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S100A4, frequently overexpressed in various human cancers, accelerates cell motility in pancreatic cancer cells

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ABSTRACT

S100A4, a member of the Ca^{2+} dependent S100 protein family, is reported to associate with metastasis through regulation of the motility and invasiveness of cancer cells. A high level of S100A4 protein has been reported in a variety of cancers, including pancreatic cancer. However, its biological role in pancreatic carcinogenesis is largely unknown. We previously reported that *S100A4* is frequently overexpressed and that RNAi-mediated knockdown induces apoptosis and suppression of cell growth, motility, and invasiveness. In this study, we analyzed the effects of forced expression of *S100A4* in pancreatic cancer cell lines without S100A4-upregulation. We used two cell lines without upregulation of S100A4 (PCI-35 and PCI-43) as well as two cell lines with highly upregulated S100A4 as the control (MIA PaCa-2 and PAN-07-JCK). Cells did not show acceleration of their growth and invasiveness after forced expression of *S100A4*, but remarkable acceleration of cell motility was observed only in PCI-35 and PCI-43. We further performed microarray analyses using PCI-35 and PCI-43 with and without forced expression of S100A4 and identified 72 and 18 genes that were 2-fold or more upregulated or downregulated, respectively, in both cell lines after forced expression of *S100A4*. Our results suggest that S100A4 is crucial for cell motility in pancreatic cancer and that some downstream genes may play important roles in cell motility.

1. Introduction

Pancreatic cancer is one of the most life-threatening diseases, and its invasive capacity and metastatic potential are crucial causes for the difficulty and/or impossibility of surgical resection. In addition, pancreatic cancers are largely resistant to both chemotherapy and radiation therapy. Diagnosis at the early stages of the disease is difficult because this disease does not show any particular symptoms. Therefore, most patients are diagnosed at an advanced disease stage, resulting in a poor prognosis [1]. Establishment of more effective methods for both diagnosis and treatment are necessary for improving the prognoses of pancreatic cancer patients. Curative treatments are limited by invasion and metastasis in many patients; thus, elucidation of molecular pathways is particularly important.

Recently, overexpression of S100A4 in various tumors has been reported [2], and its function as one of the key players in metastatic process has been gradually elucidated [3]. The S100 family members are Ca^{2+}-binding proteins characterized by the EF-hand motif. Twenty-one different human *S100* genes have been identified; most of the family member genes are clustered in chromosome 1q21. The physiological properties of these S100 proteins implicate their involvement in diverse cellular functions, including cell proliferation, differentiation, metabolism, motility, and signal transduction; therefore, targeting these molecules should be an effective strategy for cancer therapy [4]. We previously demonstrated the frequent overexpression of *S100A4* in pancreatic cancer cell lines; siRNA-mediated knockdown of *S100A4* suppressed cell proliferation and invasiveness in only those pancreatic cancer cell lines with a high level of *S100A4* expression [5]. Hence it is of great interest to analyze the changes after the introduction of *S100A4* into pancreatic cancer cells with low-level expression. Herein we report that cell motility is activated by induction of S100A4 in human pancreatic cancer.

2. Materials and methods

2.1. Pancreatic cancer cell lines and culture conditions

In this study, pancreatic cancer cell lines Panc-1, BxPC-3, MIA PaCa-2, AsPC-1, PK-1, PK-8, PK-9, PK-45P, PK-45H, PK-59, PCI-35, PCI-43, PCI-64, PAN-03-JCK, PAN-07-JCK, and PAN-08-JCK were

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used. The first four cell lines were purchased from American Type Culture Collection (Manassas, VA), and the remaining 12 were obtained from the original developers; they were analyzed in our previous report [5]. The immortalized normal human pancreatic ductal epithelial cell, HPDE [6], was kindly provided by Dr. Ming-Sound Tsao (University of Toronto) and was used as the control. All cells were maintained according to conditions recommended by the suppliers.

