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Original Article

Inhibition of epithelial to mesenchymal transition in metastatic breast carcinoma cells by c-Src suppression

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The aberrant activation of c-Src regulates multiple functions during tumor progression. This study was conducted to investigate the role of c-Src suppression in epithelial to mesenchymal transition (EMT) process in human breast carcinoma cells. c-Src suppression by PP2 (a Src-family kinase inhibitor) or small interfering RNA (siRNA) was carried out in MCF-7 and MDA-MB-231 cells. Cell migration was analyzed by wound-healing assay. The transcription and protein levels of EMT markers and transcription factors were evaluated by reverse transcription-PCR and Western blot analysis, respectively. The changed cell morphology was photographed by light microscope. c-Src suppression by PP2 or siRNA reversed the mesenchymal-like phenotype in MDA-MB-231 cells. E-cadherin was upregulated in MCF-7 and MDA-MB-231 cells after c-Src suppression, whereas vimentin was downregulated in MDA-MB-231 cells. Slug and SIP1 were downregulated after c-Src suppression in MCF-7 and MDA-MB-231 cells, whereas Twist was unchanged. These results suggest that c-Src suppression by PP2 or siRNA may inhibit EMT through regulation of different transcription factors in breast carcinoma cells that have different metastatic potential.

Keywords c-Src; PP2; siRNA; breast carcinoma; epithelial to mesenchymal transition

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Introduction

Epithelial to mesenchymal transition (EMT) is one of the key processes involved in cancer invasion and metastasis. During this process, epithelial cancer cells lose cell–cell contact and planar polarity, and acquire mesenchymal cell phenotypes, including high motility, invasiveness, and elevated resistance to apoptosis [1,2]. These cancer cells release from their neighbors, breach the basement membrane barrier, and invade neighboring tissues [3]. Loss of the epithelial cell adhesion marker E-cadherin and acquisition of the mesenchymal marker vimentin are the important hallmarks of EMT. Several transcription factors, including Slug, SIP1, and Twist have been reported to be involved in the regulation of EMT [4,5]. Expression and activation of these transcription factors could repress transcription of E-cadherin and induce EMT with the help of other transcription factors.

c-Src is a 60-kDa non-receptor tyrosine kinase and its activation is significantly associated with tumor progression and aggressive features [6–8]. Tumors with high phospho-Src (Y419) were found to be poorly differentiated and were characterized by finger-like invasive fronts [9]. Recent studies demonstrated involvement of c-Src activation in the downregulation of E-cadherin and EMT transformations [10,11]. As a selective c-Src inhibitor [12], PP2 has been reported to induce c-Src inactivation and E-cadherin upregulation in hepatocellular carcinoma cells, and head and neck squamous carcinoma cells [9,13,14]. PP2 was also shown to abrogate SIP1 upregulation induced by endoplasmic reticulum stress in the thyroid cell line PC-C13 [15]. The effect of PP2 on breast carcinoma EMT is not well understood.

Based on estrogen receptor expression, cellular phenotype (i.e. epithelial as opposed to mesenchymal markers expression), and invasion capacity breast cancer cell lines can be divided into epithelial-like cells of low invasion capacity, such as T47D and MCF-7, or mesenchymal-like cells exhibiting high invasion capacity, such as MDA-MB-231 [16]. Here, we used MCF-7 (low metastatic potential) and MDA-MB-231 (high metastatic potential) cells to study the relationship between c-Src suppression and EMT, and to provide insights into possible mechanisms.

Materials and Methods

Cell culture

All cells were obtained from American Type Culture Collection (Manassas, USA). Cells were seeded at an

initial concentration of 10^5 cells/mL in DMEM (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Pittsburgh, USA), 3.75 g/L sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

Wound-healing assay

Cells were plated on the coverslips placed in six-well culture plates (Costar, Corning, USA) in complete culture medium and grown to confluence. A wound was made by scrapping with a sterilized 200 μl pipette tip in the middle of the cell monolayer. Cells were then cultured with fresh complete culture medium containing DMSO as control or with 10 μM PP2 for 12 h. After that, complete culture medium was changed without PP2 and cells were allowed to migrate to denuded area for 6 h. Cell migration was visualized at 100× magnification and photographed using Carl Zeiss (Berlin, Germany) light microscope.

