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p8 inhibits the growth of human pancreatic cancer cells and its expression is induced through pathways involved in growth inhibition and repressed by factors promoting cell growth

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Abstract

Background: p8 is a stress-induced protein with multiple functions and biochemically related to the architectural factor HMG-I/Y. We analyzed the expression and function of p8 in pancreatic cancer-derived cells.

Methods: Expression of p8 was silenced in the human pancreatic cancer cell lines Panc-1 and BxPc-3 by infection with a retrovirus expressing p8 RNA in the antisense orientation. Cell growth was measured in control and p8-silenced cells. Influence on p8 expression of the induction of intracellular pathways promoting cellular growth or growth arrest was monitored.

Results: p8-silenced cells grew more rapidly than control cells transfected with the empty retrovirus. Activation of the Ras→Raf→MEK→ERK and JNK intracellular pathways down-regulated p8 expression. In addition, the MEK1/2 inhibitor U0126 and the JNK inhibitor SP600125 up-regulates expression of p8. Conversely, p38 or TGFβ-1 induced p8 expression whereas the specific p38 inhibitor SB203580 down-regulated p8 expression. Finally, TGFβ-1 induction was in part mediated through p38.

Conclusions: p8 inhibits the growth of human pancreatic cancer cells. p8 expression is induced through pathways involved in growth inhibition and repressed by factors that promote cell growth. These results suggest that p8 belongs to a pathway regulating the growth of pancreatic cancer cells.

Background

While studying the molecular response of the injured pancreas, we identified a new gene, called p8, whose expression is strongly induced during the acute phase of pancreatitis [1]. Further experiments have shown that p8 mRNA is activated in almost all cells in response to several

stresses [2], including minimal stresses such as after routine change of the culture medium in the absence of any added substance [3], indicating that p8 is a ubiquitous protein induced by cellular stress. The p8 gene was cloned in human, rat, mouse, and *Xenopus laevis* [1,4–6], conceptually translated from the *Drosophila melanogaster* genome

or deduced from EST libraries (*Bos taurus*, *Xenopus tropicalis*, *Zebrafish*, *Orzias latipes*, *Bombyx mori* and *Paralichthys olivaceous*). The overall degree of homology with human p8 ranged from 81 to 40%. Secondary structure prediction methods indicated that within the homologous region of the eleven proteins, there is a basic Helix-Loop-Helix secondary structure motif, characteristic of some classes of transcription factors [1]. Even though a small protein such as p8 would not need a nuclear localization signal (NLS) to be transported to the nucleus, a clear NLS can be predicted for the eleven proteins comprising a bipartite domain of positively charged aminoacids. In addition, a nuclear/cytoplasmic location has been demonstrated for human p8 upon overexpression of the recombinant protein and immunohistochemistry [4], and for recombinant *Xenopus laevis* p8 fused to green fluorescent protein [6]. Homology searching in databases did not reveal significant similarity of p8 with other proteins of known function. However, biochemical properties of the mammalian p8 proteins are shared by some high mobility group proteins (HMG) [7], particularly by the HMG-I/Y family. The overall identity of human p8 with human HMG-I/Y is only about 35%, but the molecular mass, isoelectric point, hydrophilicity plot, the resistance to denaturation after heating at 100°C and the charge separation are very similar [8]. The p8 protein seems to bind DNA weakly, as shown by electrophoretic mobility shift assay, without preference for DNA sequences. Finally, human p8 has also been shown to be a substrate for protein kinase A *in vitro* and phosphorylated p8 has a higher content of secondary structure and binding to DNA is highly increased [8]. An architectural role in transcription has been proposed for this protein, in analogy with the HMG-I/Y proteins, and a recent work seems to confirm this hypothesis [9].

