

Acquisition of Epithelial-Mesenchymal Transition Phenotype of Gemcitabine-Resistant Pancreatic Cancer Cells Is Linked with Activation of the Notch Signaling Pathway

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Abstract

Despite rapid advances in many fronts, pancreatic cancer (PC) remains one of the most difficult human malignancies to treat due, in part, to *de novo* and acquired chemoresistance and radioresistance. Gemcitabine alone or in combination with other conventional therapeutics is the standard of care for the treatment of advanced PC without any significant improvement in the overall survival of patients diagnosed with this deadly disease. Previous studies have shown that PC cells that are gemcitabine-resistant (GR) acquired epithelial-mesenchymal transition (EMT) phenotype, which is reminiscent of “cancer stem-like cells”; however, the molecular mechanism that led to EMT phenotype has not been fully investigated. The present study shows that Notch-2 and its ligand, Jagged-1, are highly up-regulated in GR cells, which is consistent with the role of the Notch signaling pathway in the acquisition of EMT and cancer stem-like cell phenotype. We also found that the down-regulation of Notch signaling was associated with decreased invasive behavior of GR cells. Moreover, down-regulation of Notch signaling by siRNA approach led to partial reversal of the EMT phenotype, resulting in the mesenchymal-epithelial transition, which was associated with decreased expression of vimentin, ZEB1, Slug, Snail, and nuclear factor- κ B. These results provide molecular evidence showing that the activation of Notch signaling is mechanistically linked with chemoresistance phenotype (EMT phenotype) of PC cells, suggesting that the inactivation of Notch signaling by novel strategies could be a potential targeted therapeutic approach for overcoming chemoresistance toward the prevention of tumor progression and/or treatment of metastatic PC. [Cancer Res 2009;69(6):2400–7]

Introduction

Pancreatic cancer (PC) is a highly aggressive malignant disease, which is ranked as the fourth leading cause of cancer-related deaths in the United States, with ~37,000 newly diagnosed cases and ~34,000 deaths per year in the United States (1). In recent years, novel treatment strategies, including diverse cytotoxic therapeutics such as chemotherapeutic drugs,

suicide genes, γ -irradiation-induced cytotoxicity, or immunotherapy have been initially encouraging, but prolonged drug exposure results in the development of acquired drug resistance impeding successful treatment (1). Thus, the aggressive behavior of PC is believed to be due to both *de novo* (intrinsic) and acquired (extrinsic) resistance to conventional therapeutics and also could be due to the lack of delivery of effective doses of the drugs in the tumor because of the existence of extensive desmoplastic stroma and lack of adequate vasculature in most PCs. Although gemcitabine monotherapy (2',2'-difluorodeoxycytidine), a deoxycytidine analogue, or its combination with other agents has become standard chemotherapy for the treatment of advanced PC, gemcitabine imparts a progression-free survival interval ranging from 0.9 to 4.2 months only (2). In summary, the effect of gemcitabine on survival has been disappointing, which could be due to many factors including intrinsic (*de novo*) drug resistance. This disappointing outcome strongly suggests that a better understanding of the mechanism by which chemoresistance arises is likely to lead to novel therapeutic strategies for the successful treatment of patients diagnosed with PC.

Emerging lines of evidence suggest molecular and phenotypic associations between chemoresistance and the acquisition of epithelial-mesenchymal transition (EMT)-like phenotype of cancer cells (3–6). This process is also believed to be reminiscent of “cancer stem-like cells” characteristics in many cancer systems (7–10). EMT has been classified as a unique process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype (mesenchymal phenotype) leading to increased motility and invasion (11, 12). The process of EMT involves loss of epithelial cell-cell junction, actin cytoskeleton reorganization, and up-regulation of mesenchymal molecular markers such as fibronectin, α -smooth muscle actin (SMA), vimentin, and N-cadherin (12–14). A disassembly of cell-cell junction, including down-regulation and relocation of E-cadherin and β -catenin from cell membrane to nucleus, results in the induction of EMT. A number of factors that transcriptionally repress E-cadherin have emerged as potent EMT drivers during normal development and cancer. These include the zinc finger Snail homologues (Snail1, Snail2/Slug, and Snail3) and several basic helix-loop-helix factors such as Twist, ZEB1, ZEB2/SIP1, and TCF3/E47/E12 (15). EMT is a dynamic process and is triggered by the interplay of extracellular signals (such as collagen) and many secreted soluble factors such as Wnt, transforming growth factor- β (TGF- β), fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, and platelet-derived growth factors. Among many of these signaling pathways, the Wnt, TGF- β ,

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Hedgehog, Notch, and nuclear factor- κ B (NF- κ B) signaling pathways are critical for EMT induction (12, 16).

Emerging lines of evidence also suggest that there is a molecular link between EMT phenotype and chemoresistance or radioresistance (3–5, 9, 10). Yang and colleagues (4) reported that oxaliplatin-resistant colorectal cancer cells underwent EMT. Paclitaxel-resistant ovarian cancer cells showed phenotypic changes consistent with EMT with decreased expression of the epithelial adhesion molecule E-cadherin and an increase in mesenchymal markers such as vimentin, α -SMA, and fibronectin (3). Moreover, tamoxifen-resistant breast cancer cells showed altered morphologic characteristic of cells similar to EMT with altered β -catenin phosphorylation (5). Recently, Rho and colleagues (17) reported phenotypic changes such as a spindle-cell shape and increased pseudopodia formation, suggesting that EMT was present in the gefitinib-resistant lung cancer cells, with a decrease in the expression of E-cadherin and an increase in the expression of vimentin, which is a mesenchymal marker. These studies clearly provide strong evidence linking chemoresistance to EMT.

