Pak1 Phosphorylation of Snail, a Master Regulator of Epithelial-to-Mesenchyme Transition, Modulates Snail's Subcellular Localization and Functions

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Abstract

The process of epithelial-mesenchymal transition plays a pivotal role in the conversion of early stage tumors into invasive malignancies, and has been shown to be regulated by the zinc finger phosphoprotein, Snail; however, no upstream signaling kinases have been shown to modulate Snail functions. Since the invasiveness of breast cancer cells is also influenced by p21-activated kinase 1 (Pak1) signaling, we investigated Pak1's potential mechanistic role in the regulation of Snail functions. We found for the first time that Pak1 promotes transcription repression activity of Snail from E-cadherin, occludin, and aromatase promoters. Pak1 regulates the repressor activity of Snail by phosphorylating on Ser^{246}. Pak1 phosphorylation of Snail supports Snail's accumulation in the nucleus as well as its repressor functions. A Ser^{246}Ala substitution in Snail or Pak1 knockdown by short interference RNA blocked Pak1-mediated Snail phosphorylation, leading to increased cytoplasmic accumulation of Snail and attenuation of Snail repressor activity in breast cancer cells. The regulation of phosphorylation and function of Snail by Pak1 represents a novel mechanism by which a signaling kinase might contribute to the process of epithelial-mesenchymal transition. (Cancer Res 2005; 65(8): 3179-84)

Introduction

The small GTPases, including Cdc42 and Rac1, have been implicated in the regulation of mammalian cell morphology and motility (1). More specifically, Rac1 induces cortical actin polymerization, which is seen as membrane ruffling and lamellipodia, and Cdc42 induces the formation of peripheral actin microspikes and filopodia (2–4). The small GTPases regulate the formation of cytoskeletal structures by means of a family of serine/threonine kinases known as p21-activated kinases (Pak). Activation of Pak1 is accompanied by the disassembly of stress fibers and focal adhesion complexes, as well as by maintenance of the integrity of the motile leading edge (5, 6). Pak1 is activated by a number of growth factors, including heregulin and epidermal growth factor, which are potent inducers of Pak1 activity and motility of breast cancer cells (7, 8). Activation of Pak1 involves autophosphorylation of several sites, including Thr^{423} within the autoinhibitory loop of the kinase (9).

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Recent data suggested that, in addition to cell motility, Pak1 is also involved in breast cancer progression. Adam et al. (10) have shown a mechanistic role for Pak1 activation in the increased cell invasion of breast cancer cells by heregulin. Furthermore, expression of a kinase-dead Pak1 mutant in the highly invasive breast cancer cell lines, MDA-MB-435 and MDA-MB-231, led to stabilization of stress fibers, enhanced cell spreading, and reduction in invasiveness (11). Conversely, hyperactivation of the Pak1 pathway by conditional expression of catalytically active T423E Pak1 in the noninvasive breast cancer cell line MCF-7 promotes cell migration and anchorage-independent growth (12). Interestingly, highly proliferative human breast cancer cell lines and tumor tissues have been shown to contain hyperactive Pak1 as well as its upstream regulator Rac3 (13). Increased Pak1 activity also correlates well with the invasiveness of human breast cancer cells and breast tumor grades (12). Expression of Pak1 in human breast tumor tissue correlates with tumor grade, with higher expression in less differentiated ductal carcinomas of the breast (grade III tumors) than in grade II and grade I tumors (14). Emerging data suggests that Pak1 may be overexpressed in human breast cancer (12, 14, 15). Inhibition of Pak1 using kinase-dead Pak1-K299R in highly invasive MDA-MB435 breast cancer cells was associated with excessive cell spreading and accumulation of mature focal points (13). Although these observations suggest that Pak1 has a role in breast cancer progression, its role in epithelial-to-mesenchymal transition (EMT) remains unknown.

The process of breast cancer invasion requires, among other steps, changes in signaling pathways and increased ability to undergo EMT. One of the critical regulators of EMT is the zinc finger protein Snail, which promotes the transcriptional repression of E-cadherin, thus allowing cells to detach from their neighbors and migrate. Most epithelial tumor cell lines exhibit an inverse correlation between levels of E-cadherin and Snail mRNA (16, 17). Repression of E-cadherin transcription seems to be specifically relevant in the late steps of epithelial tumorigenesis, since a causal relationship exists between the loss of E-cadherin and the invasive properties of some tumors. Recent studies have shown an association of Snail expression with the degree of infiltration of breast carcinomas (17). A recent study suggests that Snail function may be regulated by its intracellular location, as serine phosphorylation–dependent cytoplasmic redistribution of Snail inhibits its activity as a transcription repressor (18). A recent study showed that GSK-3β phosphorylation of Snail promotes its degradation and thus, could be another mode of Snail regulation of the metastatic potential of tumor cells (19). Here we set out to gain additional insights into Snail phosphorylation by signaling pathways. We present new evidence showing for the first time that Pak1 regulates the phosphorylation and functions of Snail in breast cancer cells.