2.2. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated using the RNeasy Midi Kit (QIAGEN, Valencia, CA). All samples were treated with RNase-free DNase (QIAGEN, Valencia, CA) during isolation according to the manufacturer's protocol. The purity and concentration of each isolated RNA was determined by spectrometry at 260 nm and 280 nm and confirmed by agarose gel electrophoresis followed by ethidium bromide staining. An aliquot of 10 μg of each total RNA was used for cDNA synthesis by SuperScript II RNase H^- reverse transcriptase (Invitrogen, Inc., San Diego, CA) according to the manufacturer's protocol. Semi-quantitative RT-PCR and quantitative RT-PCR (qRT-PCR) using the ABI PRISM 7000 (Applied Biosystems, Foster City, CA) were both performed by methods previously described [7], and the data were normalized by using the expression of the β2-microglobulin (*B2M*) mRNA as the internal control as described [8]. Control templates were PCR amplified using an amplified cDNA library [9], and the quantities were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Pittsburgh, PA). Primers and probes were designed by Primer Express software (Applied Biosystems, Foster City, CA); their nucleotide sequences are listed in Supplementary Table 1.

2.3. Forced expression of S100A4 in pancreatic cancer cell lines without overexpression

The entire coding region of S100A4 was PCR-amplified using a primer pair S100A4-F (5′-AGAAGATCTCCACCATGGCGTGCCCTCTG-GAG-3′) and S100A4-R (5′-TTACTCGAGTTTCTTCCTGGGCTGCTTAT-3′). These primers facilitate *Bgl*II and *Xho*I restriction endonuclease sites, respectively, for directional cloning; the PCR product was digested with these enzymes and ligated in pcDNA6/*Myc*-His [10]. The nucleotide sequence of the constructed S100A4 expressing plasmid was determined by the method as described [11] and was termed pcDNA6-S100A4. The plasmid was then transfected in pancreatic cancer cell lines using lipofectamine 2000 reagent (Invitrogen) according to methods by the suppliers. An empty pcDNA6/*Myc*-His plasmid was also transfected as the control.

2.4. Western blotting

A total of 2×10^5 cells were plated in 6 well dishes and allowed to adhere for 24 h; then the transfection of the S100A4 expression vector was performed as described above. After 48 h, cells were harvested, and protein concentrations in total cell lysates were measured using the DC protein assay kit (Bio-Rad, Hercules, CA). A 50 μg aliquot of the protein was subjected to Western blotting as described previously [12], using a 10–20% polyacrylamide gradient gel (Bio-Rad, Hercules, CA). Antibodies used were rabbit anti-S100A4 polyclonal antibody (Dako Cytomation, Denmark), mouse anti-β actin monoclonal antibody (Sigma, St Louis, MO), and horseradish peroxidase conjugated anti-rabbit or anti-mouse immunoglobulin antibodies (Amersham Biosciences Corp., Piscataway, NJ). For blocking conditions and concentrations of antibodies, we followed the manufacturer's recommendations. Signals were visualized by reaction with ECL Detection Reagent (Amersham Biosciences Corp., Piscataway, NJ) and digitally processed using LAS 1000 Plus with a Science Lab 99 Image Gauge (Fuji Photo Film, Minamiashigara, Japan).

2.5. Cell proliferation assay

A total of 1×10^4 cells were plated in 24-well plates 24 h after transfection. Cell number in each well was counted every 24 h up to four days; cells were harvested and their numbers were counted. These cells were stored for further investigation of S100A4 expression. All experiments were performed in triplicate.

2.6. Matrigel invasion assay

The effects of S100A4 transfection on the invasion and motility of pancreatic cancer cell lines were analyzed using Growth Factor Reduced (GFR) Matrigel™ Invasion Chambers (Becton Dickinson Labware, Franklin Lakes, NJ) by methods described previously [13]. In brief, 5×10^3 cells were seeded in the serum-free medium into the upper compartment. In the lower compartment of the chamber, medium supplemented with 10% fetal bovine serum was added. The plates were incubated in a humidified 5% CO_2 incubator at 37 °C for 60 h. The top solution was then removed, the cells on the top membrane surface were gently scraped with a cotton swab, and the cells on the bottom surface were fixed and stained with toluidine blue. Cell numbers were counted, averaged, and expressed as the rate of migrated cells compared with the mock control. All experiments were performed in triplicate.

2.7. Scratch migration assay

Pancreatic cancer cell lines were transfected in 6-well plates. After 24 h from transfection, the bottom of each well was scraped with the fine end of a 1 ml pipette tip; this time point was set as time 0. Plates were washed twice with PBS to remove detached cells and incubated with the complete growth medium. Cell migration into the wounded empty space was followed over time and photographed [14].

