Foxc1 may regulate Bim transcription in JygMC(A) cells.** (A) JygMC(A) cells were transfected with control siRNA or Foxc1-targeting siRNA (siRNA/Foxc1), and levels of expression of *Foxc1* and *Bcl2l11* mRNAs were determined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of *Hprt1*. Columns, means of duplicate determinations; bars, SDs. (B) Twelve hour after transfection, cells were cultured in serum-free medium with SB431542 (10 μM) for 48 h. Cells were then fixed and subjected to TUNEL staining. Cell nuclei were counterstained with SYTOX Green Nucleic Acid Stain. Red, TUNEL; Blue, SYTOX Green; Scale bar, 50 μm. (C) Percentages of TUNEL-positive cells among SYTOX Green-positive cells in (B) were determined. Columns, means of 10 determinations; bars, SDs. (D) Schematic representation of the TGF-β-Foxc1-Bim pathway for induction of breast cancer cell survival.

### *Roles of autocrine TGF-β signalling in the survival of breast cancer cells*

TGF-β acts as a tumour suppressor in the early stage of cancer progression, and inhibits the proliferation and induces the apoptosis of epithelial cells (*8*, *9*). However, TGF-β induces the survival of certain types of cancer cells (*26–28*). The reasons for the differences in these opposing effects of TGF-β on cell survival have yet to be fully determined, and we hypothesized that endogenous TGF-β exhibits different effects on cell survival compared to exogenously added TGF-β. In the present study, semi-quantitative RT-PCR analysis revealed that cancer cells produced TGF-β1 and TGF-β3, and that well-known target genes of TGF-β were expressed even in the absence of exogenous TGF-β stimulation (Fig. 1B and C). These findings strongly suggest that TGF-β signalling is activated in autocrine fashion, and we therefore

explored the function of endogenous TGF-β signalling in highly metastatic breast cancer cells. Since pharmacological inhibition of endogenous TGF-β signalling in breast cancer cells uniformly caused cell death (Fig. 2), it appeared that cancer cells acquire the ability to produce TGF-β to protect themselves from apoptotic stimuli.

Neutralizing antibodies to TGF-β and small molecule inhibitors, including SB431542, that inhibit the kinase activity of TβRI have been generated, and have been reported to inhibit the metastasis of certain types of cancer (*11–14*). We previously reported that inhibition of TGF-β signalling by systemic administration of adenoviruses carrying Smad7 or c-Ski cDNA resulted in prevention of metastasis of JygMC(A) cells (*15*). Interestingly, growth of primary tumours subcutaneously inoculated in mice was not significantly affected by the administration of Smad7 or c-Ski adenoviruses. Furthermore, high-throughput immunoblot analysis demonstrated increased expression of major components of adherens junctions and tight junctions, as well as decrease in migratory and invasive capacities of breast cancer cells infected with the Smad7 adenovirus. Although inhibition of EMT might be among the most important mechanisms of prevention of metastasis by TGF-β antagonists, we found that inhibitors of TGF-β signalling, such as SB431542 or A-44-03, also induced apoptosis of cancer cells. In the present study, we investigated the apoptotic signal pathway in JygMC(A) cells, and found that SB431542 promotes the transcription of the pro-apoptotic gene Bim and induces apoptosis of these cells under certain conditions. These findings thus strongly suggest that inhibition of TGF-β signalling leads to prevention of cancer metastasis by the induction of apoptosis, as well as by the inhibition of cell invasion.

Recently, inhibition of BMP signalling in cancer cells was found to diminish invasion and metastasis of various types of cancer cells (*29–35*). Specific inhibitors of BMP signalling, such as LDN-193189, have been developed (*36*). However, in contrast to TGF-β signalling, inhibition of BMP signalling in JygMC(A) cells by treatment with Noggin failed to significantly affect survival of these cells (Y. Hoshino *et al*., unpublished results). SB431542 potently inhibits the transcriptional activity induced by TβRI [activin receptor-like kinase (ALK)-5] as well as that by activin type IB receptor (ALK-4) and ALK-7, the kinase domains of which are structurally related to that of ALK-5 (*37*). However, in our previous study, we demonstrated that forced expression of dominant-negative TβRII in JygMC(A) cells resulted in an increase in number of apoptotic cells (*16*). Thus, among the many types of TGF-β family ligands, TGF-β may be the major cytokine promoting the survival of breast cancer cells.

