# Enhanced invasiveness of pancreatic adenocarcinoma cells stably transfected with cationic trypsinogen cDNA

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# ENHANCED INVASIVENESS OF PANCREATIC ADENOCARCINOMA CELLS STABLY TRANSFECTED WITH CATIONIC TRYPSINOGEN cDNA

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**Various studies have described increased expression of cationic trypsinogen in malignant tumor cells. To explore the role of secreted cationic trypsinogen in invasion by cancer cells, we introduced cationic trypsinogen cDNA into Panc-1, a pancreatic adenocarcinoma-derived cell line that lacks expression of endogeneous trypsinogen. Four independent clones (designated Panc-1-Try-7, -9, -11 and -24) stably expressing cationic trypsinogen mRNA were isolated and processed for further study. In a zymographic analysis, gelatinolytic activity for cationic trypsinogen was detectable in serum-free conditioned media obtained from all 4 transfectants but not in media from mock-transfected or parental Panc-1 cells. A Matrigel invasion assay revealed that all trypsinogen-expressing transfectants acquired significantly greater invasive ability than that shown by mock-transfected and parental Panc-1 cells. In addition, enhanced invasiveness of the transfectants was suppressed by FUT-175, a serine protease inhibitor, to the level seen in parental cells. These results provide direct evidence that cationic trypsinogen can increase the invasive ability of carcinoma cells.**


**Key words:** *pancreatic carcinoma cells; cationic trypsinogen; invasiveness; transfection; serine proteases*

Proteolytic degradation of extracellular matrix components is a process essential for tumor invasion and metastasis.[1–4] A variety of proteases produced by tumor cells are involved in matrix degradation.[1–4] These enzymes include 2 major categories: matrix metalloproteinases (MMPs) and serine proteases. Although the role of MMPs in tumor malignancy has been studied extensively,[5–9] much less is known about serine proteases, except for urokinase-type plasminogen activator (u-PA).[10–13] Although trypsin, another member of the serine protease family, also has been implicated in the spread of malignant tumor cells, the underlying molecular mechanism has not been fully elucidated.

Several previous reports have described increased amounts of trypsinogen-like enzymes in malignant tumor cells.[14–21] Koshikawa *et al.*[22] identified 1- and 2-chain forms of cationic trypsinogen in a gastric carcinoma cell line. Under the acidic condition that prevails in the extracellular space between malignant tumor cells, cationic trypsinogen is spontaneously converted to trypsin, an active form.[23] Trypsin not only degrades extracellular matrix glycoproteins[22] but also activates proenzymes of various MMP and serine proteases.[19] We previously reported that under acidic conditions cationic trypsinogen produced by human pancreatic ductal cancer spontaneously becomes activated and acquires gelatinolytic activity.[24] Furthermore, we found that serine protease inhibitors had a suppressive effect on invasion and metastasis by human pancreatic cancer cell lines.[25] However, the importance of trypsin in initiating invasion of tumor cells is still unclear; 1 major reason is that no available serine protease inhibitor can produce sufficiently selective inhibition of trypsin.

To obtain more direct evidence that trypsin potentiates tumor invasion, we transfected cationic trypsinogen cDNA into Panc-1, a pancreatic adenocarcinoma-derived cell line that normally is deficient in expression of trypsinogen. The resulting transfectants acquired enhanced invasiveness.

## MATERIAL AND METHODS

### *Cell culture*

Human pancreatic adenocarcinoma cell lines, (Capan-1, BxPC-3, AsPC-1, Panc-1 and MIAPaCa-2), were obtained from the American Type Culture Collection (Rockville, MD). These cell lines were maintained at 37°C in a 5% $CO_2$ incubator and grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% FBS, 100 U/l penicillin and 100 μg/ml streptomycin.