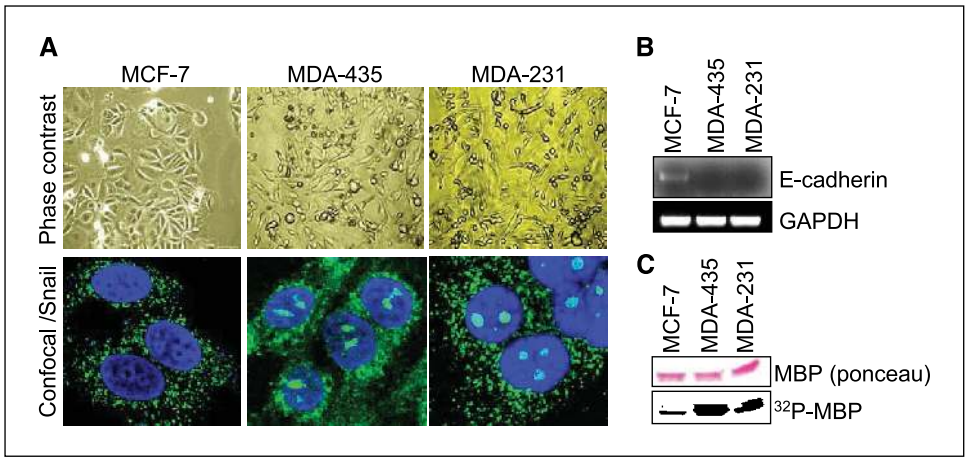

Figure 1. Status of Snail localization and Pak1 kinase activity in human breast cancer cells. *A,* morphology of MCF-7, MDA-MB-435, and MDA-MB-231 cells morphology using phase-contrast microscopy (*top*); localization of Snail in MCF-7, MDA-MB-435, and MDA-MB-231 cells by confocal microscopy (*bottom*; ×65 magnification); *B,* RT-PCR analysis of E-cadherin expression in MCF-7, MDA-MB-435, and MDA-MB-231 cells; *C,* Pak1 kinase activity in breast cancer cells. Lysates (200 μg) were immunoprecipitated with anti-Pak1 antibody, and kinase assays were done in kinase buffer using myelin basic protein as a substrate.

Materials and Methods

Cell cultures, reagents, and transfection. Breast cancer cell lines MCF-7, MDA-MB435, MDA-MD231, and 293 T cells were maintained in DMEM/F-12 (1:1) supplemented with 10% FCS. Antibodies to Pak1 and Snail were purchased from Cell Signaling Technology (Boston, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibody to E-cadherin was purchased from Zymed Laboratories (San Francisco, CA). Transient transfection studies were done using a Fugene-6 kit (Roche Biochemicals, Indianapolis, IN) in accordance with the manufacturer's instructions. Transfection with Pak1 small interfering RNAs (siRNA; Dharmacon, Lafayette, CO) was carried out in serum-free DMEM/F-12 with Lipofect-AMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. Both Pak1 siRNA and control siRNA were transfected at a concentration of 100 nmol/L.

Immunoprecipitation and kinase assay. Cells were subjected to lysis in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 10% glycerol, 1% NP40, 10 mmol/L NaF, 1 mmol/L $NaVO_4$, and a cocktail of

