

# Polyserase-1/TMPRSS9 induces pro-tumor effects in pancreatic cancer cells by activation of pro-uPA

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**Abstract.** Polyserase-1/TMPRSS9 is a type II transmembrane serine protease showing a complex molecular architecture characterized by the presence of three tandem serine protease domains in its amino acid sequence. This protease is widely expressed in mouse and human tissues, however, its functional significance is unknown in both normal and pathological conditions. In the present study, we evaluated the possible role of polyserase-1 in cancer progression. First, we showed that polyserase-1 increased the invasive capacities of PANC-1 and SK-PC-3 pancreatic cancer cells. Moreover, the presence of polyserase-1 enhanced anchorage-independent growth and diminished the adhesion capability of PANC-1 cells to different extracellular matrix components. These effects were mediated by the efficient conversion of pro-uPA to active uPA and high phosphorylation levels of ERK detected in the PANC-1 cells expressing exogenous polyserase-1. Collectively, our data suggest that polyserase-1 may be involved in cancer progression and, similarly to what has been proposed for the closely related serine proteases matriptase and TMPRSS4, inhibition of TMPRSS9 activity may contribute to the inhibition of tumor growth.

## Introduction

The tightly controlled proteolytic turnover of extracellular matrix (ECM) components in the immediate vicinity of the cell has a significant influence in the development and maintenance of tissue homeostasis (1). Proteolytic events within this pericellular microenvironment are mainly executed by extracellular or cell-surface metalloproteases and serine proteases (2). Among them, the type II transmembrane serine proteases (TTSPs), are membrane-anchored serine proteases

involved in relevant biological functions (3). Thus, enteropeptidase is produced by the duodenum and its function is essential to digest food efficiently (4). Corin contributes to blood pressure control through activation of atrial natriuretic peptide (5). Matriptase-2 participates in iron homeostasis (6), and hepsin is implicated in the process of hearing (7). These and other examples (8) illustrate the relevance of TTSPs in normal physiological processes.

The activities of the TTSPs also underlie different human disorders, including cancer. In fact, these proteases can cause an active pericellular degradation to breach tissue barriers thereby facilitating invasion of surrounding tissues (9). Moreover, this pericellular proteolysis may also trigger the activation of growth factors that promote tumor cell proliferation and migration (10). However, TTSPs can also display antitumor effects. For instance, *HPN*, the gene encoding for human hepsin, is one of the most highly upregulated in prostate cancer, and this enzyme is exclusively detected in the surface of prostate carcinoma cells (11). In contrast, low expression levels of hepsin correlate with shorter patient survival in hepatocellular carcinoma (12). Similarly, expression of matriptase was found to be markedly upregulated in different tumors of epithelial origin, including breast, lung or pancreatic carcinomas (13). However, a significant decrease in its expression was found in gastrointestinal tumors (14). *DESC1* serine protease exhibits reduced expression levels in head and neck carcinomas in comparison with matched normal tissues (15), while its exogenous expression confers pro-tumor effects to MDCK cells and it was found to be upregulated in kidney, liver, breast and brain tumors (16). Additionally, different studies have highlighted the functions of different TTSPs as regulators of cellular signaling pathways and revealed the biochemical mechanisms underlying their pro-tumor or anti-tumor effects (9).

Despite the increased understanding of the functional relevance of TTSPs, some members of this family have been characterized at the structural level while their roles in normal or pathological processes remain unknown. This is the case of polyserase-1 or transmembrane protease, serine 9 (TMPRSS9), a TTSP widely distributed in human and mouse tissues (17,18). Structurally, this enzyme shows a complex molecular architecture containing three tandem serine-protease domains called serase-1, serase-2 and serase-3 (17). The third domain