### *Construction of expression plasmid*

Complementary DNA (cDNA) containing the entire protein-coding sequence of human cationic trypsinogen was obtained by reverse transcription (RT) PCR. Primers used for PCR were based on the nucleotide sequence for human cationic trypsinogen:[26] forward, 5′-GGGTCGACACCACCATGAATCCACTCCTG-3′; and reverse, 5′-GGCTCGAGGCTTTA GCTATTGGCAGCTAT-3′. Ten microliters of cDNA, corresponding to 2 μg of total RNA, was amplified in 20 μl of a PCR reaction mixture containing 1 × LA PCR buffer (TaKaRa, Otsu, Japan), 2.5 mM $MgCl_2$, 400 μM each of dATP, dGTP, dCTP and dTTP, 0.2 μM of each primer and 2.5 units of LA Taq (TaKaRa). The reaction was carried out through 30 cycles at 98°C for 20 sec (denaturation) and 68°C for 15 min (annealing and extension). The amplified cDNA was digested with *Xho*I and *Sal*I and the resulting 754 bp fragment was ligated to the 4.5 kb *Sal*I/*Xho*I fragment from pBK-CMV (Stratagene, Los Angeles, CA) to yield the expression plasmid, pBK-CMV-Try. The cDNA insert was verified by nucleotide sequencing.

### *DNA transfection and clone isolation*

Panc-1 cells were transfected with pBK-CMV-Try and pBK-CMV by the cationic liposome method as follows. The DNA-cationic lipid component of Tfx reagent (Promega, Madison, WI) mixed with 10 μg of plasmid DNA was added to $3 \times 10^6$ Panc-1 cells per 100 mm dish. After 48 hr the medium was replaced and geneticin (G418; Sigma, St. Louis, MO) was added to a final concentration of 800 μg/ml. After 2 weeks of G418 selection, trypsinogen-expressing clones (Panc-1-Try) and mock-transfected clones (Panc-1-mock) were isolated. These clones were maintained in RPMI-1640 medium supplemented with 10% FBS and 800 μg/ml G418.

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*RT-PCR analysis*

Total RNA was extracted by the acid-phenol method[27] from exponentially growing cells and subjected to RT-PCR as described above. For detection of cationic trypsinogen mRNA, the same primers as above were used. For u-PA, primers used were: forward, 5′-ACCACCATCGAGAACCAGCC-3′ (positions 2494–2513); and reverse, 5′-AATCAGCTTCACAACAGTCA-3′ (positions 4174–4193). For MMP-2, MMP-9 and β-actin, primers were synthesized as described previously.[28] The amplified products (20 μl) were separated on a 1.2% agarose gel and stained with ethidium bromide.

*Northern analysis*

Total RNA (20 μg) from each cell line was denatured by glyoxal and electrophoresed on a 1% agarose gel. Samples then were blotted onto a nylon membrane (BioSupport; Pall, East Hills, NY) and cross-linked by ultraviolet irradiation. The membrane was prehybridized at 65°C for 2 hr in a solution of 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 × Denhardt's solution, 0.1% sodium *N*-lauroyl sarcosinate, 10 mM EDTA and 250 μg/ml denatured salmon sperm DNA. Hybridization conditions were the same as for prehybridization except for the addition of $^{32}$P-labeled probe. The probe used was the 750 bp *Xho*I/*Sal*I fragment from pBK-CMV-Try. After incubation at 65°C for 24 hr, the membrane was washed in 0.2 × SSC containing 0.1% sodium *N*-lauroyl sarcosinate at 65°C and then exposed to Kodak XAR film with an intensifying screen at −80°C for 48 h.

*Immunohistochemistry*

Cells grown on Lab-Tek chamber slides (Nunc, Naperville, IL) were fixed in a mixture of methanol and acetone (1:1) for 15 min and then air-dried. Immunostaining was performed as reported previously.[17,29] Briefly, the slides were immersed in methanol containing 0.3% $H_2O_2$ for 30 min, blocked with 3.3% normal goat serum in PBS and then incubated with anti-human pancreatic trypsinogen monoclonal antibody (1:50; Chemicon, Temecula, CA). After incubation overnight at 4°C, the slides were incubated with biotinylated goat anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) for 30 min at room temperature and then with streptavidin-biotin-peroxidase complex (Dakopatts) for 30 min at room temperature. Reaction products were developed in 0.02% 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.1% $H_2O_2$. Cells were counterstained with methyl green.