Previous studies have shown that gemcitabine-resistant (GR) PC cells acquired EMT characteristics (18). However, the exact mechanism for the acquisition of EMT phenotype of GR cells remains to be elucidated. The present study shows that the Notch signaling pathway is involved in the acquisition of EMT phenotype of GR cells. We also found that down-regulation of Notch signaling was associated with decreased invasive behavior of GR cells. Moreover, down-regulation of Notch signaling led to partial reversal of the EMT phenotype, resulting in mesenchymal-epithelial transition (MET), which was associated with decreased expression of vimentin, ZEB1, Slug, Snail, and NF- κ B. These results suggest that the increased Notch signaling is mechanistically associated with chemoresistance and EMT characteristics of PC cells, and as such, Notch pathways could be a novel target for the treatment of PC.

Materials and Methods

Cell culture. Human PC gemcitabine-resistant (GR) cell line originally was derived as described earlier (18). Briefly, L3.6pl is a gemcitabine-sensitive (GS) cell line, whereas GR cells are consistent with MIA Pa Ca-2 cells as confirmed by DNA finger-printing with EMT phenotype; however, 200 nmol/L of gemcitabine was used for maintaining the EMT phenotype of GR cells during multiple passaging. These two cell lines were cultured in MEM supplemented with 10% fetal bovine serum, L-glutamine, and 1% penicillin and streptomycin in a 5% CO₂ atmosphere at 37°C unless otherwise indicated.

Experimental reagents. Antibodies against β -catenin were purchased from Cell Signaling Technology. Antibodies against vimentin and nestin were purchased from Abcam. Primary antibodies for E-cadherin, Notch-1, Notch-2, Notch-3, Notch-4, Jagged-1, Jagged-2, Dll-1, Dll-4, urokinase-type plasminogen activator (uPA), matrix metalloproteinase (MMP)-2, Snail, Slug, vascular endothelial growth factor (VEGF), and MMP-9 were purchased from Santa Cruz Biotechnology. All other secondary antibodies were obtained from Pierce.

Real-time reverse transcription-PCR analysis for gene expression. The total RNA from GS, GR, or siRNA-transfected GR cells was isolated with Trizol (Invitrogen) and purified with RNeasy Mini Kit and RNase-free DNase Set (Qiagen) according to the manufacturer's protocols. The primers used in the PCR reaction are described before (19–22). The other primers used in the PCR reaction are ZEB1 (forward, 5'-GCACAACCAAGTGCAGAAGA-3'; reverse, 5'-GCCTGGTTCAGGAGAAGATG-3') and vimentin (forward, 5'-AGATGGCCCTTGACATTGAG-3'; reverse, 5'-TGGAA-GAGGCAGAGAAATCC-3'). Real-time PCR amplifications were done as described earlier (19).

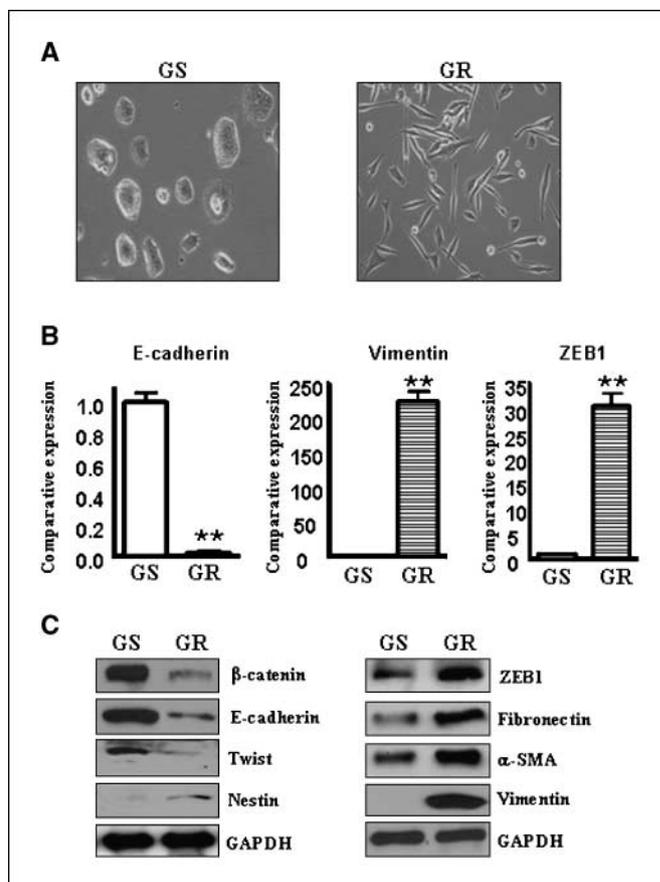


Figure 1. GR cells showed an EMT phenotype compared with the epithelial phenotype of L3.6pl GS cells. **A**, morphology of GS and GR cells. **B**, real-time RT-PCR was used to quantify E-cadherin, vimentin, and ZEB1 mRNA expression in GS and GR cells. **, $P < 0.01$, compared with GS. **C**, Western blot analysis showing the expression of markers of epithelial and mesenchymal phenotypes of cells.

Western blot analysis. Cells were lysed in lysis buffer by incubating for 20 min at 4°C. The protein concentration was determined using the Bio-Rad assay system. Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane for Western blotting as described earlier (19).