Functions of p8 appear to be multiple and complex. For example, p8 mRNA expression was strongly induced in 3T3 cells upon TGFβ-1 treatment which in turn enhances the Smad-transactivating function responsible for TGFβ-1 activity [10]. We also found that p8 is involved in cell cycle regulation since p8-deficient embryonic fibroblasts grew more rapidly and incorporated more [³H] thymidine and BrdU than p8-expressing cells [11]. Moreover, expression of p8 in breast cancer-derived cells seems to mediate the inhibition of cell growth induced by 1,25-Dihydroxyvitamin D3 [12]. On the contrary, we also reported that p8 may promote cell growth when overexpressed in Cos-7, AR42J and HeLa cells [1,4]. In addition, p8 seems to be involved in other intracellular functions such as apoptosis since p8-expressing fibroblasts are more sensitive than p8-deficient fibroblasts to the apoptosis induced by DNA damage. Also, p8 is required for endothelin-induced mesangial cell hypertrophy in diabetic kidney, in a mechanism involving ERK, JNK and PI3 kinase [13]. p8 seems to play a functional role in the initiation of LHβ gene

expression during embryonic cell differentiation [14]. Moreover, the *Drosophila melanogaster* p8 homologue is involved in response to starvation and might be activated to stop cell growth in case of nutrient deprivation [15]. Finally, a particularly attractive role in tumour progression was recently proposed for p8 [16]. Fibroblasts obtained from p8-expressing or p8-deficient animals were transformed with a retroviral vector expressing both the ras^{V12} mutated protein and the E1A adenoviral oncogene. In soft-agar assays, transformed p8-expressing cells formed colonies at high frequency, as expected, but p8-deficient transformed fibroblasts were unable to form colonies. Similarly, transformed p8-expressing cells produced tumours in all athymic nude mice when injected subcutaneously or intraperitoneally, whereas transformed p8-deficient fibroblasts did not. On the other hand, studies by another laboratory revealed that expression of the Com1 protein [17], which is identical to human p8, mediates the growth of tumour cells after metastatic establishment in a secondary organ, indicating that activated expression of Com1/p8 in metastatic cells is required for tumour progression. These results strongly suggest that p8 is involved in the cellular pathway(s) required for tumour progression and metastasis.

Our aim is to check the relevance of p8 to cancer progression in human. As a first step, we investigated in the present study the function of p8 in two cell lines derived from human pancreatic cancer. We observed that inhibition of p8 expression increased the cells growth rate. In addition, activations of the Ras→Raf→MEK→ERK and JNK intracellular pathways, which promote the growth of pancreatic cells, down-regulated p8 expression, whereas activation of p38 or TGFβ-1, which inhibit cell growth, induced its expression. It was concluded that *i/* p8 inhibits the growth of human pancreatic cancer cell lines, *ii/* p8 expression is induced through pathways involved in growth inhibition and, conversely, repressed by factors that promote cell growth.

Results

p8 is silenced in pancreatic cancer cells by infection with a retrovirus expressing p8 RNA in the antisense orientation

Panc-1 and BxPc-3 pancreatic cells were chosen for this study because, on the one hand, both cells express higher level of p8 (Figure 1) and, on the other hand, because Panc-1 is wild-type for Smad4/DPC4 and mutated for K-ras, while BxPc-3 is Smad4/DPC4 mutated and K-ras wild-type [18,19], therefore representing different mechanisms of transformation and different genetic backgrounds. K-ras and Smad4/DPC4 mutations are the major mechanisms involved in pancreatic cancer development. We inhibited p8 expression in both Panc-1 and BxPc-3 pancreatic cells by infecting cells with a retrovirus expressing the p8 asRNA (antisense RNA) and carrying the