*Gelatin zymography*

Preparation and concentration of serum-free conditioned media were performed as previously.[22] Briefly, cells were grown to semiconfluence in 100 mm dishes containing 10 ml of RPMI-1640 supplemented with 10% FBS. The cultures were washed twice with PBS and then replaced with serum-free RPMI-1640. After incubation for 48 hr, media were titrated with 1 N HCl to pH 4.8, incubated at 37°C for 3 hr and centrifuged at 15,000*g* for 15 min. The resulting supernatants were concentrated 50 times using Vivapore 10 (Vivascience, Binbrook, UK). Each conditioned medium was processed for gelatin zymography as described previously.[7,16] Authentic human trypsin (Athens Research and Technology, Athens, GA) was used as a positive control. Gelatinolytic bands were visualized by staining with Coomassie Brilliant Blue R-250 after incubation in 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ at 37°C for 20 hr.

In vitro *invasion assay*

A rapid *in vitro* invasion assay was performed as described previously[30] using Transwell chambers with polycarbonate membrane filters with pores 8 μm in diameter (Becton Dickinson Labware, Bedford, MA). Basement membrane complex (Matrigel; Becton Dickinson Labware) containing laminin, collagen IV and proteoglycan as major components was applied to the membrane filter of the upper chamber (10 μg/filter) and the filters were dried under a laminar flow hood to produce Matrigel-coated filters.

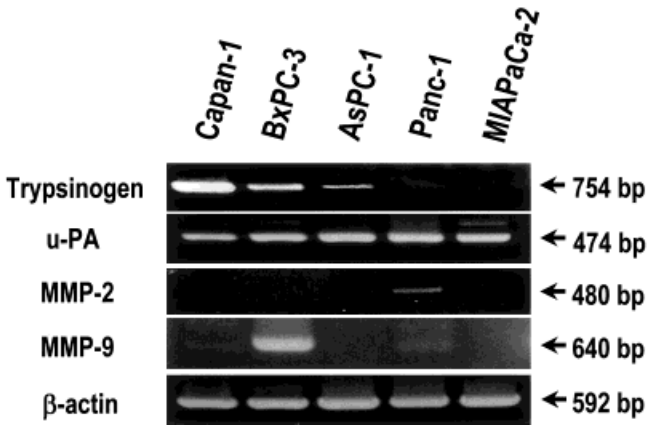



**FIGURE 1** – RT-PCR analysis of human pancreatic cancer cell lines for various proteolytic enzymes. Total RNA from Capan-1, BxPC-3, AsPC-1, Panc-1 and MIAPaCa-2 cells (left to right) was reverse-transcribed and amplified using specific primers for cationic trypsinogen, u-PA, MMP-2 and MMP-9. PCR products were separated on a 1.2% agarose gel and visualized by staining with ethidium bromide. β-Actin gene expression was used to confirm that equal amounts of RNA were used in each RT-PCR reaction. The size of PCR products is indicated in base pairs.