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is predicted as catalytically inactive due to the presence of an alanine residue instead of the serine residue present in the active site of this type of enzyme. Serase-1B is a splice variant of TMPRSS9 containing only one serase domain which efficiently activates pro-uPA (18). Active uPA converts plasminogen to plasmin, an enzyme that cleaves different ECM components and activates latent collagenases. These effects contribute to the invasiveness of tumor cells of different origin, including pancreatic carcinomas. In fact, uPA exhibits marked overexpression in this type of tumor, and its detection provides prognostic information (19). Different reports have emphasized the pro-tumor activities of different TTSPs in pancreatic cancer (9,20,21). Activation (22) or upregulation (23) of pro-uPA may underlie the pro-tumor effects caused by these serine proteases. In the present study, we investigated the effects of the exogenous expression of polyserase-1 in pancreas-derived tumor cells (24,25). Collectively, our data suggest that polyserase-1 exerts pro-tumor effects through a mechanism involving activation of pro-uPA and phosphorylation of ERK.

## Materials and methods

**Cell lines and culture conditions.** The cell line PANC-1 was purchased from Cell Lines Service and the SK-PC-3 cell line was kindly provided by Dr Francisco Real (National Cancer Research Centre, Madrid, Spain). Cells were routinely maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

**Transfection and western blot analysis.** The cell lines were transfected with the plasmid pcDNA3-FLAG-poly1, which contains the complete sequence of polyserase-1 and a FLAG epitope at the amino terminal region (17). Cells transfected with an empty pcDNA3 vector were employed as a control. Transfections were performed using the Lipofectamine system (Invitrogen) following the manufacturer's recommendations. Cells were selected in the presence of G-418 (Sigma-Aldrich) at 400 µg/ml. For western blot analysis, the proteins were separated on SDS/PAGE, and blotted onto PVDF membranes. Detection of exogenous polyserase-1 was performed using an anti-FLAG antibody (Sigma Aldrich), and the H-140 antibody from Santa Cruz Biotechnology was used to detect uPA. Antibodies to detect p-ERK and ERK were from Cell Signalling Technology.

**qRT-PCR.** Total RNA from cell cultures was obtained using the RNeasy Mini kit (Qiagen) following the manufacturer's recommendations. For cDNA synthesis, 2 µg of RNA was used as a template and the system ThermoScript RT-PCR (Invitrogen) was employed following the protocol recommended by the manufacturer. cDNA was then used as a template for quantitative PCR using TaqMan probe HS00917112\_m1, TaqMan Master Mix reagent and the detection system 7300 Real-Time PCR system (Applied Biosystems). Expression of human β-actin was employed as endogenous control. Data analysis was performed using the computer program ABI PRISM 7900HT, and RQ Manager 1.2 (Applied Biosystems).

**Cell invasion assay.** *In vitro* invasion potential was evaluated using 24-well Matrigel-coated invasion chambers with a 8-µm pore size (BD Biosciences). Cells (6x10<sup>4</sup>) were allowed to migrate for 24 h using 10% FBS as a chemoattractant. Cells that reached the lower surface were fixed and stained with crystal violet. Three independent experiments were performed with triplicates for each condition. Cells were counted in eight randomly selected microscopic fields.

**Soft agar colony formation.** Anchorage-independent growth was examined using CytoSelect™ 96-Well In Vitro Tumor Sensitivity Assay kit (Cell Biolabs) following the manufacturer's instructions. Cell density was 6x10<sup>3</sup> cells/ml, and assays were performed for 8 days at 37°C and 5% CO<sub>2</sub> using 24-well Ultra-low attachment plates (Costar). Colonies were examined and photographed with a Leica microscope EZ 2.0 (Leica Microsystems).

**Cell adhesion assay.** The adhesion capacity of the different cell lines was analyzed using a colorimetric ECM Cell Adhesion Array kit (EMD Biosciences) following the manufacturer's instructions. Briefly, 2x10<sup>5</sup> cells were incubated for 2 h. The cells were then lysed, and the absorbance was measured at 485 nm using a Synergy H4 Hybrid reader. All data are the mean of three independent experiments.