Immunofluorescence microscopy. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in PBS and 10% goat serum blocking solution for 1 h. The cells were incubated for 2 h with anti-Notch-2, anti-Notch-4, anti-Jagged-1, anti-E-cadherin, anti-vimentin, anti- β -catenin, and anti-F-actin in 5% goat serum and were analyzed as described earlier (13).

uPA activity assay. The culture medium of the GS or GR cells grown in six-well plates was collected. After collection, the medium was spun at 800 \times *g* for 3 min at 4°C to remove cell debris. The supernatant was either frozen at -20°C for uPA assay later or assayed immediately using commercially available ELISA kits (American Diagnostica, Inc.).

MMP-9 activity assay. The GS or GR cells were seeded in six-well plates and incubated at 37°C. After 24 h, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 48 h. MMP-9 activity in the medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay Kit (R&D Systems, Inc.) according to the manufacturer's protocol.

Plasmids and transfections. GS and GR cells were transfected with Notch-2 siRNA, Notch-4 siRNA, Jagged-1 siRNA, and siRNA control (Santa Cruz Biotechnology), respectively, using Lipofectamine 2000 as described earlier (19).

Cell attachment and detachment assays. Cell attachment assay was done as follows. GS, GR, and GR cells transfected with siRNA were seeded in 24-well plates at 5×10^4 per well. After 1-h incubation, unattached cells were removed, and the attached cells were counted after trypsinization. The data were presented as a percentage of the cells attached to the plate compared with total cells. For cell detachment assay, the cells were seeded in 24-well plates at 5×10^4 per well. After 24-h incubation, the medium was removed and the cells were incubated with 0.05% trypsin for 3 min to detach the cells from the culture plates. The medium containing 10% fetal bovine serum was added into the cells to inactivate the trypsin and the detached cells were collected into tubes. The remaining cells were incubated with 0.25% trypsin to detach all the cells and collected into fresh tubes. The cells were counted and the data were presented as a percentage of the detached cells to total cells.

Cell migration and invasion assays. Cell migration was assessed using 24-well inserts (BD Biosciences) with 8- μ m pores according to the manufacturer's protocol. The invasive activity of the GR siRNA transfected cells was tested by using the BD BioCoat Tumor Invasion Assay System (BD Biosciences) as described earlier (19).

Data analyses. Experiments presented in the figures are representative of three or more different repetitions. The data are presented as the mean values \pm SE. Comparisons between groups were evaluated by a two-tailed Student's *t* test. *P* < 0.05 was considered statistically significant.

Results

GR cells display morphologic changes consistent with EMT.

As shown in Fig. 1A, the GR cells displayed elongated, irregular fibroblastoid morphology. In contrast, GS cells had a rounded shape, typical of an epithelial cobblestone appearance, and these cells grew in clusters. These changes in phenotype suggested that GR cells have undergone the EMT as reported previously (18). To further confirm whether GR cells underwent EMT, we

determined the expression of markers of epithelial and mesenchymal phenotypes. We found that E-cadherin, β -catenin, and Twist expression was greatly reduced in GR cells (Fig. 1B and C). However, elevated levels of nestin, ZEB1, α -SMA, vimentin, and fibronectin were observed in GR cells, suggesting that the expression of these factors may be important to gemcitabine-induced EMT of PC cells.

To further confirm whether GR cells are truly EMT-like cells, we assessed the expression and localization of E-cadherin and β -catenin using immunofluorescence staining. GR cells acquired their elongated shape, which was consistent with β -catenin nuclear translocation and relocation of E-cadherin from cell plasma membrane to the nuclear compartment (Fig. 2). The results from immunostaining also indicate that GR cells had increased levels of expression of vimentin. F-actin reorganization and a diffuse pattern were also observed in GR cells, which were correlated with EMT phenotype. Having confirmed that the GR cells are EMT-like cells, we investigated the role of Notch signaling especially because Notch signaling is known to play important roles during normal development as well as in the process of EMT that is reminiscent of cancer stem-like cells (23, 24).

Activation of the Notch pathway is involved in EMT in GR cells.

The Notch pathway is involved in the EMT induction during tumor progression and converts polarized epithelial cells into motile, invasive cells (23). To determine whether the Notch pathway is involved in GR cells, we assessed the levels of the Notch pathway at mRNA and protein levels by real-time reverse transcription-PCR (RT-PCR) and Western blotting, respectively. GR cells displayed an increased activation of Notch-2 and Jagged-1 at mRNA and protein levels. Notch-4 expression was also increased in GR cells. However, other Notch receptors and ligands were not