Reverse transcription–PCR (RT–PCR) analysis

Total RNA was extracted by Trizol reagent (Invitrogen) based on the suggested protocol. RNA (5 μg) was used to synthesize the first-strand cDNA with the superscript first-strand synthesis system (Invitrogen) for RT–PCR according to the manufacturer's recommendation. Gene-specific primers for RT–PCR analyses were synthesized commercially by AuGCT Biotechnology (Beijing, China) as shown in **Table 1**.

Western blot analysis

Cell lysates (20 μg) of each sample were separated by 12.5% SDS–PAGE. Proteins were transferred onto PVDF membrane (Millipore, Billerica, USA) and incubated with primary antibodies at optimal dilution at 4°C overnight, followed by incubating with secondary antibody (according to different primary antibodies, HRP-conjugated goat anti-mouse, anti-rabbit, and rabbit anti-goat IgG were used, respectively, Jackson ImmunoResearch, West Grove, USA) at 37°C for 1 h. The immunoblot was visualized with Enhanced chemiluminescence detection kit (Vigorous, Beijing, China).

Polyclonal antibodies to phospho-Src (Y419), phospho-FAK (Y576/577), and FAK (focal adhesion kinase) were obtained from Cell Signaling Technologies (Cambridge, USA). Monoclonal antibodies to c-Src and vimentin were obtained from Cell Signaling Technologies. Monoclonal antibody to Slug was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Polyclonal antibodies to E-cadherin and SIP1 were obtained from Santa Cruz Biotechnology. Monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Proteintech Group (Chicago, USA).

c-Src silencing by RNA interference (RNAi)

Small interfering RNAs (siRNA) were synthesized by Genepharma Company (Shanghai, China). The target sequences for c-Src siRNAs were: 5′-AACAAGAGCAA GCCCAAGGAT-3′ (52–71 bp) and 5′-AAGCACTACAA GATCCGCAAG-3′ (607–628 bp). Scramble siRNA duplexes were used as a negative control. Cells were transfected with 100 nM *c-Src* siRNAs or control siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the instructions of the manufacturers, and transfection was carried out for 4 h. After that, culture medium was replaced with complete DMEM supplemented with 10% FBS. Cells were collected after transfection for 48 h and silencing effects were evaluated by Western blot analysis. The morphology of cells was visualized at 200× magnification and

Table 1 Primer sequences used in this paper

Gene	Accession No.	Primer sequence (5′ → 3′)
E-cadherin	NM 004360.3	(F) TTCCTCCCAATACATCTCCC
		(R) TTGATTTTGTAGTCACCCACC
Vimentin	NM 003380.2	(F) CTCTTCCAAACTTTTCCTCCC
		(R) AGTTTCGTTGATAACCTGTCC
Slug	NM 003068.3	(F) CGCCTCCAAAAAGCCAAAC
		(R) CGGTAGTCCACACAGTGATG
Twist	NM 000474.3	(F) GGAGTCCGCAGTCTTACGAG
		(R) TCTGGAGGACCTGGTAGAGG
SIP1	NM 014795.2	(F) AGTCCATGCGAACTGCCATCTGAT
		(R) CTGGACCATCTACAGAGGCTTGTA
GAPDH	NM 002046.3	(F) GGGAGCCAAAAGGGTCATCATC
		(R) CCATGCCAGTGAGCTTCCCGTTC

F, forward primer; R, reverse primer.

photographed using Carl Zeiss light microscope. All the experiments were repeated at least three times.

Statistical analysis

All statistical analyses were performed using SigmaStat software (version 3.5). Differences between different groups were assessed by one way ANOVA. $*P < 0.05$ was considered to indicate statistical significance.

Results

PP2 inhibits phosphorylation of c-Src in breast carcinoma cells

Previous research indicated that PP2 inhibited cell proliferation in a dose-dependent manner, but we detected no significant difference in cell viability when treated with 10 μM PP2 (data not shown). Thus, MCF-7 and MDA-MB-231 cells were treated with 10 μM PP2 for the subsequent experiments. Phosphorylation of c-Src was greatly inhibited by PP2 in MCF-7 and MDA-MB-231 cells [**Fig. 1**(**A**)]. In contrast, the total c-Src levels did not change after PP2 treatment.