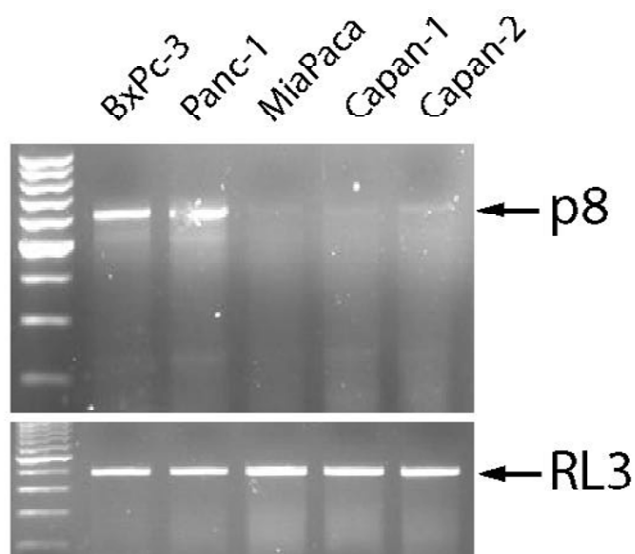


Figure 1
Expression of the p8 mRNA in pancreatic cell lines. RNA from BxPc-3, Panc-1, MiaPaca, Capan-1 and Capan-2 was extracted using Trizol procedures and 1 μ g RNA was analyzed by RT-PCR using specific primers for p8 and RL3 as a control as described in Material and Methods.

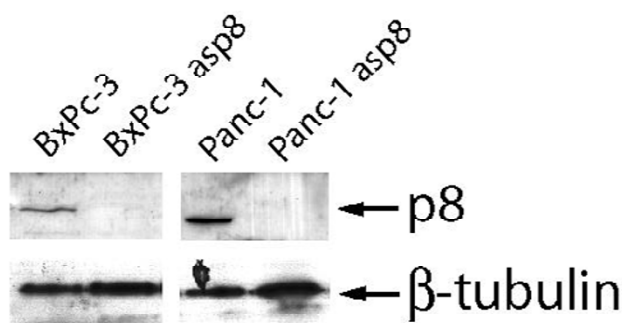


Figure 2
p8-silenced Panc-1 and BxPc-3 pancreatic cells. p8 expression was inhibited in both Panc-1 and BxPc-3 pancreatic cells by using an antisense strategy based on an infection with a retrovirus expressing the p8 asRNA (antisense RNA). The pLPC empty vector was used as a control. Cells were infected with both p8 asRNA or empty retrovirus and the antibiotic-selected cells were analyzed by Western blot to evaluate the intracellular amounts of p8 protein. β -tubulin was used as a housekeeping control.

puromycin resistance. The antibiotic-selected cells were analyzed by Western blotting to evaluate the intracellular

amounts of p8 protein. As shown in Figure 2, the p8 protein was clearly visible in both Panc-1 and BxPc-3 pancreatic cells infected with the empty retrovirus but almost undetectable in cells infected with the retrovirus encoding the p8 asRNA showed, indicating that our anti-sense strategy is efficient to silence p8 gene expression in pancreatic cancer cells. Preliminary studies had been conducted to select the best strategy to inhibit p8 expression. We compared the efficacy of the stable transfection of a siRNA, using a retroviral expression vector to the asRNA strategy described above. In our hands, the antisense strategy worked best, as judged from Western blot assessment of p8 protein expression (data not shown).

p8-silenced pancreatic cells grow more rapidly

We compared in the two cell lines the influence on growth parameters of blocking p8 expression with the p8 asRNA. Figure 3 shows that both Panc-1 and BxPc-3 cells in which p8 has been silenced grew more rapidly than cells infected with the empty vector suggesting that inhibition by p8 of pancreatic cancer cell growth is independent from the mechanism of transformation and genetic background.

Serum-stimulated cellular growth down-regulates p8 expression

Fetal calf serum, which contains a complex mix of growth factors, can be used as inducer of cell growth. As shown in Figure 4 expression of p8 mRNA was down-regulated in both Panc-1 and BxPc-3 when the cells were shifted from culture media containing 0.1% fetal calf serum to media containing 10% FCS. p8 protein showed a similar behavior. These results show that p8 expression is down-regulated in growing pancreatic cells.

The Ras \rightarrow Raf \rightarrow MEK \rightarrow ERK pathway down-regulates p8 expression in pancreatic cancer cells