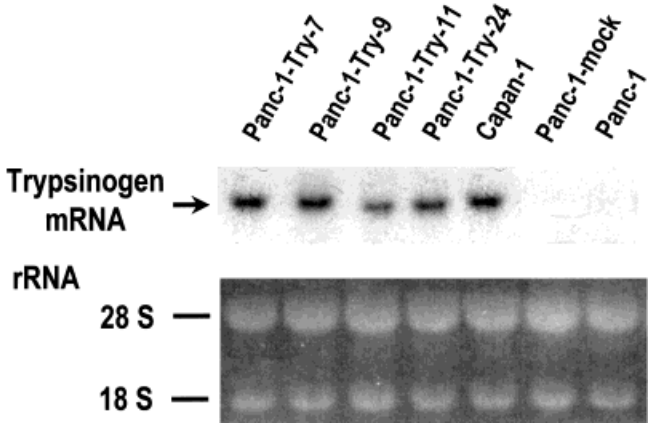



**FIGURE 2** – Northern analysis of Panc-1 cells transfected with human cationic trypsinogen cDNA. Top: Autoradiogram obtained by hybridization with a $^{32}$P-labeled cDNA probe specific for human cationic trypsinogen. Left to right: Panc-1 clones stably transfected with cationic trypsinogen cDNA (Panc-1-Try-7, -9, -11 and -24), Capan-1 cells as a control, Panc-1-mock cells and parental Panc-1 cells. Expression of cationic trypsinogen mRNA (arrow) is detectable in all Panc-1-Try clones and Capan-1 cells, but not in Panc-1-mock or parental cells. Bottom: Ethidium bromide staining of 28S and 18S ribosomal RNA (rRNA).

Subsequently the filters were exposed to 500 μl of warm culture medium and rehydrated at 37°C for 1.5 hr. Cell suspensions were adjusted to a concentration of $10^6$ cells/ml with 0.1% BSA and the final volume of 500 μl ($5 \times 10^6$ cells/filter) was immediately placed in the Matrigel-treated upper chamber. The lower chamber was filled with 1 ml of conditioned medium. After incubation at 37°C for 24 hr in a 5% $CO_2$ incubator, the cells on the upper surface of the filter were completely removed with cotton swabs. The filter was then stained with hematoxylin and eosin and the invasive potential of the cells was estimated using a light microscope by counting the number of cells that had migrated to the lower surface of the filter in 10 different areas. Each assay was performed in triplicate and repeated 3 times.