**Statistical analysis.** Statistical analysis was performed using the GraphPad Prism 5.0 software. Data are represented as means ± SE. The occurrence of significant differences was determined with the Student's-Welch t-test. P-values <0.05 were considered to indicate statistically significant differences.

## Results

**Exogenous expression of TMPRSS9/polyserase-1 in pancreatic cancer cells.** To examine whether polyserase-1 affects the tumorigenic phenotype of pancreatic cancer cells, we employed two cell lines, PANC-1 and SK-PC-3, to perform further functional analysis. These cell lines do not endogenously express TMPRSS9 as assessed by qRT-PCR; therefore, cells were transfected using the full-length cDNA for polyserase-1 (17) (Fig. 1A). Expression of exogenous polyserase-1 was assessed using qRT-PCR and western blot analysis by detection of the FLAG epitope at the amino-terminal region of the recombinant protein (Fig. 1A). Cells that stably expressed TMPRSS9, referred to as PANC-1/TMPRSS9 and SK-PC-3/TMPRSS9, respectively, were chosen for further evaluation of potential phenotypic changes. PANC-1 and SK-PC-3 cells transfected with an empty vector, PANC-1c and SK-PC-3c, respectively, did not show immunoreactivity for the antibodies against FLAG epitope and were used as negative controls.

**Polyserase-1 increases invasive properties of pancreatic cancer cells.** Invasive capacity of the PANC-1/TMPRSS9 and SK-PC-3/TMPRSS9 cells was examined using Matrigel-coated invasion chambers (Fig. 1B). Average cell numbers penetrating the Matrigel per field were 312 in the case of PANC-1/TMPRSS9 cells and 62 in the case of SK-PC-3/TMPRSS9 cells. However, only 102 PANC-1c and 10 SK-PC-3c invasive cells per field were observed in this assay (Fig. 1B). These data indicated that