Most human pancreatic cancers harbor mutations in the K-ras oncogene, which happens relatively early in pancreatic tumorigenesis [20]. The oncogenic mutation of the K-ras gene stabilizes the Ras protein in a GTP-bound form, which is constitutively active and make the cells grow more rapidly. Contrary to the activated Ras protein, p8 inhibits cell growth (Figure 3). We looked whether the Ras \rightarrow Raf \rightarrow MEK \rightarrow ERK pathway was also involved in the regulation of p8 expression, and which step(s) were critical. Figure 5 shown that expression of a mutated form of the Ras protein (ras^{V12}) in BxPc-3 cells, which are wild-type for ras, resulted in decreased p8 mRNA concentration and protein level suggesting that the activated ras inhibits p8 expression. Figure 6 shows that overexpression of Raf, but not of Raf301 (a negative mutant of Raf), and of ERK also inhibited the expression of the p8-CAT construct in Panc-1 as well as in BxPc-3. Finally, the MEK1/2 specific inhibitor U0126 [21] activated p8 mRNA expression in pancreatic cells whether they carry mutated ras (Panc-1)

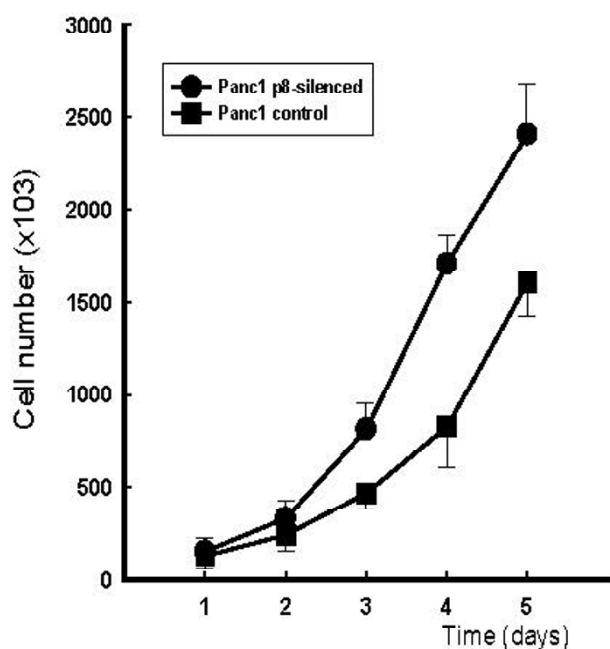
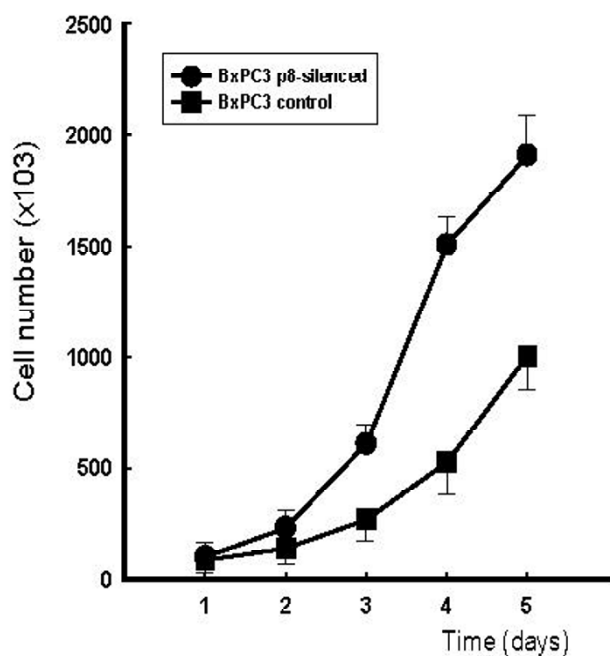


Figure 3
Growth curves of BxPc-3 and Panc-1 cells, native or p8-silenced. One hundred thousand cells per well were plated in a series of 35-mm culture dishes. The cell number was estimated daily in triplicate (from 1 to 5 days) in a haemocytometer. Within an experiment, each point was determined at least two times and the experiment was repeated three times with similar results.