### *Function of TGF-β-induced Foxc1 in apoptosis of cancer cells*

Transcriptional repressor Dec1 was previously reported as a downstream target of TGF-β signalling which promotes the survival of breast cancer cells (*16*). We also identify Bim as an inducer of apoptosis in breast cancer cells using microarray analysis in this study. Ohgushi *et al*. also reported that TGF-β mediates physiological apoptosis in gastric epithelial cells through the induction of Bim (*38*). Thus, we initially attempted to examine the association between Dec1 and Bim in JygMC(A) cells. However, we found that overexpression of Dec1 in JygMC(A) cells did not affect the expression of Bim, and that overexpression of Foxc1 did not alter the expression level of *Dec1* mRNA (Y. Hoshino, unpublished results). TGF-β-Dec1 pathway and TGF-β-Foxc-Bim pathway might contribute to the survival of JygMC(A) cells independently.

In the present study, Foxc1 was extracted using microarray analysis as a candidate gene regulating the expression of Bim (Fig. 5A). Foxc1 is a member of the forkhead family of transcription factors, characterized by their molecular arrangement of two wings connecting β strands flanking one of three α helices (*39*). This helix-turn-helix structure comprises the evolutionarily conserved forkhead domain of 110 amino acids through which the FOX proteins are able to interact with DNA and translocate to cell nuclei (*40*). *Foxc1*$^{-/-}$ homozygotes die peri- or post-natally due to hemorrhagic hydrocephalus, skeletal hypoplasia and eye defects, including malformation of the eyelids and cornea and hypoplasia of the iris (*25*, *41*, *42*). Moreover, Foxc1 plays a crucial role in eye development that depends on TGF-β signalling and promotes the survival of corneal endothelial cells (*43*).

Recently, the roles of Foxc1 in the progression of cancer have been documented. Zhou *et al*. reported that the expression of Foxc1 in endometrial cancer cells and ovarian cancer cells is positively regulated by TGF-β1 and induces cell cycle arrest in these cells (*44*). Methylation of the Foxc1 promoter has been recognized as a therapeutic biomarker for breast cancer (*45*, *46*). Ray *et al*. recently reported that expression of Foxc1 in basal-like breast cancer cells is an important negative prognostic factor, and that it accelerates cell motility and invasion (*47*). This is in contrast to our finding that expression of Foxc1 in JygMC(A) cells promotes apoptosis of these cells (Fig. 6B and C). These differences in findings may be due to differences in cell types tested and/or experimental conditions. Since Foxc1 forms transcriptional complexes with different factors, and regulates the expression of many target genes, it may exhibit various biological responses in context-dependent fashion.

In the present study, we demonstrated that silencing of *Foxc1* mRNA in JygMC(A) cells resulted in reduction of *Bcl2l11* mRNA, which promoted cell survival even in serum-free conditions (Fig. 6). We also examined whether forced expression of Foxc1 in JygMC(A) cells increased Bim expression. However, adenoviral gene transfer of *Foxc1* to JygMC(A) cells did not decrease the level of expression of Bim in cells (Y. Hoshino, unpublished results). Since Foxo3a, a member of the Fox family of transcription factors, could activate target genes in cooperation with Runt related transcription factor (RUNX) (*48*), it appeared that up-regulation of *Bcl2l11* mRNA in apoptotic cells might require the recruitment of another unidentified transcription factor, and that

induction of apoptotic signal is carefully regulated by complex mechanisms.

In conclusion, the findings of the present study show that endogenous TGF-β signalling is transduced in breast cancer cells, and that it aids the survival of cancer cells through the TGF-β-Foxc1-Bim pathway (Fig. 6D). Inhibitor of TGF-β up-regulated Bim expression in breast cancer cells, and effectively induced apoptosis of these cells. SB431542 or related chemicals might thus be useful in the treatment of certain types of breast cancer.

## Supplementary Data

Supplementary Data are available at *JB* online.

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### Conflict of interest

None declared.

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