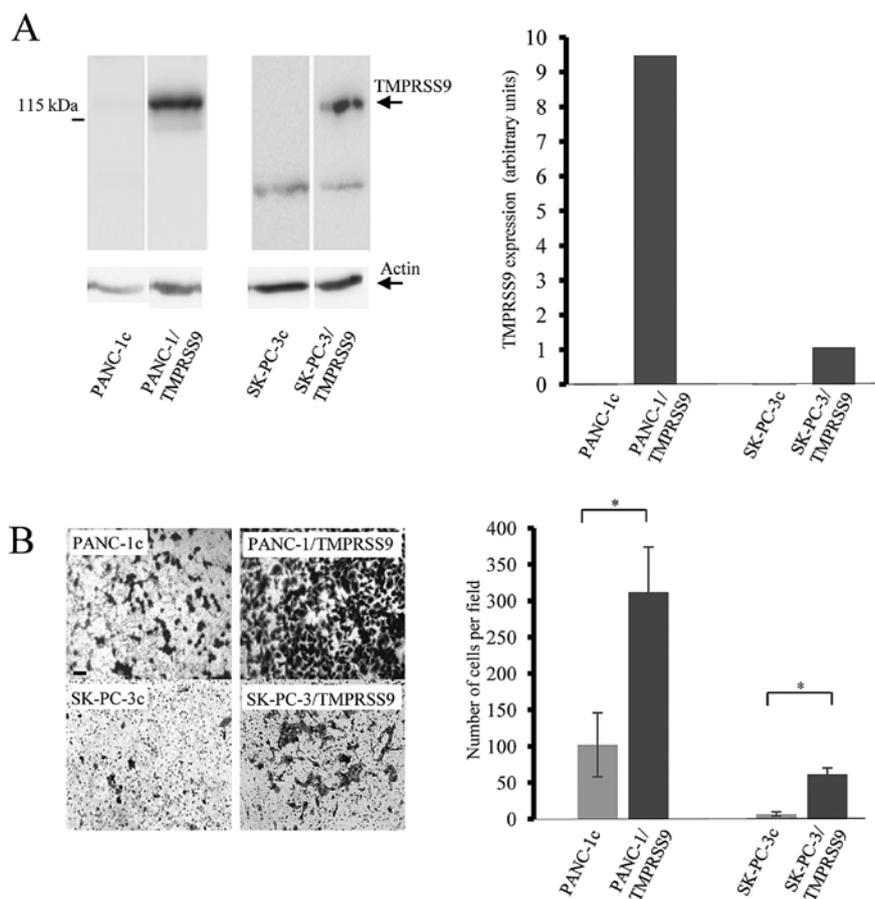


Figure 1. Selection of PANC-1 and SK-PC-3 stable transfectants and invasion assay. (A) Left: western blot analysis of PANC-1 and SK-PC-3 cells expressing exogenous polyserase-1/TMPRSS9, indicated as PANC-1/TMPRSS9 and SK-PC-3/TMPRSS9, respectively. PANC-1c and SK-PC-3c indicate the control cells, transfected with an empty vector. The molecular weight marker is indicated on the left. Actin was used as a loading control. Right: qRT-PCR for mRNA expression levels of TMPRSS9 in the indicated cells. (B) Cell invasion assay using Matrigel-coated invasion chambers. Left: representative microscopic images of the indicated cells. Scale bar, 50  $\mu$ m. Right: cells that reached the lower surface were counted, and the numbers are graphically represented. \* $p < 0.05$ .

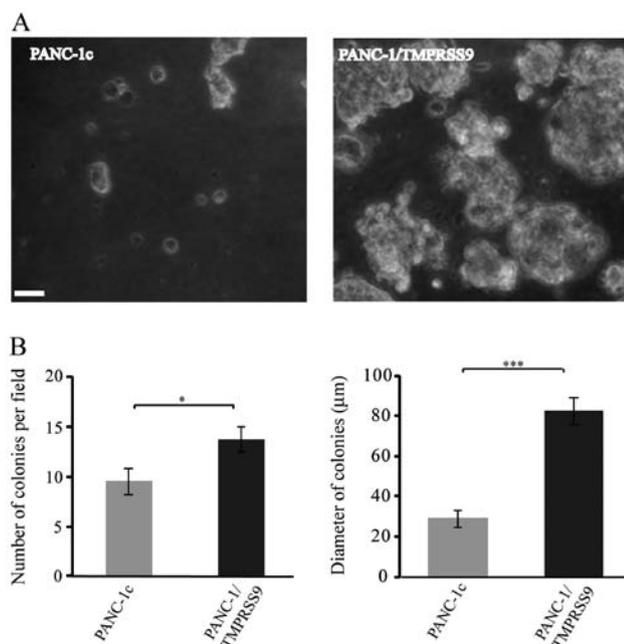


Figure 2. Polyserase-1/TMPRSS9 increases anchorage-independent growth of PANC-1 cells. (A) Representative microscopic images of colonies formed by PANC-1c and PANC-1/TMPRSS9 cells. Scale bar, 50  $\mu$ m. (B) Graphical representation of the number and diameter of the colonies. \* $p < 0.05$ , \*\*\* $p < 0.005$ .

the presence of polyserase-1 markedly increases the invasive capacity of both PANC-1 and SK-PC-3 cells.

*Polyserase-1 enhances anchorage-independent growth of PANC-1 cells.* Results obtained from invasive assays prompted us to perform new cell-based assays using PANC-1, a cell line commonly employed as an *in vitro* model of pancreatic cancer for tumorigenicity studies since its invasive and proliferative phenotypes have been widely characterized (24). Thus, we carried out a soft-agar colony formation assay to compare the PANC-1/TMPRSS9 and PANC-1c cells and, as shown in Fig. 2A, the colonies formed by cells expressing polyserase-1 were more numerous (14 vs. 9) and much larger (82  $\mu$ m vs. 30  $\mu$ m in diameter) than those formed by the PANC-1c cells after 8 days of growth (Fig. 2B). These data are also consistent with the hypothesis that polyserase-1 acts a pro-tumor protease.