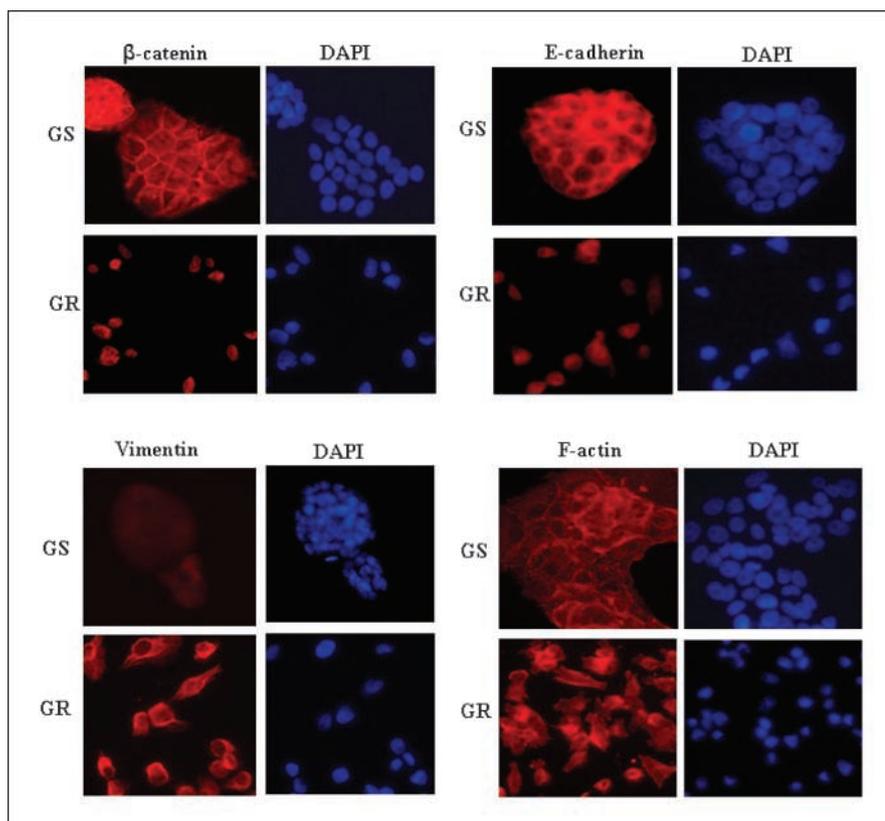


Figure 2. Immunofluorescence staining for the expression and cellular localization of β -catenin, E-cadherin, vimentin, and F-actin.

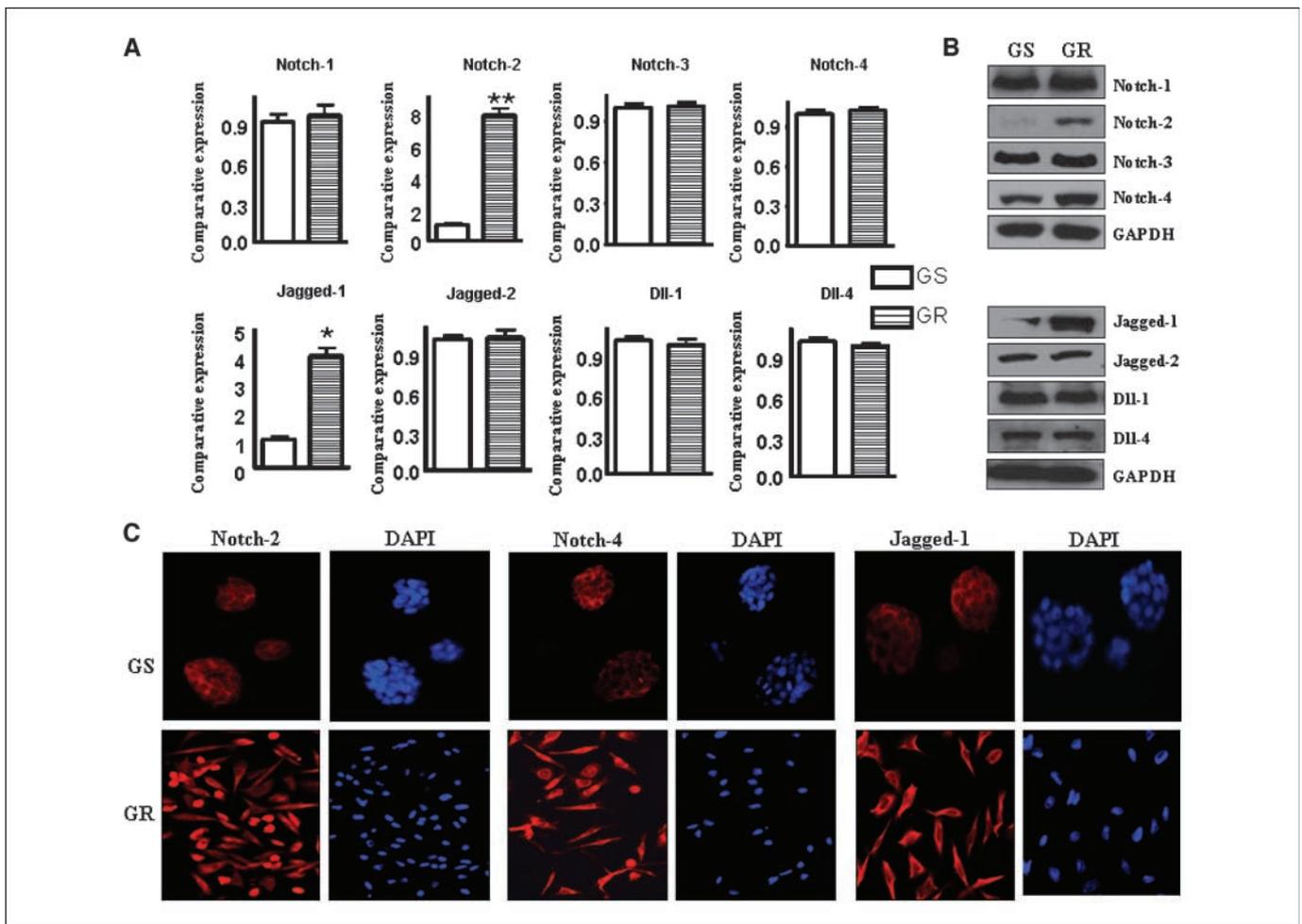


Figure 3. The Notch pathway is up-regulated in GR cells. *A*, real-time RT-PCR was used to quantify Notch pathway genes at the mRNA levels in GS and GR cells. *, $P < 0.05$; **, $P < 0.01$, compared with GS. *B*, Western blot analysis showing the expression of Notch pathway–related proteins. *C*, immunofluorescence staining for the expression and cellular localization for Notch-2, Notch-4, and Jagged-1.

significantly changed (Fig. 3*A* and *B*). To further confirm whether the Notch pathway is involved in EMT, we determined the expression and localization of Notch-2, Notch-4, and Jagged-1 using immunofluorescence staining. We found that Notch-2 and Notch-4 were up-regulated in nuclear compartment in GR cells. We also found that the expression of Jagged-1 was increased in GR cells compared with GS cells (Fig. 3*C*).