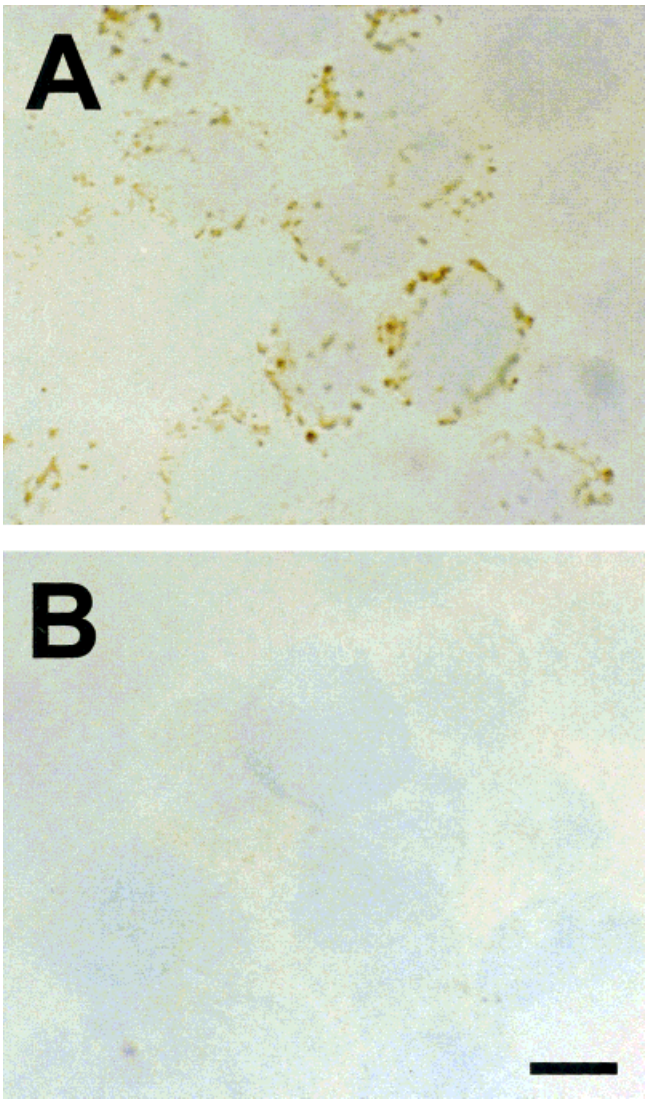



**FIGURE 3** – Immunoperoxidase staining for trypsinogen in Panc-1-derived transfectants. Panc-1-Try cells (*a*) and Panc-1-mock cells (*b*) were incubated with anti-human pancreatic trypsinogen monoclonal antibody. Immunoreactivity was visualized by the avidin-biotin complex method. Trypsinogen immunoreactivity is apparent in the cytoplasm of Panc-1-Try cells, but not Panc-1-mock cells. Scale bar = 10 μm.

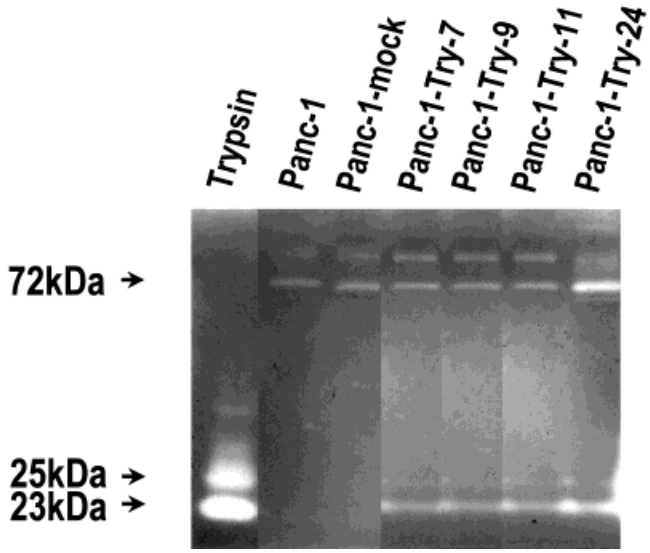



**FIGURE 4** – Zymographic analysis for proteolytic enzymes secreted into media from Panc-1-derived cell lines. Left to right: Authentic human trypsin used as a size standard, parental Panc-1, Panc-1-mock and Panc-1-Try-7, -9, -11 and 24 cells. Panc-1-Try clones exhibit gelatinolytic activity corresponding to cationic trypsin (25 and 23 kDa). Additional gelatinolytic activity, consistent with MMP-2 (72 kDa) and an unknown protease (around 92 kDa) is present in all cell lines examined.

In some experiments invasiveness of cell lines was examined in the presence of a serine protease inhibitor, 6-amidino-2-naphthyl-*p*-guanidinobenzoate dimethane-sulfonate (FUT-175; Torii Pharmaceuticals, Tokyo, Japan). The cytotoxic effect of FUT-175 was assessed by the methylthiazol tetrazolium bromide (MTT) colorimetric assay.[31]

### *Statistical analysis*

Data are expressed as the mean ± SD. Group differences were examined using unpaired *t*-tests. A *p*-value of $< 0.05$ was considered to indicate a significant difference.

## RESULTS

### *Establishment of cell lines stably transfected with trypsinogen cDNA*

To identify cell lines suitable for cDNA transfection, we screened various pancreatic adenocarcinoma cell lines (Capan-1, BxPC-3, AsPC-1, Panc-1 and MIAPaCa-2) for expression of tumor-associated protease genes. As shown in Figure 1, RT-PCR analysis revealed a substantial amount of u-PA mRNA in all cell lines. The mRNA encoding pancreatic trypsinogen was detected in Capan-1, BxPC-3 and AsPC-1 cells, but not in Panc-1 or MIAPaCa-2 cells (Fig. 1). Although they lacked trypsinogen mRNA, Panc-1 cells exhibited expression of transcripts for u-PA and MMP-2. Based on these observations, we chose Panc-1 cells to determine whether trypsinogen expressed from transfected cDNA could influence invasive behavior of cells that did not normally express trypsinogen.