*Presence of polyserase-1 activates pro-uPA and increases phosphorylation levels of ERK.* Different mechanisms have been proposed to underlie the pro-tumor properties associated with the activities of TTSPs, including activation of pro-uPA or modulation of the ERK signaling pathway (23). We examined whether exogenous polyserase-1 also affects these mechanisms in PANC-1 cells. Toward this end, we performed

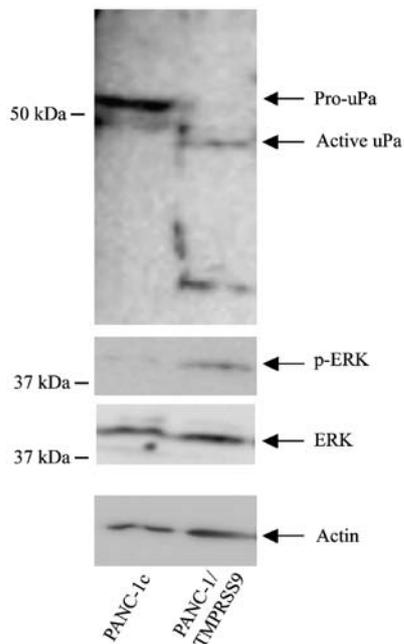


Figure 3. Western blot analysis showing pro-uPA activation and levels of phosphorylated ERK (p-ERK) and total ERK (ERK) in PANC-1c and PANC-1/TMPRSS9 cells. Molecular weight markers are indicated on the left. Actin was used as a loading control.

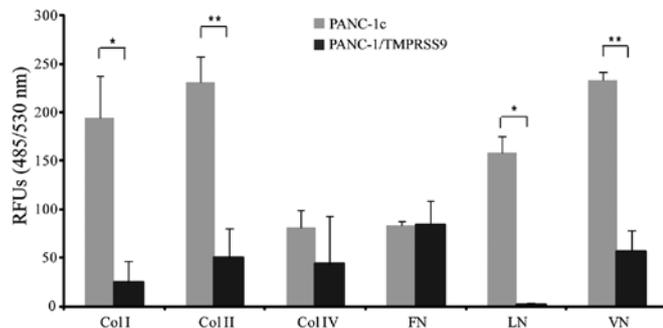


Figure 4. Presence of polyserase-1/TMPRSS9 modifies the adhesion profile of PANC-1 cells to different ECM components. Col I, type I collagen; Col II, type II collagen; Col IV, type IV collagen; FN, fibronectin; LN, laminin; VN, vitronectin. \* $p < 0.05$ , \*\* $p < 0.01$ .

western blot analysis that revealed a significant activation of pro-uPA and high levels of phosphorylated ERK (p-ERK) in the PANC-1/TMPRSS9 but not in the PANC-1c cells (Fig. 3). These results suggest that TMPRSS9 may contribute to induce cancer cell invasion by enhancing the activation of survival pathways in tumor cells.

#### *Polyserase-1 alters the adhesive properties of PANC-1 cells.*

To evaluate whether polyserase-1 alters adhesion of PANC-1 cells to different ECM substrates, we performed a fluorometric assay using PANC-1/TMPRSS9 and PANC-1c cells. The results clearly indicated that the presence of polyserase-1 reduced the capacity of PANC-1 cells to bind to collagens as well as to vitronectin, and completely abolished adherence to laminin (Fig. 4). However, polyserase-1 did not alter the binding capacity of PANC-1 cells to fibronectin. Overall, these results

indicated that polyserase-1 induced changes in cell adhesion which eventually leads to increased tumor progression.