Notch downstream target, NF- κ B, and its downstream genes contribute to EMT characteristics. The Notch pathway has been reported to strongly regulate NF- κ B activity and induce expression of several NF- κ B subunits (25). NF- κ B has been identified as a central mediator of EMT in cancer progression (12). Therefore, we assessed whether the expression of Notch downstream target NF- κ B subunit p65 is altered in GR cells or not. We found that p65 was up-regulated in GR cells. The expressions of MMP-9, MMP-2, uPA, and VEGF are known to be transcriptionally regulated by NF- κ B and have been reported to play important roles in tumor migration and invasion (26). We therefore investigated whether MMP-9, MMP-2, uPA, and VEGF correlate with the acquisition of EMT characteristics in GR cells. Indeed, we found that both MMP-9 mRNA and protein levels were dramatically increased in the GR cells (Fig. 4*A* and *B*). Next, we examined whether the GR cells have increased MMP-9 activity or not. We found a dramatic increase in

the activity of MMP-9 in GR cells (Fig. 4*C*). The uPA and uPA receptor (uPAR) are known to regulate the MMP-9 activity in PC (27), and our data showed that both uPA mRNA and protein levels are significantly increased in the GR cells (Fig. 4*A* and *B*). Most importantly, GR cells showed higher uPA activity (Fig. 4*B*). However, we did not find any changes in the expression of MMP-2 or VEGF. Our results clearly showed up-regulation of MMP-9 and uPA in GR cells, results consistent with the up-regulation of NF- κ B induced by the activation of the Notch signaling pathway. To obtain direct proof in support of the role of Notch in EMT of GR cells, we used Notch inactivation strategies in GR cells.

Down-regulation of Notch signaling induces reversal of EMT to MET in GR cells. We have shown that GR cells have a fibroblast-like morphology that is typical of mesenchymal phenotype of cells associated with the loss of epithelial markers. Therefore, to further confirm a direct mechanistic role of Notch in GR cells containing EMT characteristics, we decreased the expression of Notch-2 and Jagged-1 using specific siRNAs. As shown in Fig. 5*A*, specific siRNAs for Notch-2 or Jagged-1 were effective in reducing the expression of these proteins compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (used as protein loading control). Subsequently, we assessed whether EMT phenotype was reversed in

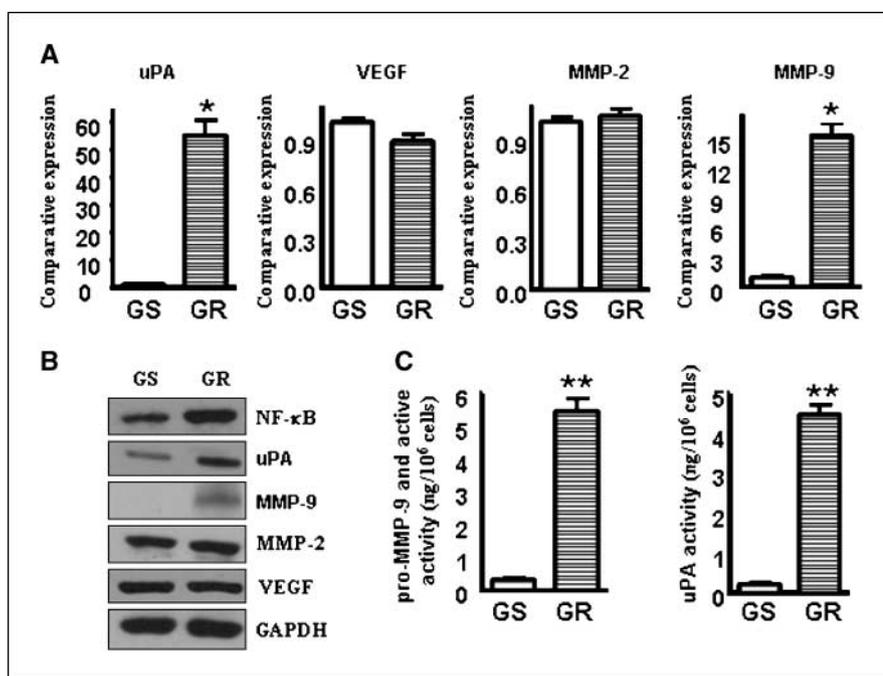


Figure 4. MMP-9 and uPA were up-regulated in GR cells. *A*, real-time RT-PCR reaction was used to quantify uPA, VEGF, MMP-2, and MMP-9 mRNA expression in GS and GR cells. *, $P < 0.05$, compared with GS. *B*, Western blot analysis showing that MMP-9 and uPA protein levels were significantly increased in GR cells. *C*, left, MMP-9 activity assay showing that MMP-9 was up-regulated in GR cells; right, uPA assay showing that uPA level in culture medium was up-regulated in GR cells.

GR cells transfected with Notch-2 and Jagged-1 siRNAs. We found that GR cells transfected with Notch-2 or Jagged-1 siRNA displayed round cell-like morphology 3 days after transfection (Fig. 5B). The results from real-time RT-PCR showed that the expression of E-cadherin was significantly increased, which clearly suggests that the down-regulation of Notch-2 or Jagged-1 expression in GR cells results in the reversal of EMT phenotype to MET phenotype (Fig. 5C). However, down-regulation of Notch-4 by siRNA transfection did not show any sign of the reversal of EMT (data not shown). We also found that the pattern of expression of mesenchymal markers was changed in GR cells transfected with Notch-2 and Jagged-1 siRNAs, showing a dramatic reduction in the expression of vimentin, which is consistent with the acquisition of MET phenotype of GR cells (Fig. 5C and D).