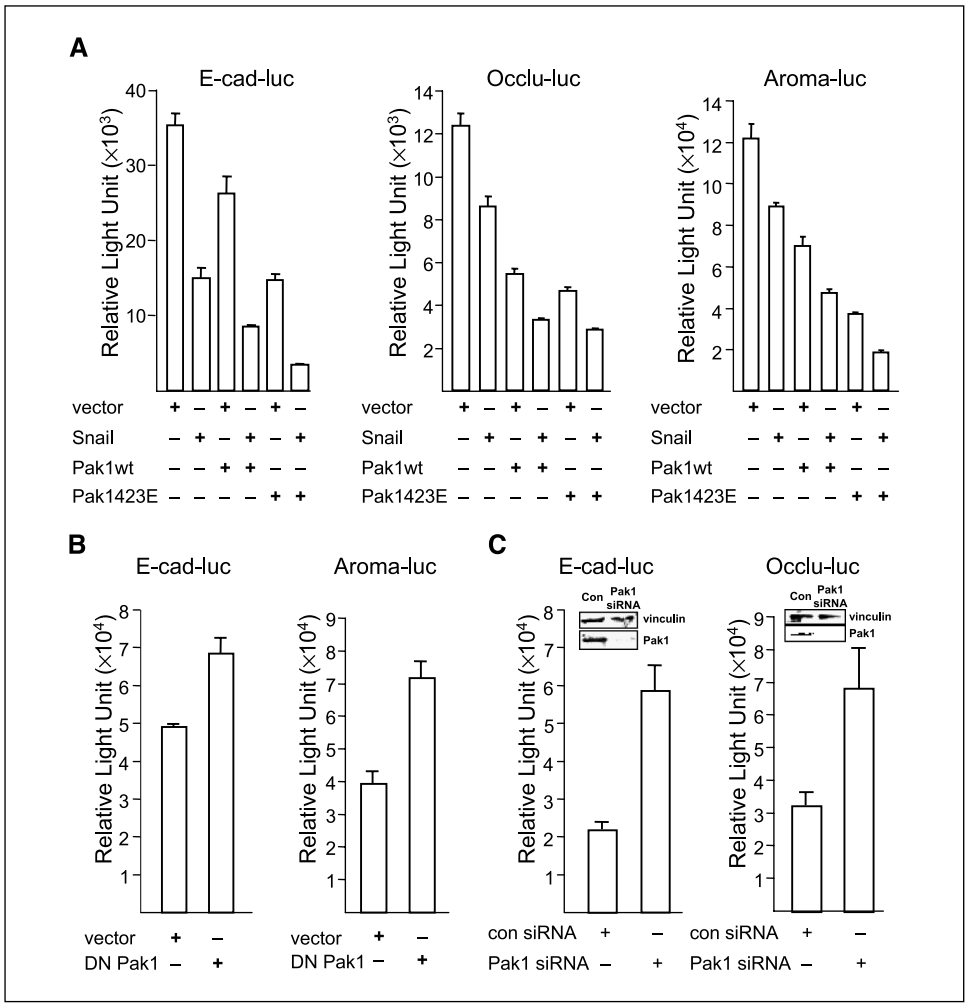

Figure 2. Pak1-induced repression of the promoter activities of Snail target genes E-cadherin, occludin, and aromatase. *A,* E-cadherin, occludin, and aromatase luciferase reporter assay: 100 ng of luciferase reporter constructs carrying human E-cadherin, occludin, or aromatase promoter were transfected into MCF-7 cells singly or together with the Snail expression vector with/without cotransfection of wild-type Pak1 or constitutively active form Pak1 (423E-Pak1), respectively; *B,* E-cadherin and aromatase luciferase reporter assay: 100 ng of luciferase reporter constructs carrying human E-cadherin or occludin promoters was transfected into MCF-7 cells singly or together with the dominant-negative Pak1 autoinhibitory fragment (residues 83-149) expression vector, respectively; *C,* E-cadherin and occludin luciferase reporter assay after siRNA-mediated inhibition of Pak1 expression. Transfection with Pak1 siRNA was carried out in serum-free DMEM/F-12 with LipofectAMINE into MCF-7 cells according to the manufacturer's specifications. Either Pak1 siRNA or control siRNA was transfected at a concentration of 100 nmol/L. Two days after siRNA transfection, 100 ng of luciferase reporter constructs carrying human E-cadherin or occludin promoter were transfected, respectively. The inhibition of Pak1 expression mediated by Pak1 siRNA was shown by Western blotting, and equal loading was indicated by anti-vinculin antibody (*inset*). All assays were done in triplicate and all results correspond to the average of three independent experiments. *Columns*, mean; *bars*, ± SD.

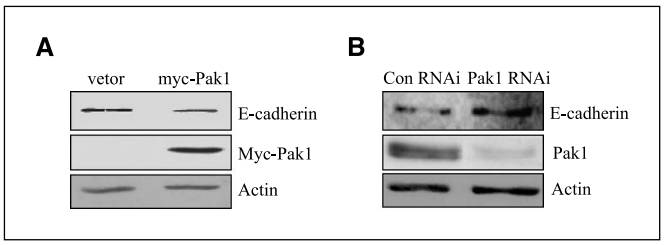

Figure 3. Pak1 Regulation on the expression of E-cadherin. *A,* down-regulation of E-cadherin expression in MCF-7 cells by transient transfection of myc-tagged Pak; *B,* up-regulation of E-cadherin expression in MDA-MB-435 cells by inhibition of Pak1 expression mediated by Pak1 siRNA.

protease inhibitors. In *in vitro* kinase assays using myelin basic protein as the substrate, Pak1 was immunoprecipitated from 200 μL of the cell lysates with Pak1 antibody as described elsewhere (9). When indicated, the immunocomplex was washed with kinase buffer [20 mmol/L HEPES (pH 7.4), 1 mmol/L DTT, 10 mmol/L $MnCl_2$, and 10 mmol/L $MgCl_2$].