2.8. Microarray analysis

Gene expression profiling was analyzed by methods described previously [15] using the Agilent Whole Human Genome

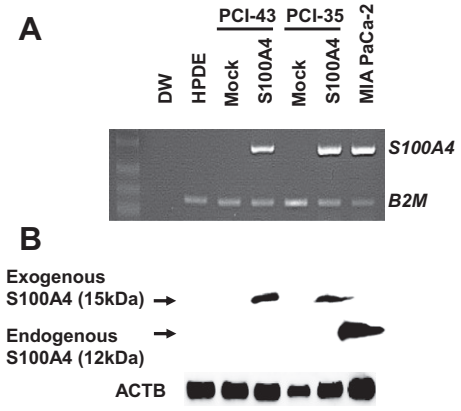

Fig. 1. (A) Results of RT-PCR after transfection of S100A4-expression vector pcDNA6-S100A4 in pancreatic cancer cells. MIA PaCa-2 was used as the cell line with a very high level of S100A4 expression, and *B2M* was used as the internal control. (B) Western blotting confirmed the expression of exogenous S100A4 after transfection of pcDNA6-S100A4 in pancreatic cancer cells. MIA PaCa-2 was used as the positive control, and ACTB was used as the internal control.

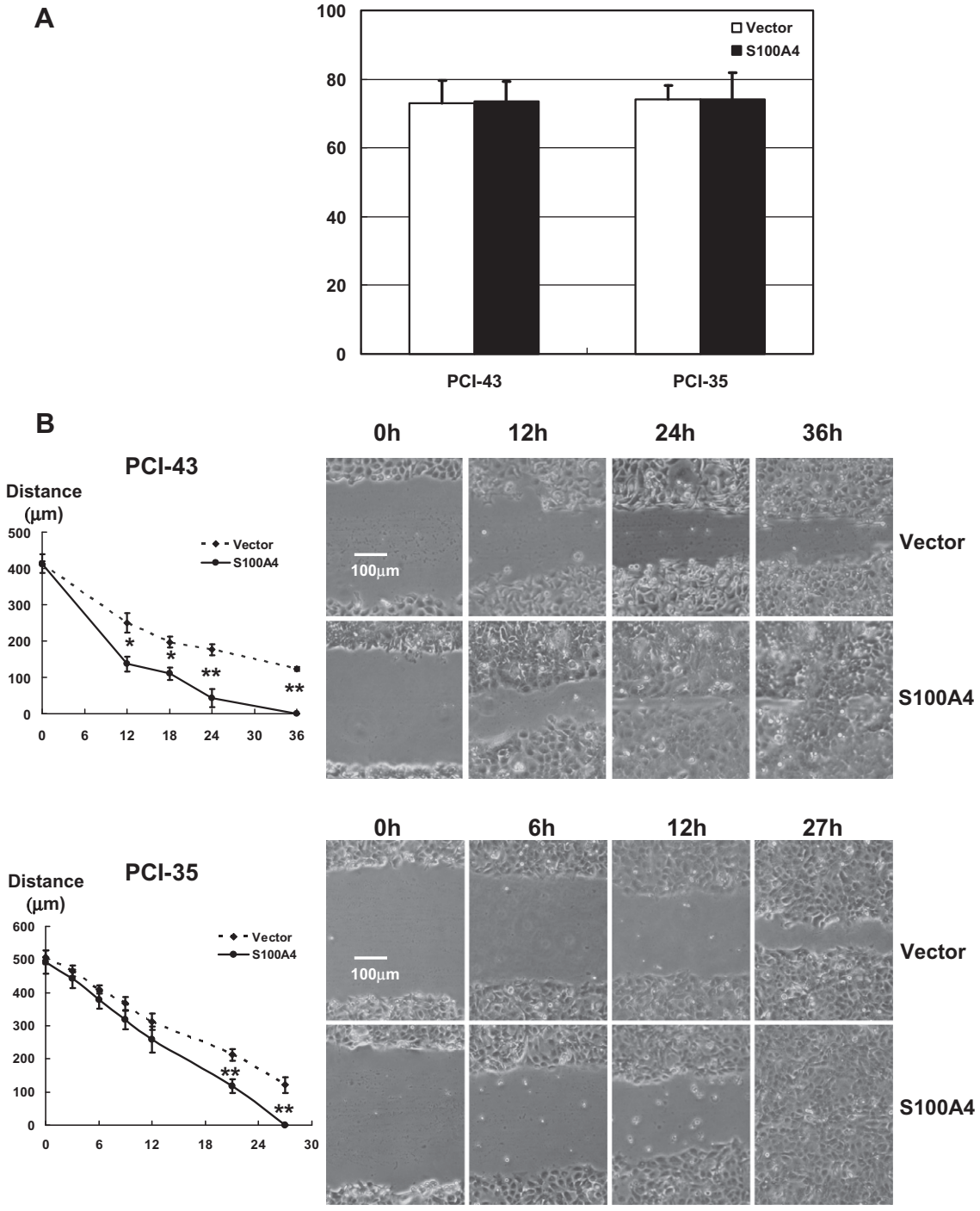

Fig. 2. (A) Invasion assay demonstrated no differences after forced expression of S100A4. Open and closed bars denote empty vector (Vector) and S100A4-expression vector (S100A4) transfected cells, respectively. (B) Results of the scratch assay. Cells were seeded at 24 h after the transfection, and 24 h after the cell-seeding, the bottom of each well was scraped with the fine end of a 1-ml pipette tip. This time point was set as time 0. Distances were measured at various time points and are graphically demonstrated at the left; then, statistical analyses were done. Significant differences between cell migrations were observed in both PCI-43 and PCI-35; $*$ and $**$ denote $P < 0.05$ and $P < 0.001$, respectively. Representative pictures are shown in the right columns.

(4×44 K). The slides were scanned using a GenePix 4000A scanner (Axon Instruments, Union City, CA), and the obtained results were analyzed using GeneSpring 8.0 software (Silicon Genetics, Redwood City, CA) according to methods described previously [15]. Microarray data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under the accession number: GSE39493.

2.9. Statistical analyses

All experiments were performed in duplicate or triplicate. A two-tailed Student's *t*-test was used for statistical analyses of comparative data using Microsoft Excel software (Microsoft Corporation, Tokyo, Japan). Values of $P < 0.05$ were considered as significant.

3. Results and discussion