PP2 inhibits migration of MCF-7 and MDA-MB-231 cells

A wound-healing assay was carried out to assess how PP2 affected the migration of cells. After treatment with 10 μM PP2 for 12 h, cells were allowed to migrate into the denuded areas for 6 h. The migration in both cell lines was inhibited, as fewer cells migrated to the wound after 10 μM PP2 treatment compared with the control [**Fig. 1**(**B**)]. It is well known that FAK expression and activation is linked to migratory activity and its phosphorylation at Y576/577 by c-Src is important in mediating downstream signal pathways [17]. PP2 inhibited phosphorylation of FAK in both cell lines [**Fig. 1**(**C**)].

Changes in the expression of epithelial and mesenchymal markers caused by PP2 treatment

In the wound-healing assay, high metastatic MDA-MB-231 cells became compact and tended to cluster with each other after PP2 treatment. But low metastatic MCF-7 cells treated with the same concentration of PP2 maintained the same epithelial morphology [**Fig. 1**(**B**)]. The morphological changes indicated a possible role of PP2 in EMT process in MDA-MB-231 cells, but not in MCF-7 cells. We further examined the expression of the epithelial marker E-cadherin and the mesenchymal marker vimentin after PP2 treatment in the two breast carcinoma cell lines. The human embryonic kidney epithelial cell line HEK293 was used as a control epithelial cell. PP2 significantly induced E-cadherin at the transcriptional and protein levels in MCF-7 and MDA-MB-231 cells, but PP2 reduced

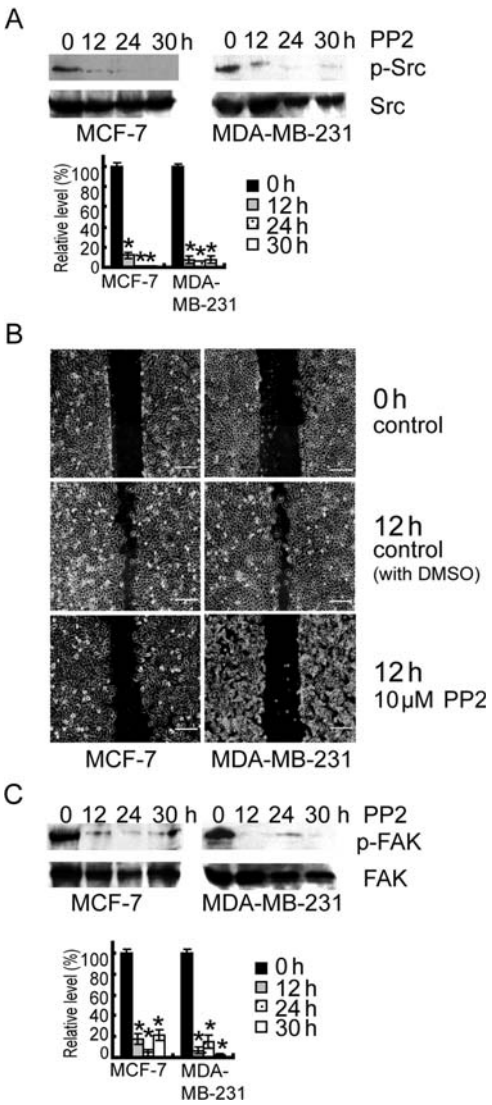

Figure 1 PP2 inhibited phosphorylation of c-Src and cell migration (A) Western blot analysis of phospho-Src (p-Src) and c-Src after PP2 treatment in MCF-7 and MDA-MB-231 cells. Cells were collected before or after 10 μM PP2 treatment for 12, 24, and 30 h. Total protein lysates (20 μg) were separated by SDS–PAGE followed by Western blot analysis using antibodies specific to phospho-Src (Y419) and c-Src. (B) A wound-healing assay was performed to determine the effect of PP2 on cell migration. After treatment with 10 μM PP2 for 12 h, cells were allowed to migrate to denuded area for 6 h. Cell migration was visualized at 100× magnification and photographed with Carl Zeiss light microscope. Scale bar = 100 μm. Results are the representative of at least three separate experiments. (C) Western blot analysis of phospho-FAK (p-FAK) and FAK after PP2 treatment in MCF-7 and MDA-MB-231 cells. Western blot results were quantified by densitometry analysis. Data were calculated as the mean optical density of treated samples as a percent of untreated cells normalized against the total protein level of c-Src and FAK, respectively.