Plasmid construction. We generated Snail constructs tagged with Gal4, glutathione S-transferase (GST), or HA by amplifying the selected region of Snail and then subcloning it into the *Bam*HI and *Xho*I sites of the Gal4 (Clontech, Palo Alto, CA), pGEX5X (Amersham Pharmacia, Piscataway, NJ), or pcDNA3.1 (Invitrogen) vectors. We generated mutations in the expression vector for full-length Snail by site-directed mutagenesis using the Quick Change kit (Stratagene, La Jolla, CA), and the sequences of resulting mutant Snail constructs were verified by direct sequencing at the M.D. Anderson Cancer Center core facility.

***In vitro* phosphorylation.** Using GST-Filamin as the positive control and GST-Snail protein (4 μg each) as the substrate, this assay was done in HEPES buffer (50 mmol/L HEPES, 10 mmol/L $MgCl_2$, 2 mmol/L $MnCl_2$, 0.2 mmol/L DTT) containing 100 ng of purified bacterially expressed GST-Pak1 enzyme, 10 μCi of γ-[^{32}P]-ATP, and 25 μmol/L of cold ATP (9).

***In vivo* phosphorylation.** 293 T cells were transfected with either Snail expression vector or the construct with point mutation S246A and cotransfected with wild-type Pak1 or a constitutively active form of Pak1 (423E). Cells were labeled with [^{32}P]orthophosphoric acid overnight. Twenty-four hours after transfection, cell lysates were immunoprecipitated with an anti-HA monoclonal antibody (mAb) and then separated by 10% SDS-PAGE. Labeling was visualized by autoradiography with a PhosphorImager (9).

Promoter-reporter assays. Subconfluent human breast cancer cells, cultured in six-well plates, were transiently cotransfected with 100 ng of E-cadherin, occludin (20), or aromatase (21) promoter-luciferase reporter, and cotransfected in the presence or absence of 500 ng Snail, Pak1, or T423E-Pak1 (12), together with 20 ng of β-galactosidase, using the Fugene-6 reagent according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Twenty-four hours after transfection, cells were subjected to lysis with passive lysis buffer, and luciferase assay was done using the luciferase reporter assay kit (Promega, Madison, WI). β-Galactosidase activity was used to normalize the transfection. Each transfection was done in triplicate wells.

Reverse transcription-PCR. Total RNA was isolated from MCF-7, MDA-MB435, and MDA-MD231 breast cancer cells using the TRIzol Reagent (Life Technologies, Inc., Grand Island, NY), and 100 ng of each RNA sample was analyzed by reverse transcription (RT)-PCR. Isolated RNA was reverse-transcribed and amplified using the One-Step RT-PCR System (Promega).