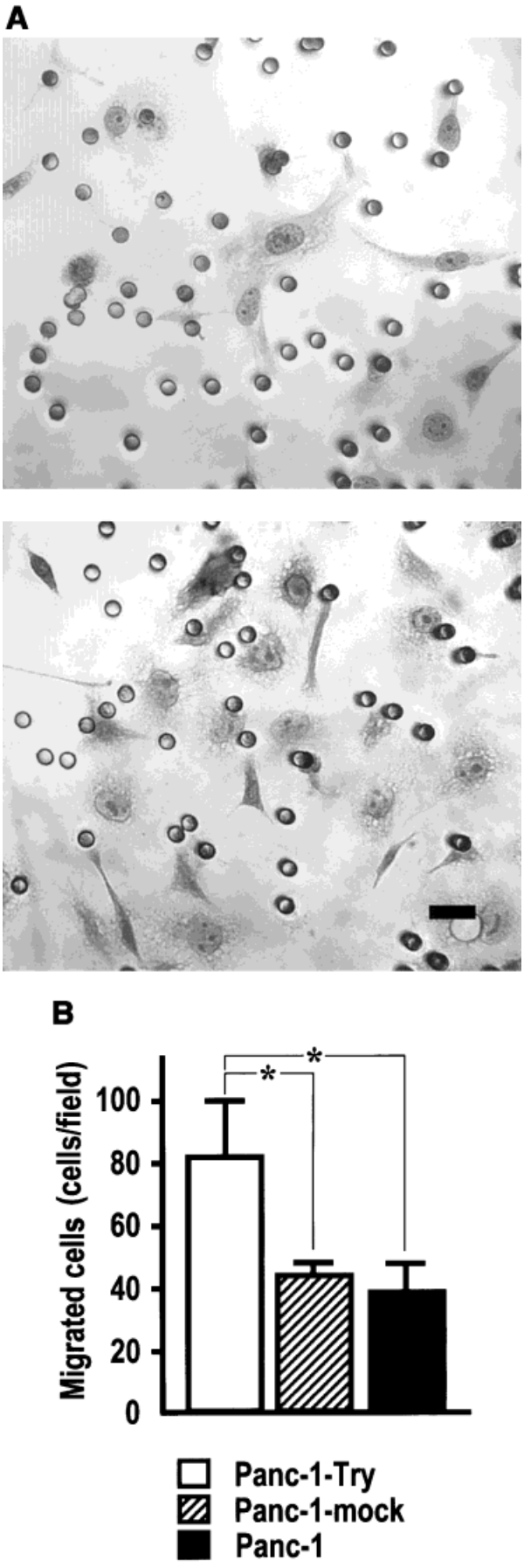
After transfecting Panc-1 cells with pBK-CMV-Try, an expression plasmid for human trypsinogen, we isolated 25 independent G418-resistant clones and processed the cells for Nothern analysis. Four of these clones, designated Panc-1-Try-7, -9, -11 and -24, expressed a 0.75 kb transcript that hybridized with trypsinogen cDNA (Fig. 2); the size was consistent with that expected from the expression construct. In contrast, no hybridizing band was detectable in mock-transfected (Panc-1-mock) or parental Panc-1 cells (Fig. 2). The levels of trypsinogen mRNA in the transfectants were almost the same as that in Capan-1 cells, which retain invasion properties similar to those observed in pancreatic tumors *in vivo*[25] (Fig. 2).

### *Immunocytochemistry*

Expression of exogenous trypsinogen was examined at the protein level. Cells stably transfected with trypsinogen cDNA displayed intense cytoplasmic immunoreactivity for pancreatic trypsinogen (Fig. 3*a*), whereas no immunoreactivity was observed in Panc-1-mock cells (Fig. 3*b*) or parental Panc-1 cells (data not shown).