3.1. Expression analyses of S100A4 before and after the introduction by RT-PCR and Western blotting

An association between poor prognosis and S100A4 overexpression has been reported in a variety of cancers, including pancreatic cancer [2]. We first examined the expression of S100A4 in 16 pancreatic cancer cell lines as well as in HPDE, an immortalized normal human pancreatic ductal epithelial cell line, and the results as shown in Supplementary Fig. 1 were in good agreement with our previous report [5]. Then we selected PCI-43 and PCI-35 as the representative cell lines without overexpression of S100A4; the expression levels of these cells were also comparable to HPDE in our previous report [5]. In addition to these two cell lines, MIA PaCa-2 and PAN-07-JCK were also selected as the representative cell lines with very high level of expression of S100A4. Then we introduced S100A4-expression vector. Results of RT-PCR and Western blotting, as shown in Fig. 1, demonstrated that the S100A4 was successfully introduced and expressed in cell lines PCI-43 and PCI-35. It is notable that no significant morphological differences were evident after the introduction of S100A4 (data not shown).

3.2. Forced expression of S100A4 promotes in vitro cell migration, but not cell growth and invasion

To address the question of whether forced expression of S100A4 stimulates cell growth, we performed proliferation assay by counting the number of cells. The results are shown in Supplementary Fig. 2; forced expression did not promote cellular proliferations.

To elucidate the effects of forced expression of S100A4 on invasion and/or metastasis, we performed an invasion assay using Growth Factor Reduced (GFR) Matrigel™ Invasion Chambers; no significant differences were observed (Fig. 2A). We further performed an *in vitro* scratch assay to elucidate the ability of cell migration after induction of S100A4. As shown in Fig. 2B, the gaps within the S100A4 introduced cell lines were narrower than the control (empty vector-transfected) cells. These results were digitalized, and significant differences were observed ($P < 0.001$); aberrant activation of S100A4 promotes cell migration in pancreatic cancer cells.