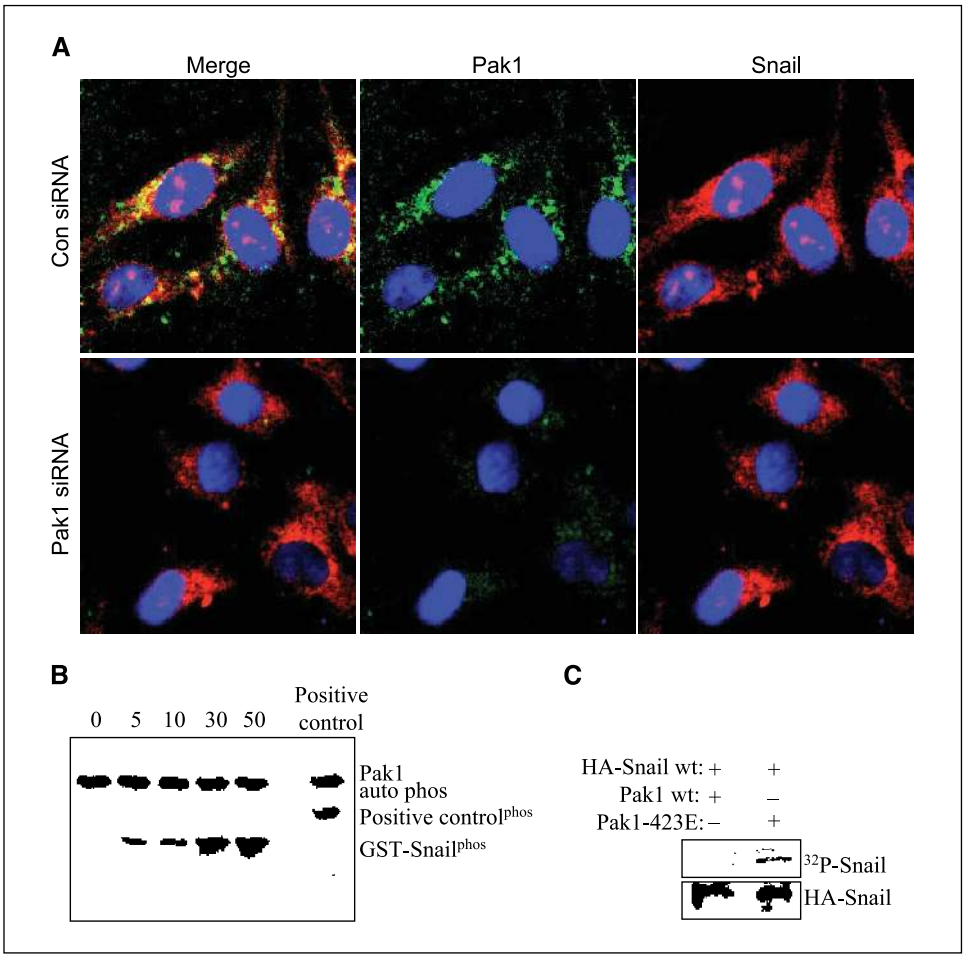

Figure 4. Pak1 regulation of Snail subcellular localization and phosphorylation. *A,* confocal microscopic analysis of Snail (*red*) localization in MDA-MB-231 cells as affected by inhibition of Pak1 expression (*green*) with Pak1 siRNA (×65 magnification); *B,* Pak1 phosphorylated Snail in a dose-dependent manner. Filamin was used as the positive control in this *in vitro* phosphorylation assay; *C, in vivo* phosphorylation of Snail by Pak1. 293 T cells were serum-starved for 1 day and then transfected with Snail expression vector together with either wild-type Pak1 or constitutively active form of Pak1 (423E). Cells were labeled with [^{32}P]orthophosphoric acid and cell lysates were immunoprecipitated with an anti-HA mAb. Phosphorylation was visualized by autoradiography with a PhosphoImager. Equal loading was indicated by Ponceau staining (*bottom*).

Primer sequences used were E-cadherin forward (5′-GGC CTG AAG TGA CTC GTA ACG A-3′) and E-cadherin reverse (5′-GCT CAG ACT AGC AGC TTC GGA AC-3′), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward (5′-CCA TCT TCC AGG AGC GAG ATC-3′) and GAPDH reverse (5′-CGT TCA GCT CAG GGA TGA CC-3′). Amplification was conducted in 20 μL reactions each containing 50 ng total RNA, 0.2 mmol/L deoxynucleotide triphosphate, 1 mmol/L $MgCl_2$, 1 μmol/L of each primer, 0.5 units of avian myeloblastosis virus, 0.5 units of *Tfl* DNA polymerase, and reaction buffer. The cycling conditions included an initial incubation at 48°C for 45 minutes and second incubation at 94°C for 2 minutes, followed by 35 cycles comprising 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 68°C. The final extension was 7 minutes at 68°C. The RT-PCR product for E-cadherin is 203 bp, and 451 bp for GAPDH. RT-PCR products were separated on 2% agarose gels.

Immunofluorescence and confocal studies. The cellular location of proteins was determined by using indirect immunofluorescence (9). Briefly, cells grown on glass coverslips were fixed in methanol at −20°C for 6 minutes. Cells were incubated with primary antibodies for 2 hours at room temperature, washed thrice with PBS, and then incubated with 546Alexa (red)-, 633Alexa (blue)-, or 488 Alexa (green)-labeled secondary antibodies (Molecular Probes, Orlando, FL). The DNA dye Topro-3 was used to costain the DNA, which gives an emission in the far-red segment of the light spectrum and color-coded in blue (Molecular Probes). Confocal analysis was done with a Zeiss laser-scanning confocal microscope using established methods of processing of the same section for each detector (the three excitations corresponding to 546, 488, or 633 nm), comparing the sections pixel by pixel.