E-cadherin in HEK293 cells [**Fig. 2**(**A**,**B**)]. In addition, vimentin was downregulated after PP2 treatment in MDA-MB-231 cells, whereas it was undetectable in MCF-7 and unchanged in HEK293 [**Fig. 2**(**A**,**B**)].

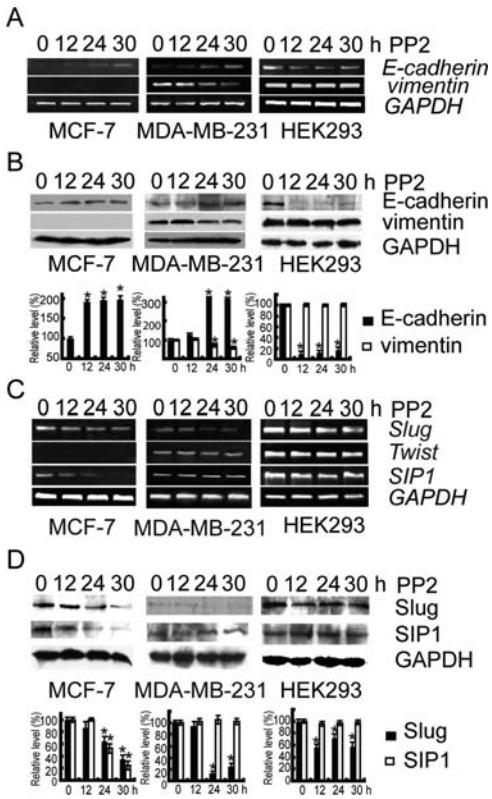

Figure 2 PP2 affected transcription and expression of E-cadherin, vimentin, Slug, SIP1, and Twist (A) and (C) MCF-7 cells, MDA-MB-231, and HEK293 cells were incubated with 10 μM PP2 for indicated times. Following incubation, cells were harvested. The indicated gene transcription was analyzed by RT–PCR. *GADPH* was used as internal control. (B) and (D) Following incubation with 10 μM PP2 for indicated times, cells were harvested and lysates were separated by SDS–PAGE. Proteins were detected using indicated antibodies. GADPH was used as internal control. The accompanying graphs under each figure show the relative amounts of Western blot bands measured by densitometry. Data were calculated as the mean optical density of treated samples as a percent of untreated cells normalized against GAPDH.

PP2 affects transcription factors that involved in EMT

To study the upstream effectors that regulate transcription of E-cadherin and vimentin, we focused on three well-known transcription factors, Slug, SIP1, and Twist. We chose Slug because it has been proposed to be a likely *in vivo* repressor of E-cadherin when compared with Snail in breast carcinomas [18,19]. In MCF-7 cells, Slug and SIP1 were decreased at the transcriptional and protein levels after PP2 treatment, whereas Twist was undetectable. In contrast, only Slug was downregulated after PP2 treatment in MDA-MB-231 cells, whereas SIP1 and Twist were mainly unchanged [**Fig. 2**(**C**,**D**)]. In addition, Slug was also slightly decreased by PP2 in HEK293, whereas SIP1 and Twist were unchanged.

Suppression of c-Src by RNAi

To confirm the relationship between c-Src suppression and EMT, we used siRNA strategy to deplete c-Src. Western blot analysis demonstrated that c-Src was significantly suppressed in two siRNA clones in all cell lines. But there was no difference between parental control cells and mock cells [**Fig. 3**(**A**)].

Knockdown of c-Src by RNAi inhibited EMT and affected expression of transcription factors

After knockdown of c-Src by RNAi, we observed morphological changes of MDA-MB-231 cells from elongated and spindle shape to rounded and epithelial-like shape [**Fig. 3**(**B**)]. In contrast, no obvious morphological changes were observed in MCF-7 and HEK293 cells. Further studies showed that knockdown of c-Src induced E-cadherin in both MCF-7 and MDA-MB-231 cells, but significantly inhibited vimentin in MDA-MB-231 cells [**Fig. 3**(**C**)]. In HEK293 cells, vimentin was unchanged after c-Src silencing, whereas E-cadherin was downregulated. In addition, Slug and SIP1 were down-regulated after c-Src silencing in MCF-7 and MDA-MB-231 cells, but mainly unchanged in HEK293 cells [**Fig. 3**(**C**)].