3.3. Microarray analyses after induction of S100A4

In this study, activation of cell proliferation and motility were demonstrated after the forced expression of S100A4. However, the molecular mechanisms are not well understood. Therefore, we next analyzed gene expression profiles before and 40 h after the induction of S100A4 in pancreatic cancer cell lines PCI-35 and PCI-43 in duplicated experiments; 770 and 733 genes, respectively, were selected as the genes upregulated by 2-fold or more (Fig. 3A). To identify relevant genes that are associated with increased S100A4 expression in pancreatic cancer, we selected 72 genes that are commonly upregulated in both cell lines as the candidate upregulated genes. These genes are listed in Supplementary Table 2. Using the geometric mean, 19 of these genes showed more than 5-fold upregulation. In the same manner, 18 downregulated genes were also selected after induction of S100A4 into PCI-35 and PCI-43 as the commonly downregulated genes in both cell lines (Supplementary Table 3); by geometric mean, two genes showed more than 5-fold downregulation.

From these genes, we further selected the 10 upregulated genes as listed in Table S1 based upon their putative functions. We then performed RT-PCR experiments for the 10 selected upregulated genes using the primers listed in Supplementary Table 1. Results are shown in Fig. 3B. We further analyzed four genes, *IFI27*, *NOV*, *TXNIP*, and *GEM*, by qRT-PCR and found that *IFI27* showed significant upregulation after the forced expression of S100A4 (see Fig. 3C). S100A4 showed upregulation at the time point of 24 h, whereas *IFI27* showed upregulation at time points of 48 h or later. Both the upregulation of cell growth and the migration started rather quickly, as shown in Fig. 2, and the timing for upregulation of *IFI27* was a somewhat later event. Upregulation of S100A4 itself directly or with factors other than IFI27 may play important roles in cell growth and/or migration in pancreatic cancer cells. It is notable that this gene was 4.12-fold downregulated after siRNA-mediated knockdown in our previous study [5].

IFI27, interferon-alpha-inducible protein 27, was isolated by Rasmussen et al. in 1993 from estradiol-treated human breast carcinoma cell, and was mapped to 14q32 [16]. Upregulation of *IFI27* has been observed in several cancers, including psoriatic skin [17], pyothorax-associated lymphoma [18], ovarian cancer [19], and breast cancer [16,20]. *IFI27* seems to be one of the downstream genes involved after upregulation of S100A4, and further investigation is necessary.

3.4. Future studies and conclusion

In this study, we investigated the biological responses in pancreatic cancer cells after upregulation of S100A4 and found that cell motility was upregulated. Previously, Tabata et al. reported [5] that *S100A4* frequently overexpresses in human pancreatic cancer cells and that suppression of S100A4 induces cell apoptosis and suppresses motility. In the present study, however, forced expression of S100A4 in cells without S100A4-upregulation only promoted migration, and cell growth and invasiveness were not changed. These results suggest that pancreatic cancer cells without

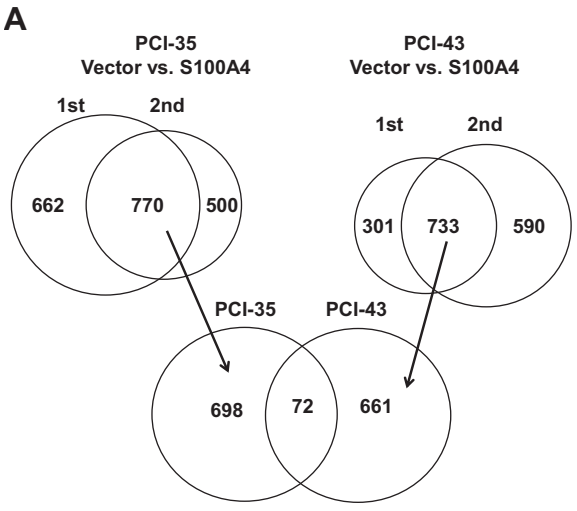

Fig. 3. (A) Gene expression profiles after induction of S100A4. A Venn diagram of the genes shows that 770 and 733 genes were reproducibly upregulated in PCI-35 and PCI-43, respectively, and 72 genes showed common upregulation in both cell lines. (B) Results of RT-PCR in the 10 genes selected with empty vector transfection (vec) and pcDNA6-S100A4 transfection at indicated time points. PCR bands of the selected genes are indicated by arrows, and arrowheads indicate *B2M* products as the internal control. No expression of either *CDH9* or *NR1I3* was observed even after the pcDNA6-S100A4 transfection. (C) Results of qRT-PCR of S100A4 and *IFI27*. *B2M* was used as the internal control. Expression levels were normalized using the empty vector-transfected PCI-43 cells. S100A4 was significantly upregulated at 24 h after the transfection, but *IFI27* showed upregulation at 48 h and 72 h in PCI-43 and PCI-35, respectively. Statistical differences denoted by ∗, ∗∗, and ∗∗∗ are $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