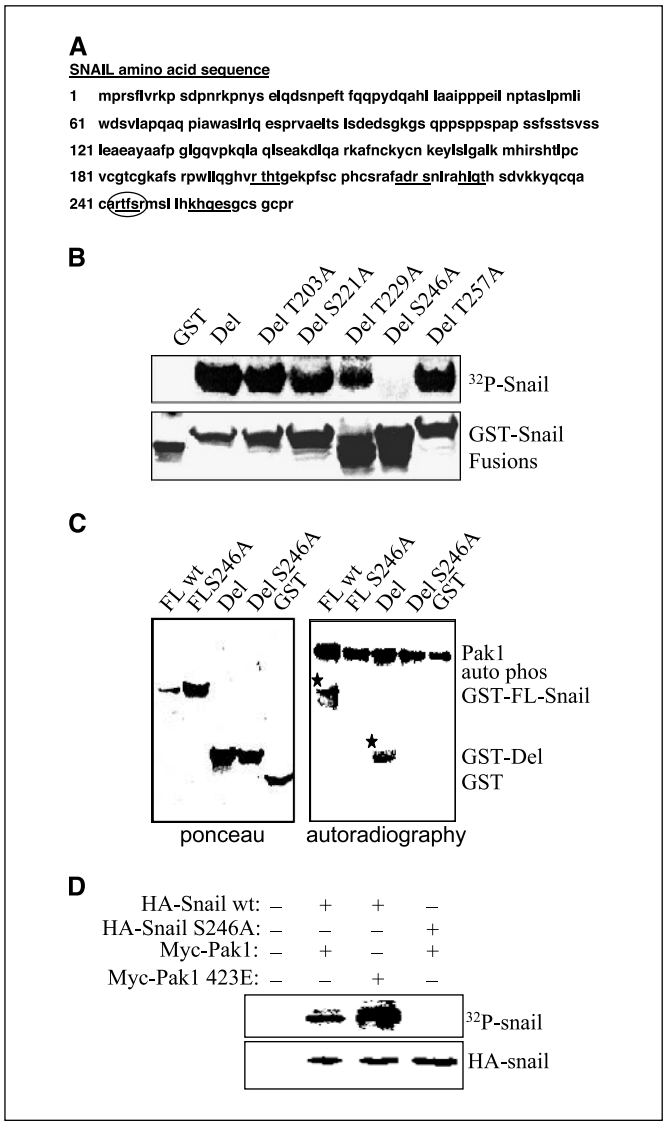

Figure 5. Phosphorylation of Snail on Ser^{246} by Pak1. *A,* snail amino acid sequence showing the possible Pak1 phosphorylation sites. Potential Pak1 phosphorylation sites are underlined and the identified Pak1 phosphorylation site is encircled; *B, in vitro* phosphorylation of Snail deletion constructs by Pak1. All five different point mutation constructs were made from a Snail deletion construct (amino acids 181-264). Equal loading is indicated by Ponceau staining (*bottom*); *C, in vitro* phosphorylation of Snail full-length and deletion constructs with/without point mutation of S246A by Pak1. The equal loading was indicated by Ponceau staining (*left*); *D, in vivo* phosphorylation of Snail on Ser^{246} by Pak1. 293 T cells cultured in DMEM/F-12 (1:1) supplemented with 10% FCS were transfected with either Snail expression vector or the construct with point mutation S246A and cotransfected with wild-type Pak1 or a constitutively active form of Pak1 (423E). Cells were labeled with [^{32}P]orthophosphoric acid and cell lysates were immunoprecipitated with an anti-HA mAb. Phosphorylation was visualized by autoradiography with a PhosphorImager. Equal loading is indicated by Ponceau staining (*bottom*).

Results and Discussion

The process of EMT, the hallmark of invasiveness, is characterized by distinct spindle-like morphologic alteration. To understand the significance of signaling pathways in EMT, we initially compared the morphologies of densely plated invasive breast cancer MDA-MB231 and MDA-MB435 cells with the morphology of noninvasive MCF-7 cells. As expected, invasive breast cancer cells exhibited spindle-like morphology with fewer cell-cell interactions than MCF-7 cells (Fig. 1*A*). Since repression of E-cadherin transcription is particularly relevant in the late steps of epithelial tumorigenesis, both MDA-MB231 and MDA-MB435 cells contained substantially lower levels of E-cadherin than MCF-7 cells (Fig. 1*B*). Since recent data have implicated Pak1 signaling in breast cancer invasiveness (13–15), we next evaluated Pak1 activity in these breast cancer cell lines. MDA-MB231 and MDA-MB435 cells contained significantly greater levels of Pak1 activity than MCF-7 cells (Fig. 1*C*).

To explore a potential effect of Pak1 signaling upon the functions of Snail, we next examined the influence of Pak1 upon the ability of Snail to repress E-cadherin promoter activity. Our results showed that both wild-type Pak1 and catalytically active T423E-Pak1 (423E) had an inhibitory effect on E-cadherin promoter activity (Fig. 2*A*, left). Furthermore, Pak1 enhanced the ability of Snail to repress E-cadherin promoter activity (Fig. 2*A*, left). To evaluate the generality of these findings, we examined the effect of Pak1 on two additional Snail-regulated genes, tight junction membrane protein occludin (20) and estrogen synthetase enzyme aromatase (21). Interestingly, we found that Pak1 expression also promotes the ability of Snail to repress transcription from both occludin- and aromatase-promoter reporters (Fig. 2*A*, middle and right). Consistent with a role for Pak1 in modulating Snail activity, inhibition of Pak1 activity with a dominant-negative Pak1 autoinhibitory fragment (residues 83-149) relieved, at least in part, the repression effects of Snail on E-cadherin and occludin promoter activity (Fig. 2*B*). We next showed that inhibition of the Pak1 pathway by Pak1-specific siRNA resulted in increased transcriptional activity from E-cadherin and occludin promoters (Fig. 2*C*). To validate these findings, we investigated whether Pak1 regulates the expression of endogenous E-cadherin, the Snail target gene, in breast cancer cell lines. Convincingly, we noticed that E-cadherin expression in MCF-7 cells was down-regulated upon transfection of myc-tagged Pak1 (Fig. 3*A*), and conversely, E-cadherin expression in MDA-MB-435 cells was up-regulated through inhibition of Pak1 expression mediated by Pak1