Discussion

In this study, we used two well-characterized breast carcinoma cell lines to study the relationship between c-Src and EMT. MCF-7 cells displayed an epithelial morphology, whereas MDA-MB-231 displayed typical mesenchymal morphology. After Src inhibition by PP2, the migration of both cells was inhibited and the phosphorylation of FAK was suppressed. In addition, MDA-MB-231 cells became compact and tended to cluster with each other after PP2 treatment. This morphological change indicates that c-Src suppression by PP2 may inhibit EMT.

The following study on epithelial and mesenchymal markers revealed that PP2 affected the expression of E-cadherin and vimentin in breast carcinoma cells. E-cadherin is an important protein for cell adhesion and is required for the formation of epithelia in the embryo and to maintain epithelial homeostasis in adult. Loss of E-cadherin increases tumor cell invasiveness *in vitro* and contributes to the transition of adenoma to carcinoma in animal models [4]. Vimentin is a predominant intermediated filament protein in mesenchymal cells and plays key roles in maintaining cytoarchitecture and cell migration [20]. c-Src suppression by PP2 or siRNA induced E-cadherin and inhibited vimentin in breast carcinoma cells. Restore of E-cadherin by PP2 was also observed in other cancer cells [13,15,21]. An indirect immunoperoxidase procedure was used for E-cadherin expression in MDA-MB-231 cells. After PP2 treatment or c-Src silencing by siRNA, E-cadherin was up-regulated on the cytomembrane in MDA-MB-231 cells (Supplementary Fig. S1). But E-cadherin was downregulated after c-Src suppression in