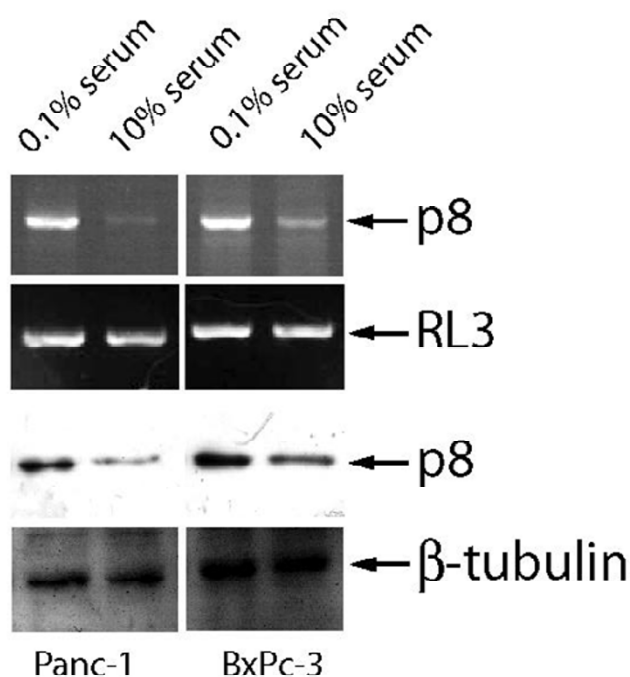


Figure 4
Cell growth arrest by serum deprivation induces p8 expression in Panc-1 and BxPc-3 cells. Panc-1 and BxPc-3 cells were cultivated in 10-cm Petri plates for 24 hours in a culture medium containing 0.1% fetal calf serum. Then, the culture medium was replaced by culture medium with 10% fetal calf serum to start cell growth or by fresh culture medium with 0.1% fetal calf serum to maintain arrest. Twenty four hours later cells were recovered and RNA and proteins prepared and p8 mRNA and p8 protein expression were estimated by RT-PCR and Western blot respectively as described in Material and Methods. RL3 mRNA expression was used as control in RT-PCR experiments and β -tubulin as a housekeeping control protein in Western blots.

or wild-type (BxPc-3). Similar results were observed when expression of the p8 protein was monitored by Western blotting (Figure 7).

Activation of the JNK pathway down-regulates p8 expression in pancreatic cancer cells

c-Jun NH₂-terminal kinase (JNK) is another major MAPK pathway which converts extracellular signals into expression of specific target genes through phosphorylation and activation of transcription factors. JNK activation has been implicated in various, often opposite cellular responses, such as cell proliferation, transformation and apoptosis. As shown in Figure 8, overexpression of JNK down-regulates the gene reporter activity of the p8-CAT construct in Panc-1 cells. Similar results were found in BxPc-3 cells.

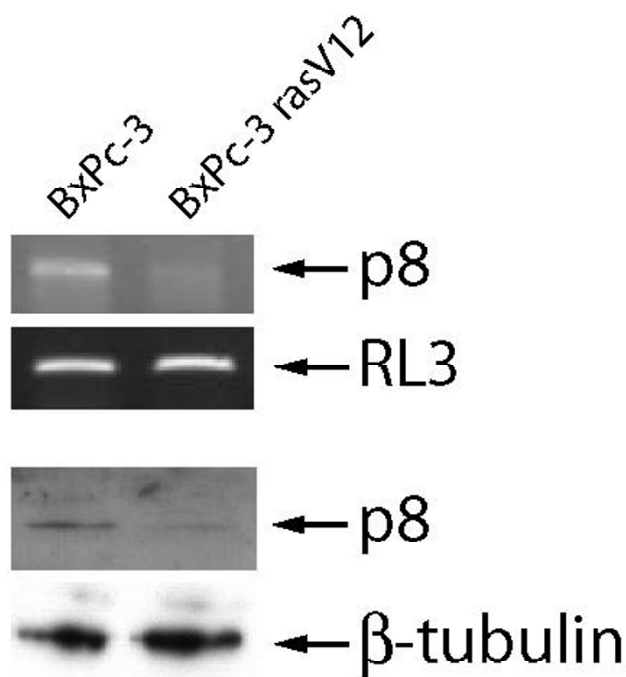


Figure 5
p8 expression is regulated by the ras^{V12} mutated oncogene. BxPc-3 cells were transduced with pLPC-ras^{V12} and pLPC (control) retrovirus and selected with puromycin. Expression of p8 mRNA and p8 protein was estimated by RT-PCR and Western blot respectively as described in Material and Methods. RL3 mRNA expression was used as control in RT-PCR experiments and β -tubulin was used as a housekeeping protein control in Western blot experiments.