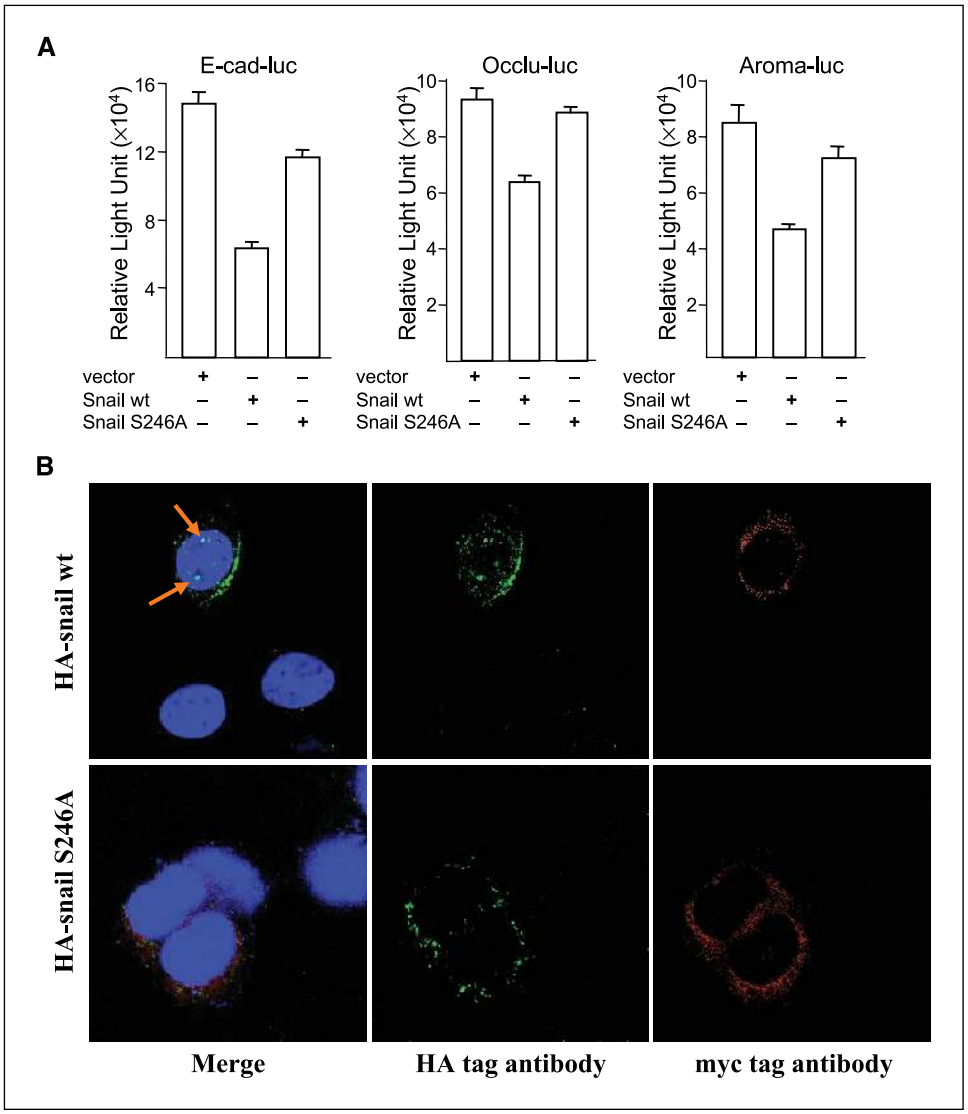

Figure 6. Pak1 phosphorylation is essential for Snail function. *A,* E-cadherin, occludin and aromatase luciferase reporter assays: 100 ng of luciferase reporter constructs carrying human E-cadherin, occludin, or aromatase promoter were transfected into MCF-7 cells singly or together with either the Snail expression vector or the construct with point mutation of S246A, respectively. All these luciferase reporter assays were done in triplicate, and all results correspond to the average of three independent experiments. *Columns*, mean; *bars*, ± SD; *B,* confocal microscopic analysis of localization of Snail-246A. MCF-7 cells were transfected with either the Snail expression vector (*red, top*) or the construct with point mutation of S246A (*red, bottom*) as indicated, together with myc-tagged Pak1 (*green*). Twenty-four hours after transfection, cells were fixed with methanol for 5 minutes at −20°C, and localization of Snail was analyzed by using indirect immunofluorescence and counterstained for nuclear DNA (*blue*) with Topro 3 (×65 magnification).

siRNA (Fig. 3*B*). Since the significance of E-cadherin in the process of EMT is well established, these results suggested that Pak1 might play its role in breast cancer progression and also in EMT by regulating the expression of E-cadherin via Snail.