## Discussion

Proteolytic enzymes have been traditionally associated with tumor progression. In fact, proteases produced by cancer cells contribute not only to destroy the ECM barrier but also to actively release growth factors to control cell survival. However, recently, a growing number of proteases have also been associated with tumor-protective functions (26). One future challenge includes the identification of the precise functions of the proteolytic enzymes related to tumorigenesis. In this regard, we evaluated the potential contribution of polyserase-1/TMPRSS9 to tumorigenesis employing cell-based assays. This unique enzyme belongs to the TTSP family of membrane-anchored serine proteases and is widely distributed in human and mouse tissues, yet its functional relevance is largely unknown (18). Polyserase-1 possesses three serine-protease domains in tandem each of which shows a high degree of identity with other members of the TTSP family, particularly with those belonging to the matriptase subfamily (17). Different TTSPs such as TMPRSS4, initially referred to as TMPRSS3 (20) and matriptase (21) have been shown to display pro-tumor activity in various types of tumors, including pancreatic carcinoma, one of the most aggressive cancers. In the present study, we demonstrated that polyserase-1 also acts as a pro-tumor protease. Thus, the presence of this enzyme increased the invasive properties of both SK-PC-3 and PANC-1 pancreatic cancer cells using Matrigel-coated invasion chambers. Moreover, different reports have illustrated the usefulness of PANC-1 to examine the effect of the overexpression (27) or downregulation (28) of particular genes on anchorage-independent growth, a hallmark of malignant transformation. Using similar approaches, we also found that the presence of polyserase-1 considerably enhanced this effect in PANC-1 cells.

Serase-1B, a spliced variant of polyserase-1 containing one serine protease domain, is able to activate pro-uPA (18), a serine protease largely involved in tumor progression. PANC-1 cells expressing polyserase-1 were also found to exhibit significant pro-uPA activation when compared to PANC-1 control cells. Furthermore, the presence of polyserase-1 also increased levels of ERK phosphorylation in this cell line. Similar effects have been previously associated to pro-tumor TTSPs. For example, TMPRSS4 upregulates uPA expression and activation in the DU145 prostate cancer and the NCI-H322 lung cancer cell lines by a mechanism involving modulation of the ERK signaling pathway (23). Matriptase is also able to activate pro-uPA in the ovarian cancer cell line HRA (29) and the monocytic leukemia cell line THP-1 (22). On the other hand, induction of invasive and migratory capacities of PANC-1 cells has been linked to activation of ERK by phosphorylation (30). Collectively, our data suggest that TMPRSS9 exerts pro-tumor effects on PANC-1 cancer cells overcoming apoptotic signals induced in anchorage-dependent cells when detaching from the surrounding ECM.

ECM components also affect the adhesive properties of tumor cells. In the case of PANC-1 cells, adhesion capacity to type I, II and IV collagens, laminin, fibronectin and vitronectin

has been previously assessed (24). Our data indicated that polyserase-1 reduces the adhesion capacity of PANC-1 cells to collagens, laminin and vitronectin which may contribute to the promotion of cell migration and invasion (31). In reference to other TTSPs, overexpression of matriptase and TMPRSS4 was also found to alter the adhesion profile of tumor cells to ECM components thereby contributing to facilitate their invasive and metastatic properties (32,33).

In summary, in the present study, we provide initial evidence suggesting that polyserase-1 induces pro-tumor activities in pancreatic cancer cells and that uPA and ERK may participate in these processes. Consequently, polyserase-1 may be involved in tumor progression, and inhibition of its activity could contribute to prevent progression of this malignant tumor. Further exhaustive *in vivo* functional studies will help to shed light on the participation of polyserase-1 in tumorigenesis. In this regard, generation of mice lacking TMPRSS9 may be a useful tool to understand the precise role of this polyserase-1 not only in pancreatic carcinoma but also in tumors from different origins.

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