### *Gelatin zymography*

To examine whether expressed trypsinogen was secreted extracellularly and was convertible to an active form, we analyzed gelatinolytic activity in serum-free conditioned medium prepared from each cell line. Consistent with authentic trypsin, gelatinolytic bands at 25 and 23 kDa were detected in media from Panc-1-Try-7,




-9, -11 and -24 cells but not in Panc-1-mock or parental Panc-1 cells (Fig. 4). These 2 bands agreed well with those reported for the activated forms of 2- and 1-chain cationic trypsinogen, respectively.[22] In addition, all supernatants exhibited gelatinolytic activity at 72 kDa, which corresponded to MMP-2, in good agreement with data obtained by RT-PCR (Fig. 2). We also detected a gelatinolytic band at around 92 kDa. The identity of this protease is currently unknown.

*Invasive ability of transfected cell lines*

The invasive ability of transfected cell lines was evaluated by a Matrigel invasion assay. The trypsinogen-expressing transfectants invaded Matrigel more readily than Panc-1-mock cells (Fig. 5). No change in cell shape was detected in the transfected cells (Fig. 5*a*). As shown in Figure 5*b*, Panc-1-Try cells exhibited significantly higher invasive ability (81 $\pm$ 19 cells/field, $n = 4$) than Panc-1-mock cells (42 $\pm$ 4 cells/field, $n = 3$) or parental Panc-1 cells (38 $\pm$ 6 cells/field, $n = 3$) in a 24 hr assay ($p < 0.05$, unpaired *t*-test).

Figure 6 shows the effects of FUT-175, a potent inhibitor of serine protease,[32] on cell invasion. Significant inhibition ($p < 0.05$) was observed in Panc-1-Try clones at concentrations as low as 0.1 $\mu$M; in contrast, invasiveness of Panc-1-mock and parental Panc-1 cells remained unchanged. In an MTT colorimetric assay, FUT-175 (0.1, 1 and 10 $\mu$M) had no cytotoxic effect (data not shown). These results indicate that the suppressive effect on invasiveness resulted from inhibition of trypsin, not from nonspecific cytotoxicity.

## DISCUSSION

In this study we stably transfected pancreatic adenocarcinoma cells with cDNA encoding human cationic trypsinogen. The expression of cationic trypsinogen in the established transfectants was confirmed by Northern blot and immunocytochemical analyses. Furthermore, active trypsin was detected by gelatin zymography only in conditioned media from these transfectants, although the secretion of trypsinogen was not steadily detectable by Western blot analysis (data not shown), presumably due to insufficient sensitivity. Compared with mock-transfected and parental Panc-1 cells, significant enhancement of invasiveness was observed in all transfectants examined. We further demonstrated that enhanced invasiveness was suppressed by FUT-175, a potent inhibitor of serine protease. These data indicate that trypsin expressed from the transfected cDNA strongly potentiated the invasive ability of Panc-1 cells.

To date, several reports have documented increased expression of trypsin or trypsin-like enzymes in a variety of cancer cell lines and tissues.[14–19,21] This activated trypsin extensively degraded laminin, fibronectin, gelatin and certain collagens.[14–16] By zymographic analysis, we found that basement membrane complex Matrigel was also degraded by trypsin (unpublished data). Trypsinogen is thus thought to participate in both degradation of extracellular matrix components and activation of other pro-enzymes such as pro-MMP and serine proteases.[14–19] Trypsin also can stimulate tumor growth and enhance tumor adhesion to extra-