Treatment of these cells with the JNK specific inhibitor SP600125 [22] up-regulates expression of the p8 mRNA and p8 protein (Figure 9). These results show that the JNK pathway is involved in the regulation of p8 expression.

The p38 pathway up-regulates p8 expression in pancreatic cancer cells

The p38 signal transduction pathway also plays an essential role in regulating several cell functions including growth, response to inflammation, differentiation and apoptosis. In fact, in pancreatic cancer cells, p38 is a strong inhibitor of proliferation [23] contrary to the Ras \rightarrow Raf \rightarrow MEK \rightarrow ERK and JNK pathways. We therefore analyzed the putative role of the p38 pathway in regulating p8 expression in pancreatic cancer cells. Figure 10 shows that over-expression of the plasmid encoding p38 significantly increases p8-CAT activity in Panc-1 as well as in BxPc-3 cells. Then, cells were treated with SB203580, a specific inhibitor of p38 [24], and p8 expression was

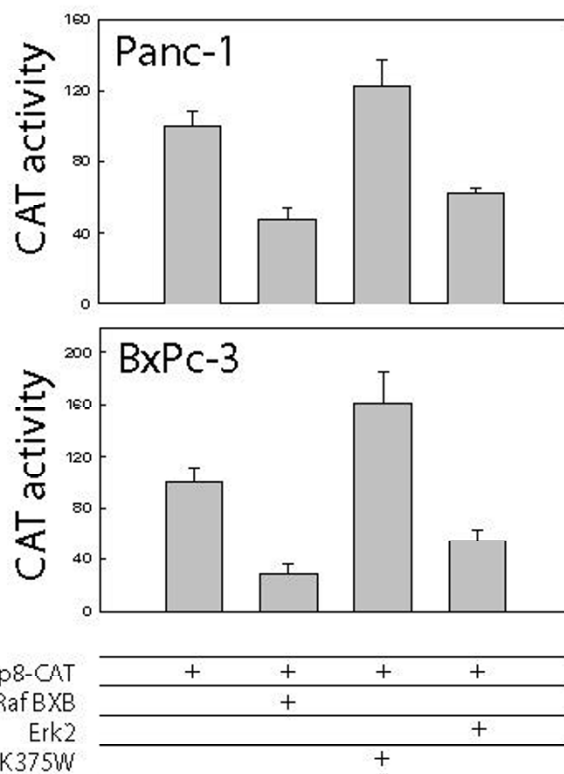


Figure 6
p8 transcription is repressed by the Raf \rightarrow MEK \rightarrow ERK pathway. Panc-1 and BxPc-3 were cultivated in 30 mm diameter culture dishes for 24 hours and then transiently transfected with 0.5 μ g of p8-CAT and 0.5 μ g of pCMV/ β gal plasmids. Expression plasmids pcDNA RAF BXB encoding the wild-type RAF, pcDNA RAF 301 K375W encoding a dominant negative RAF, and pcDNAIII HA ERK2 encoding wild-type ERK2 (0.5 μ g) were co-transfected with p8-CAT and pCMV/ β gal plasmids as indicated. Cell extracts were prepared 24 hours after transfection and CAT and β -galactosidase activities were measured. CAT activity was normalized to β -galactosidase activity. Experiments were carried out in triplicate and repeated two or three times.

measured. p8 mRNA as well as the encoded protein were down-regulated after inhibition of the p38 activity (Figure 11). These results indicate that the p38 pathway is a positive regulator of p8 expression in pancreatic cancer cells.