It is known that E-cadherin is a downstream target of ZEB1, and that ZEB1 is a transcriptional repressor to the expression of which that down-regulates E-cadherin, resulting in the induction of the EMT phenotype. Therefore, we sought to assess the expression levels of ZEB1 in GR cells. As documented in Fig. 1, the expression of ZEB1 was increased in GR cells, consistent with up-regulation of E-cadherin and the acquisition of EMT phenotype. We subsequently observed that ZEB1 expression was down-regulated in GR cells transfected with Notch-2 and Jagged-1 siRNAs (Fig. 5D). Recently, the Notch pathway was found to up-regulate Snail and Slug expression (24, 28). Slug is essential for Notch-mediated EMT by repressing E-cadherin expression, which results in β -catenin activation (24, 28). Therefore, we assessed the expression of Snail and Slug in GR cells transfected with Notch-2 and Jagged-1 siRNAs. As we expected, the expressions of Snail and Slug were down-regulated in Notch-2 siRNA- and Jagged-1 siRNA-transfected cells (Fig. 5D).

Next, we assessed whether the increased levels in the expression of NF- κ B in GR cells, as seen in Fig. 4, could be down-regulated in GR cells transfected with Notch-2 and Jagged-1 siRNAs. Moreover, because it is known that NF- κ B (p65) could up-regulate ZEB1 expression, we also assessed the interrelationships among Notch-2,

Jagged-1, NF- κ B, and ZEB1 in GR cells transfected with Notch-2 and Jagged-1 siRNAs. Our results showed that the expression of NF- κ B (p65 protein) was down-regulated in GR cells transfected with Notch-2 and Jagged-1 siRNAs (Fig. 5D). These results strongly suggest that down-regulation of the Notch pathway contributes to down-regulation of NF- κ B and ZEB1, resulting in the up-regulation of E-cadherin and the reversal of EMT phenotype to MET phenotype.

Down-regulation of Notch signaling reduces the detachment and attachment and inhibits the migration and invasion of GR cells. It is well known that cell detachment from the matrix where the tumor grows in the microenvironment and attachment to the secondary site is the "hallmark" of cell migration and invasion during metastatic process. We found that GR cells have increased capacity for attachment and detachment (Fig. 6A). Because the activation of Notch can lead to increased migration and invasion, we sought to assess the ability of GR cells for attachment and detachment and their ability for migration and invasion when transfected with Notch-2 and Jagged-1 siRNAs. As expected, we found that GR cells transfected with Notch-2 and Jagged-1 siRNAs displayed decreased detachment and attachment (Fig. 6B). More importantly, down-regulation of Notch-2 and Jagged-1 by siRNA markedly reduced the migratory and invasive ability of GR cells (Fig. 6C), which is consistent with the cell attachment and detachment findings. These results clearly suggest that down-regulation of the Notch pathway down-regulates NF- κ B and ZEB1 and consequently up-regulates E-cadherin, resulting in the reversal of the EMT phenotype to a MET phenotype with less cell migration and invasion characteristics.

Discussion

Despite rapid advances in the diagnostic and surgical procedures, PC remains one of the most difficult human malignancies to treat. This may be due to the intrinsic (*de novo*) and extrinsic (therapy-induced) chemoresistant or radioresistant

behaviors of PC, and as of yet, no systemic therapy has been proved effective in improving the overall survival of patients diagnosed with this deadly disease (1). It is believed that both *de novo* and acquired resistance to therapy could be overcome by understanding the mechanisms by which PC becomes chemoresistant, which will improve the overall survival of patients. Therefore, in this study, we have used a GS and a GR PC cell line to investigate the molecular mechanism of resistance and associated cellular behaviors. Previous studies have shown that GR cells acquired EMT phenotype, which is reminiscent of cancer stem-like cells, which was associated with spindle-shaped morphology and enhanced pseudopodia formation. The GR cells also showed decreased expression of the epithelial adhesion molecule E-cadherin and an increase in the expression of mesenchymal markers such as vimentin. However, the mechanism(s) governing the acquisition of EMT phenotype by GR cells have not been fully elucidated.

Many of signaling pathways, including the Notch and NF- κ B signaling pathways, are found to be critical for EMT induction (12, 16, 28). The activation of Notch signaling is known to regulate the expression of its target genes and, thus, plays important roles in growth and development, including regulation of proliferation and apoptosis (29–31). Therefore, not surprisingly, alterations in Notch signaling are associated with tumorigenesis (19, 29, 30). Moreover, it has been reported that Notch is involved in the EMT induction during tumor progression and converts polarized epithelial cells into motile, invasive cells (24). Overexpression of Jagged-1 and Notch-1 induces the expression of Slug

and correlates with poor prognosis in various human cancers (24). Slug is also essential for Notch-mediated EMT by repressing E-cadherin expression, which results in β -catenin activation (24). In addition, Niessen and colleagues (32) showed that Slug is directly up-regulated by Notch in endothelial cells and that Slug expression is required for Notch-mediated repression of the vascular endothelial cadherin promoter and for promoting migration of transformed endothelial cells. Our results are consistent with these findings.