**FIGURE 5** – *In vitro* Matrigel invasion assay. (*a*) Photomicrographs showing Panc-1-Try-24 (top) and parental Panc-1 cells (bottom) that migrated to the lower surface of the Matrigel-coated filter. Cells had been plated on the opposite surface of these filters and incubated for 24 hr before staining with hematoxylin and eosin. Many more Panc-1-Try-24 cells have invaded the filter than parental cells. Scale bar = 20 $\mu$m. (*b*) Comparison of invasive ability of Panc-1-Try clones, Panc-1-mock and parental Panc-1 cells. Migrated cells such as Panc-1 were counted. Panc-1-Try-7, -9, -11 and -24 cells were used for the assay. Each value represents the mean $\pm$ SD of 3 independent assays done in triplicate. *, $p < 0.05$ compared with Panc-1-Try clones by unpaired *t*-test.

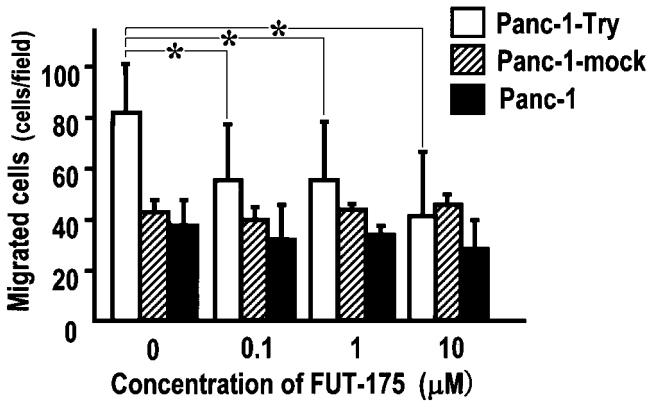



**FIGURE 6** – Effect of a serine protease inhibitor on invasiveness of Panc-1-Try clones, Panc-1-mock and parental Panc-1 cells. Cells were cultured on Matrigel-coated filters in the presence of FUT-175 at the indicated concentrations. After 24 hr, cells that migrated were counted as in Figure 5. Each value represents the mean ± SD of 3 independent assays performed in triplicate. *, $p < 0.05$ compared with control (untreated) cells by an unpaired *t*-test.

cellular matrix components.[20,21] In previous studies, we demonstrated in pancreatic cancer cell lines that hepatic metastasis and invasive ability were correlated with amounts of cationic trypsinogen and were inhibited by a serine protease inhibitor.[24,25]

Considering the results of this earlier work, secreted cationic trypsin is likely to degrade extracellular matrix components in several ways, including activating trypsinogen itself as well as latent proenzyme forms of other proteases. Notably, Panc-1 cells express u-PA and MMP-2 (Figs. 1,4), but despite this expression Panc-1 cells show less invasiveness than other cell lines. These observations suggest that transgene-derived trypsinogen may have enhanced tumor cell invasiveness by activating these proteases. Since trypsinogen is released into the extracellular space of carcinoma cells in an inactive form, activation represents a critical regulatory step in degradation of the extracellular matrix.

In this regard, 2 possible mechanisms have been proposed: autoactivation under acidic conditions[23,33,34] and activation by lysosomal cysteine proteases such as cathepsin B.[23,35] The extracellular pH within malignant tumors is known to be lower than that in normal tissue.[36] In breast cancer cells, Montcourrier *et al.*[37] have demonstrated that both the vacuolar type proton pump (V-ATPase) and lactic acid production contribute to acidification of the extracellular milieu. We have shown that V-ATPase is overexpressed in carcinoma-derived cell lines including Panc-1 cells, as well as in human pancreatic cancer tissue.[38] This overexpression of V-ATPase may promote autoactivation of trypsinogen expressed by Panc-1-derived transfectants. Although the precise mechanism for activation of trypsinogen from transfectants is not yet defined, our established transfectants should serve as a useful model for further analysis.

In summary, using a heterologous expression system, we demonstrated that cationic trypsinogen could potentiate invasion by carcinoma cells. Our results suggest that trypsin may act *in vivo* as a key factor in tumor invasion and possibly metastasis.

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