TGF β -1 up-regulates p8 expression in pancreatic cancer cells

The most prominent biological activity of TGF β -1 is its potent inhibition of cell growth in a wide variety of cell types including pancreatic cells. TGF β -1 signals are sent through two types of transmembrane serine/threonine

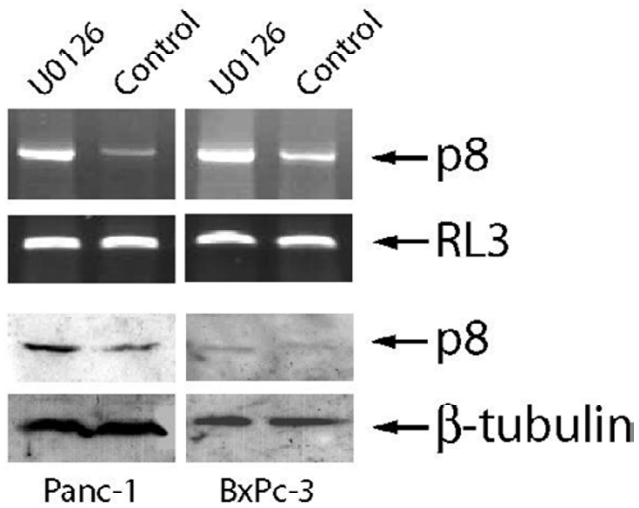


Figure 7
The U0126 MEK1/2 inhibitor induces p8 expression in Panc-1 and BxPc-3 cell. Panc-1 and BxPc-3 cells were treated with 10 μ M of U0126 for 12 hours. Total RNA and protein were prepared and p8 mRNA and p8 protein expression were measured by RT-PCR and Western blot respectively as described in Material and Methods. RL3 mRNA expression was used as control on RT-PCR experiments and β -tubulin as a housekeeping control protein in Western blot.

kinase receptors. In fact, TGF β -1 binds and brings together the type I and type II receptors. In the resulting complex, the constitutively active TGF β -1 type II receptor phosphorylates the type I receptor, which then plays a major role in transducing the signal to downstream components to affect gene expression through phosphorylation of SMAD proteins. Phosphorylated receptor-regulated SMADs then form heteromeric complexes with the common partner SMAD4. These heteromeric complexes then move to the nucleus, where SMAD4 will bind DNA and contribute to transcriptional activation. In general, pancreatic cancer cells present with defects in TGF β -1 signaling and are resistant to TGF β -1-mediated growth suppression. Since TGF β -1 and p8 are inhibitors of pancreatic cell growth we analyzed whether p8 could mediate, at least in part, the effect of TGF β -1. First, we found that treatment of Panc-1 cells with TGF β -1 increased p8 mRNA levels and p8 protein as judged by Western blot (Figure 12). Then, to confirm that overexpression is regulated at the transcriptional level, we analyzed the effect of some constructs expressing constitutively activated type I TGF β receptor, dominant negative type II TGF β receptor, a dominant negative of Smad4 and the wild-type Smad4 on the p8-CAT activity. As expected, the constitutively activated type I TGF β receptor but not the dominant negative type II TGF β receptor