Recently, the Notch pathway was found to up-regulate Snail expression by recruitment of the intracellular Notch to Snail promoter (28). Moreover, Notch potentiates hypoxia-inducible factor-1 α recruitment to the lysyl oxidase promoter and elevates the hypoxia-induced up-regulation of lysyl oxidase, which stabilizes the Snail protein (28). Consistent with these findings, we found that GR cells displayed an increased activation of Notch-2 and Jagged-1 both at the mRNA and protein levels, which was consistent with EMT characteristics of GR cells. To gain further insight whether Notch could directly regulate the expression of the above-mentioned epithelial marker genes, we down-regulated the expression of Notch-2 and Jagged-1 by transfection of GR cells with specific siRNAs. The GR cells transfected with Notch-2 and Jagged-1 siRNAs led to an increase in the expression of E-cadherin and reduced the expression of vimentin and ZEB1. The expressions of Snail and Slug were also down-regulated by Notch-2 and Jagged-1 siRNAs. Taken together, these results suggest that the activation of Notch, Jagged-1, and ZEB1 could mediate the induction of EMT phenotype, as observed in GR cells,

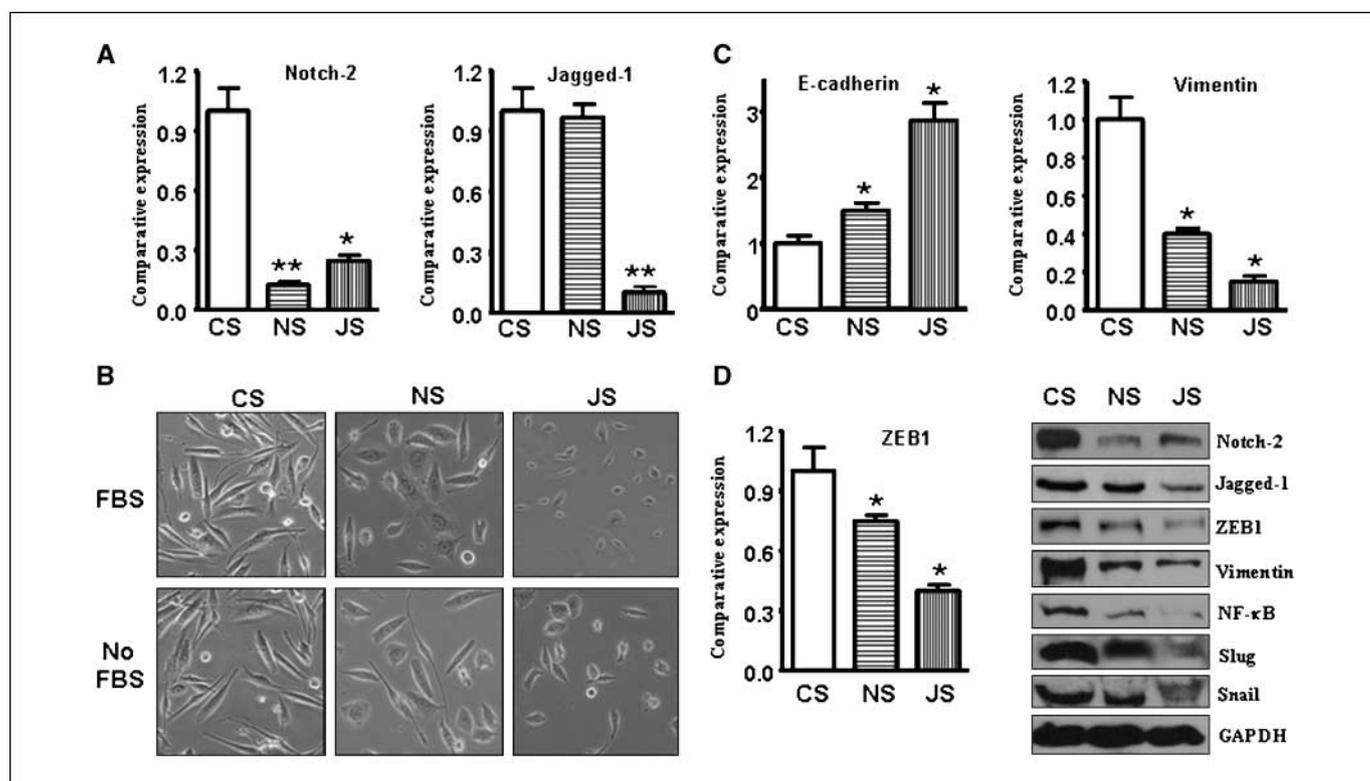


Figure 5. The Notch pathway contributes to the regulation of molecular markers of EMT in GR cells. CS, control siRNA; NS, Notch-2 siRNA; JS, Jagged-1 siRNA. A, real-time RT-PCR was used to quantify Notch-2 and Jagged-1 mRNA expression in GR cells transfected with specific siRNA. *, $P < 0.05$; **, $P < 0.01$, compared with control siRNA. B, down-regulation of Notch-2 and Jagged-1 could cause reversal of EMT phenotype of GR cells. C and D, GR cells transfected with control siRNA or Notch-2 siRNA or Jagged-1 siRNA were assessed for the expression of markers of epithelial and mesenchymal phenotypes using real-time RT-PCR and Western blot analysis, respectively.