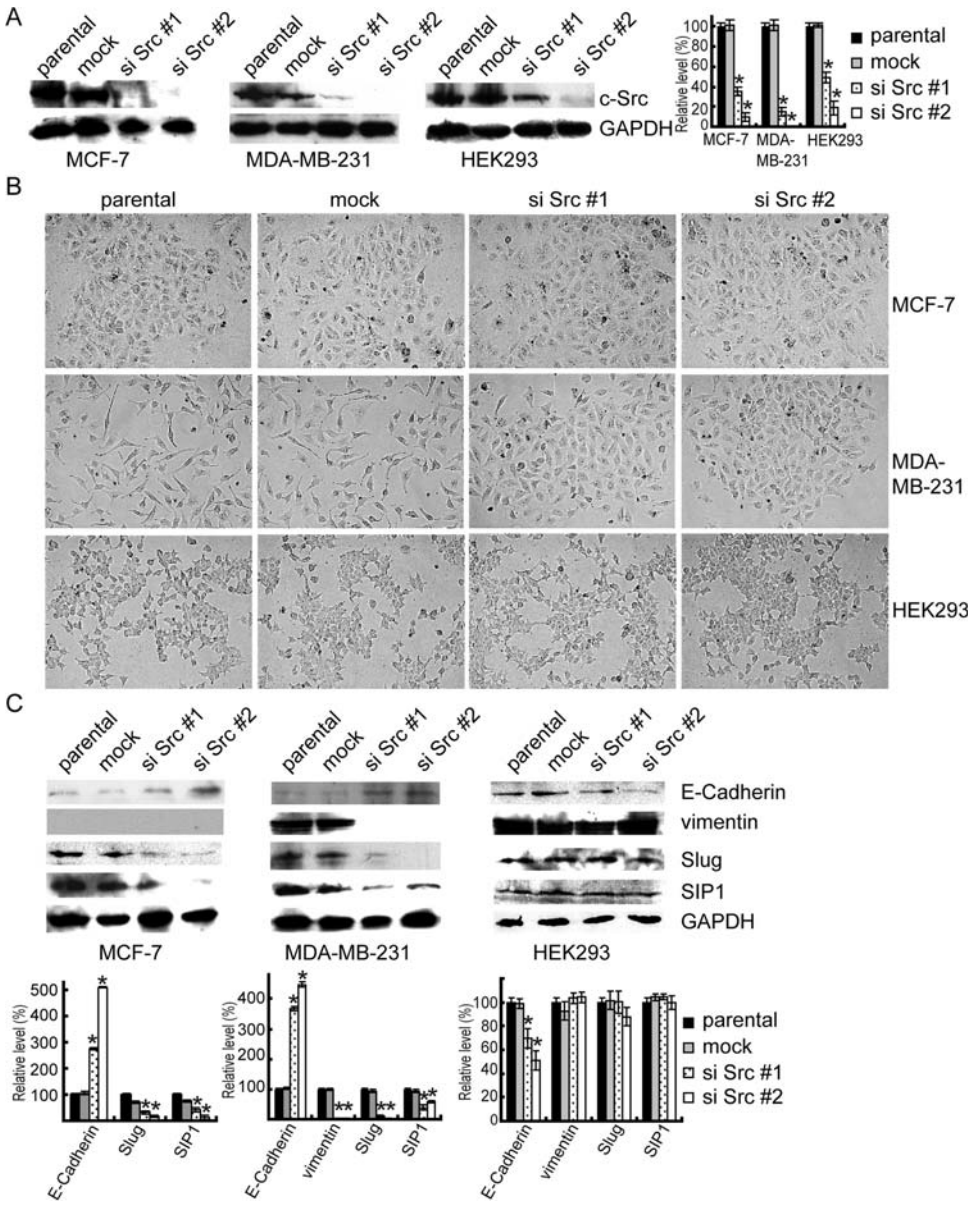

Figure 3 Knockdown of c-Src by siRNA inhibits EMT (A) c-Src knockdown by siRNA. Western blot analysis indicated that c-Src protein levels were suppressed in two siRNA clones compared to that of parental cells (no treatment) or mock cells (scramble siRNAs). (B) Morphology of cells after c-Src siRNA transfection was visualized at 200× magnification and photographed with Carl Zeiss light microscope. (C) Western blot analysis for E-cadherin, vimentin, Slug, and SIP1 after c-Src silencing. GADPH was used as internal control. The accompanying graphs under each figure show the relative amounts of Western blot bands measured by densitometry. Data were calculated as the mean optical density of treated samples as a percent of parental cells normalized against GAPDH.

HEK293 cells and the mechanism needs to be further studied.

The transcription factors that regulate E-cadherin and vimentin were further investigated. Slug is a mediator of EMT in mouse and human invasive carcinoma cells [22]. SIP1 is a zinc finger protein and has similar repressor effect in E-cadherin transcription as Slug. Twist was recently shown to induce EMT through firstly repression of E-cadherin and increase of vimentin [5]. Both Slug and SIP1 were downregulated after c-Src inhibition by PP2 or RNAi in breast carcinoma cells. But Twist was invariant in MDA-MB-231 and HEK293 cells while undetectable in MCF-7 cells. These results suggest that c-Src inhibition may regulate E-cadherin and vimentin through Slug and SIP1 in breast carcinoma cells. Twist may not involve in the c-Src-mediated EMT in breast carcinoma cells. It was reported that c-Src might regulate EMT though Slug in Slug-overexpressing MDCK cells [23]. c-Src-dependent activation of FAK is required for delocalization of membrane-bound E-cadherin through Snail [24]. Twist and Snail were independently regulated, but exerted inhibitory effect to suppress E-cadherin transcription [25]. The possible mechanism of the results is that these transcription factors may be regulated through different pathways.

MAPK acted as the upstream of Snail/Slug and is required for the GSK3β-mediated Snail protein stabilization [26], whereas MAPK is the direct downstream of c-Src. In *Drosophila*, a NF-κB-like transcription factor named Dorsal was known as upstream regulator of Twist, and Twist may be the downstream target of NF-κB in mammals [27]. But NF-κB does not always act in the same direction as c-Src [28]. The different signaling pathways may contribute to the different changes of Snail, Slug and Twist after c-Src inhibition.

In conclusion, c-Src may play important roles in EMT process in metastatic breast carcinoma cells. c-Src suppression by PP2 or siRNA inhibited EMT and reversed the mesenchymal-like phenotype in high metastatic MDA-MB-231 cells. The results of these studies provide the first characterizations of molecular and biological effects of c-Src suppression on EMT in metastatic breast carcinoma cells.

Supplementary data

Supplementary data is available at *ABBS* online.

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