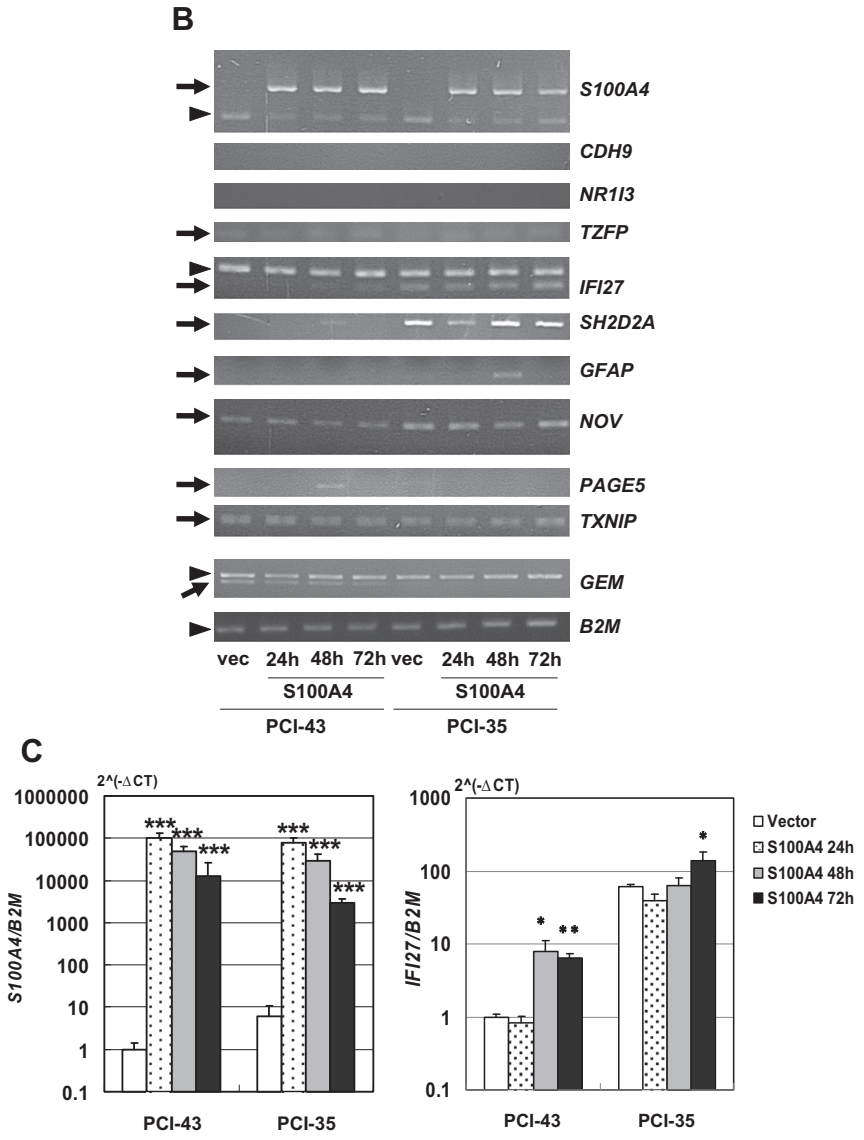

Fig. 3. (*continued*)

upregulation of S100A4 acquired cell growth and invasiveness by factors other than S100A4. However, it is possible that restriction of the cells from migration will open the way to find inhibition methods against metastasis; it is particularly important for clinical management of patients with pancreatic cancer. Because metastasis is frequent issues in pancreatic cancer patients and because these features of this cancer result in shortened prognoses and lowered QOL, future studies may provide a valuable clue(s) for improving the clinical management of patients with pancreatic cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.048.

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