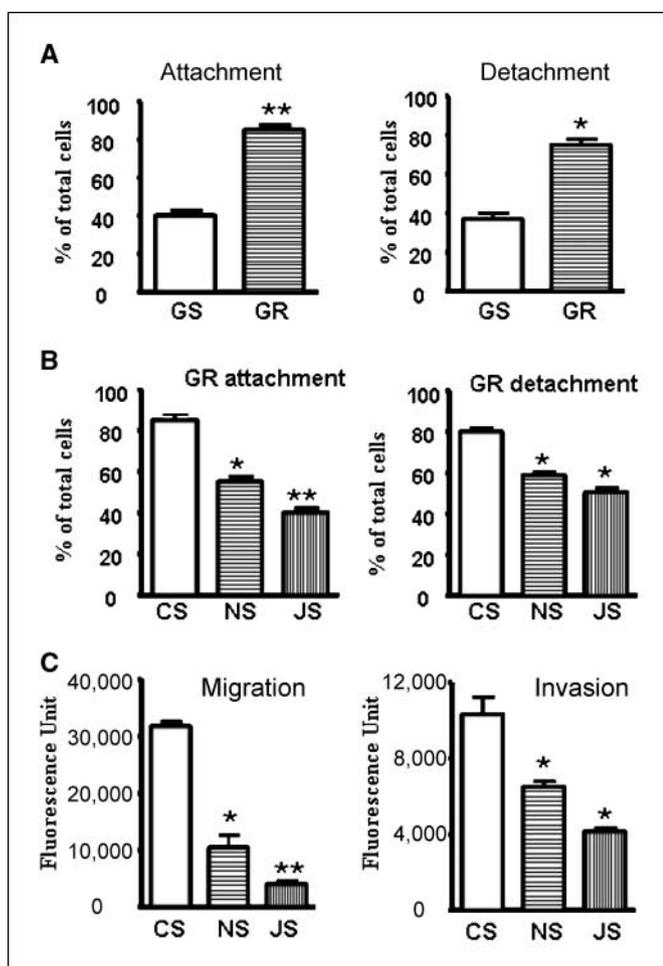


Figure 6. Down-regulation of Notch-2 and Jagged-1 inhibits cell migration and invasion and reduces attachment and detachment of GR cells. *, $P < 0.05$; **, $P < 0.01$, compared with control. **A**, GR cells showed increased cell attachment and detached. **B**, down-regulation of Notch-2 or Jagged-1 in GR cells dramatically decreased the attachment and detachment of cells. **C**, transfection of GR cells with Notch-2 siRNA or Jagged-1 siRNA inhibited migration and invasion.

and that the down-regulation of Notch signaling could be useful for the reversal of the EMT phenotype. However, our results raised a question with respect to how the activation of Notch-2 could regulate the expression of epithelial and mesenchymal molecular markers and whether Notch signaling could cross-talk with other signaling molecules critical to EMT. To answer this question, we determined the expression of NF- κ B in GR cells. The rationale was based on the fact that Notch was reported to cross-talk with NF- κ B (33). Constitutive levels of Notch activity are essential to maintain NF- κ B activity in various cell types (34, 35). Recently, Fukushima and colleagues (36) reported that Notch-2 could enhance NF- κ B transcriptional activity. Indeed, we found that the expression of NF- κ B protein (p65 subunit of NF- κ B) was significantly increased in GR cells compared with GS cells. Moreover, the down-regulation of Notch-2 and Jagged-1 by siRNA transfection resulted in decreased NF- κ B in GR cells, suggesting a molecular link or cross talk between Notch and NF- κ B, and thus the activation of these signaling pathways seems to be mechanistically associated with the acquisition of EMT phenotype of GR cells.

The activation of NF- κ B is known to play critical roles in the processes of EMT and tumor cell invasion and metastasis (12). Emerging evidence also suggests that the activation of NF- κ B is mechanistically linked with the processes of EMT via regulation of the expression of a transcription factor, ZEB1, which negatively regulates the transcription of E-cadherin, resulting in the loss of E-cadherin in EMT-like cells (37), and our findings are consistent with this mechanism. Although the activation of NF- κ B is associated with EMT, it is not known what downstream gene of NF- κ B is mechanistically associated with EMT. A candidate for such a gene is *MMP-9*, a well-described NF- κ B downstream target gene. Thus, we assessed the role of NF- κ B downstream genes in the processes of EMT and found that *MMP-9* expression and its activity were significantly increased in GR cells, which is consistent with the activation of Notch and NF- κ B signaling. Because uPA and uPAR are known to regulate the activity of *MMP-9* activity in PC cells (27), and uPA and its receptor (uPAR) are important genes in the processes of tumor cell invasion and metastasis (38, 39), we assessed the expression of uPA in our system. We found that GR cells have higher expression of uPA, which is consistent with previous findings (18). Therefore, our results suggest that GR cells could potentiate aggressive behavior due, in part, to the up-regulation of *MMP-9* and uPA expression during the acquisition of EMT phenotype.

Because the processes of EMT have been linked with cell migration and invasion, we hypothesized that GR cells transfected with Notch-2 and Jagged-1 siRNAs could lead to decreased migration and invasion compared with cells transfected with control siRNA. We found that transfection of GR cells with Notch-2 and Jagged-1 siRNAs significantly inhibited cell attachment and detachment, as well as cell migration and invasion. These results are consistent with the mechanistic role of Notch signaling in the processes of EMT and that the inactivation of Notch signaling leads to the reversal of EMT to MET phenotype with less invasive characteristics. Collectively, our results provide strong evidence suggesting that the inactivation of Notch signaling by novel therapeutic strategies could be clinically important in overcoming drug resistance and the reversal of EMT phenotype, which is likely to improve the overall survival of patients diagnosed with PC.

In conclusion, our results provided, for the first time, strong molecular evidence showing that GR cells underwent EMT partly due to the activation of Notch signaling. Therefore, we believe that novel strategies by which Notch signaling could be down-regulated may become an important approach for the prevention of tumor progression and/or treatment of invasive and metastatic PC for which there is currently no curative therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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