To gain deeper insight into the mechanism of Pak1 regulation of Snail functions, we next examined the effect of knocked-down Pak1 expression with siRNA upon the subcellular localization of Snail in MDA-MB231 cells by scanning confocal microscopy. We found that inhibition of Pak1 expression leads to almost complete disappearance of nuclear Snail in MDA-MB231 cells (Fig. 4*A*). The increased level of Pak1 activity has been shown to correlate well with the invasiveness of human breast cancer cells and tumors (13–15); hyperactivation of the Pak1 pathway in the noninvasive breast cancer cell line MCF-7 promotes cell invasiveness (11), raising the possibility that Pak1 signaling might affect the phosphorylation status of Snail and could influence the functions of Snail.

To directly test the above hypothesis, we first identified five potential Pak1 phosphorylation sites in Snail (Fig. 5*A*, underlined). To determine if Snail was a kinase substrate of Pak1, an *in vitro* kinase assay was carried out using purified Pak1 enzyme and purified GST-Snail. Interestingly, we noticed dose-dependent phosphorylation of Snail by Pak1 enzyme (Fig. 4*B*), suggesting that Snail might be a novel substrate of Pak1. To confirm this finding, we then did the *in vivo* Snail phosphorylation assay. We cotransfected serum-starved 293 T cells with HA-tagged Snail, with or without catalytically active T423E Pak1, and cells were metabolically labeled with [^{32}P]orthophosphoric acid. Results showed that HA-Snail can be phosphorylated by catalytically active T423E Pak1, instead of wild-type Pak1, in serum-starved conditions (Fig. 4*C*). To map the Pak1 phosphorylation site in Snail, we next substituted all potential serine or threonine residues with alanine in the potential Pak1 phosphorylation sites in Snail by site-directed mutagenesis and generated Snail mutants Thr^{203}Ala, Ser^{221}Ala, Thr^{229}Ala, Thr^{257}Ala, and Ser^{246}Ala to test *in vitro* (Fig. 5*B* and *C*) and in an *in vivo* phosphorylation assay (Fig. 5*D*). The site of Pak1 phosphorylation on Snail was identified as Ser^{246}.

To show the functionality of the Pak1 phosphorylation site in Snail, we next compared the repression activity of Snail Ser^{246}Ala mutant with that of wild-type Snail against a Snail-target gene reporter assay. We found that Snail-mediated repression of transcription from the E-cadherin, occludin and aromatase

promoters were significantly relieved by the mutant Snail Ser^{246}Ala (Fig. 6*A*), suggesting a mechanistic role for Pak1 phosphorylation of Snail on Ser^{246} in regulating the functions of Snail. To delineate the potential mechanism by which Pak1 phosphorylation regulates Snail function, we examined the subcellular localization of Snail in MCF-7 cells, expressing myc-Pak1 with either wild-type HA-Snail or mutant HA-Snail Ser^{246}Ala (Fig. 6*B*). We found that the wild-type Snail localizes in both the cytoplasm and in the nucleus, whereas the mutant Snail Ser^{246}Ala preferentially localized in the cytoplasmic compartment. These results suggested that Pak1 phosphorylation of Snail modulates its transcriptional activity by promoting increased accumulation of Snail in the nucleus, an increasing accepted mode of influencing transcription functions of coregulators such as p53, C-terminal Binding Protein, and Forkhead transcription factor.

Since Pak1 has now been shown to be present in both the cytoplasmic and the nuclear compartments (9–12), and because Pak1 also contains nuclear localization signal, which subcellular compartment is responsible for Pak1 phosphorylation of Snail and/or if Snail phosphorylation also influences its import or export from the nucleus remain unknown. Since Snail contains a nuclear export sequence (18), phosphorylation of an adjacent serine-rich sequence may make this nuclear export sequence accessible to the CRM1 transporter, thus facilitating translocation of the Snail protein to the nucleus. These issues have not been investigated here, and will be addressed in detail in our planned future studies. In summary, results presented here show for the first time that Pak1 phosphorylation of Snail on Ser^{246} promotes Snail's nuclear accumulation and consequently its repressor activity in the nucleus. Since Pak1 is a major signaling nodule of extracellular stimuli and growth factor pathways that are widely implicated in the process of EMT, it is possible that Pak1 signaling or its upstream regulators might constitute an important modifier of EMT by directly phosphorylating Snail on Ser^{246}.

Acknowledgments

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