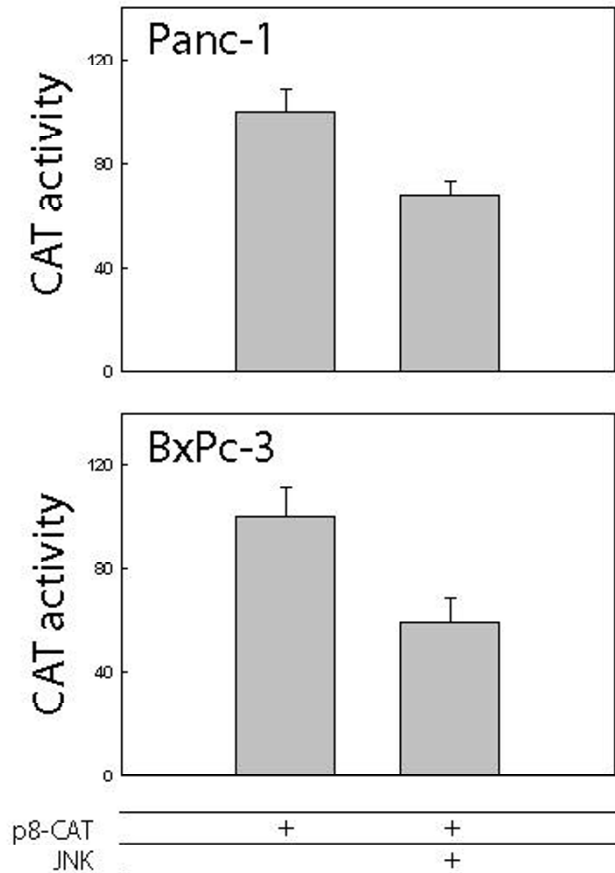


Figure 8
p8 transcription is repressed through the JNK pathway. Panc-1 and BxPc-3 were cultivated in 30 mm diameter culture dishes for 24 hours and then transiently transfected with 0.5 μ g of p8-CAT and 0.5 μ g of pCMV/ β gal plasmids. Expression plasmid pcDNAIIIIB HA JNK encoding the wild-type JNK (0.5 μ g) was co-transfected with p8-CAT and pCMV/ β gal plasmids as indicated. Cell extracts were prepared 24 hours after transfection and CAT and β -galactosidase activities were estimated. CAT activity was normalized to β -galactosidase activity. Experiments were carried out in triplicate and repeated two times.

increased CAT activity. Also, expression of the Smad4, contrary to that of the negative mutant, induced p8 transcription (Figure 13). Together, these results indicate that p8 is positively regulated by TGF β -1.

Beside the Smad proteins, TGF β -1 also activates the p38 MAPK pathway in pancreas-derived cells, which may play an important role in TGF β -1 induced genes [25]. Therefore, we analyzed the p38-dependent effect of TGF β -1 on p8 transcription. As shown in Figure 13, inhibition of p38

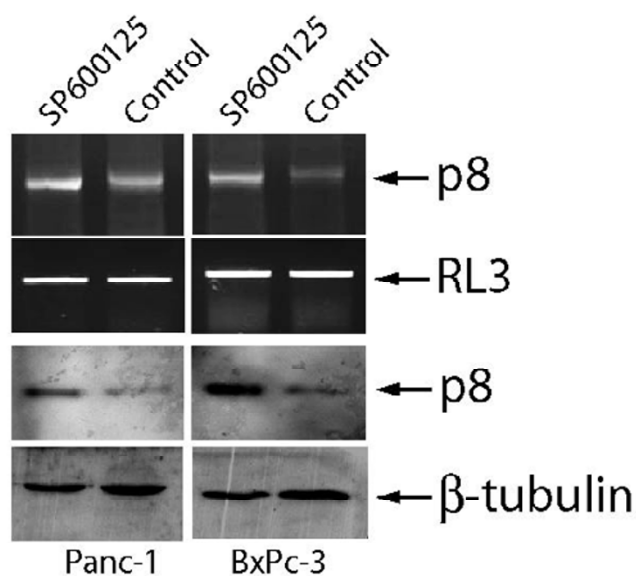


Figure 9
The SP600125 JNK inhibitor increases p8 expression in Panc-1 and BxPc-3 cells. Panc-1 and BxPc-3 cells were treated with 10 μ M of SP600125 for 12 hours and total RNA and protein were prepared and p8 mRNA and p8 protein expression were estimated by RT-PCR and Western blot respectively as described in Material and Methods. RL3 mRNA expression was used as control in RT-PCR experiments and β -tubulin was used as a housekeeping control protein in Western blots.

activity with the SB203580 specific inhibitor decreased about 40% the activity of TGF β -1 on the p8 promoter indicating that the effect of TGF β -1 on p8 promoter is mediated by both p38-dependent and p38-independent pathways.

Discussion

Pancreatic adenocarcinoma is the fourth leading cause of death from malignant diseases [26]. The aggressive nature of the neoplasia, the lack of early detection, and the limited response to treatments such as chemotherapy and radiotherapy contribute to the high mortality rate of the disease. Therefore, a better understanding of the molecular mechanism leading to pancreatic cancer remains a major goal because it may help proposing strategies for earlier diagnosis and better treatment. The most commonly altered genes in pancreatic adenocarcinoma are K-ras (75 to 100%), p16INK4a (95%), p53 (50 to 75%) and DPC4 (50%) [27–31]. Whereas K-ras is a proto-oncogene all the others are tumour suppressor genes. Additional genes have been found altered at lower frequency. Panc-1 and BxPc-3 pancreatic cells were chosen

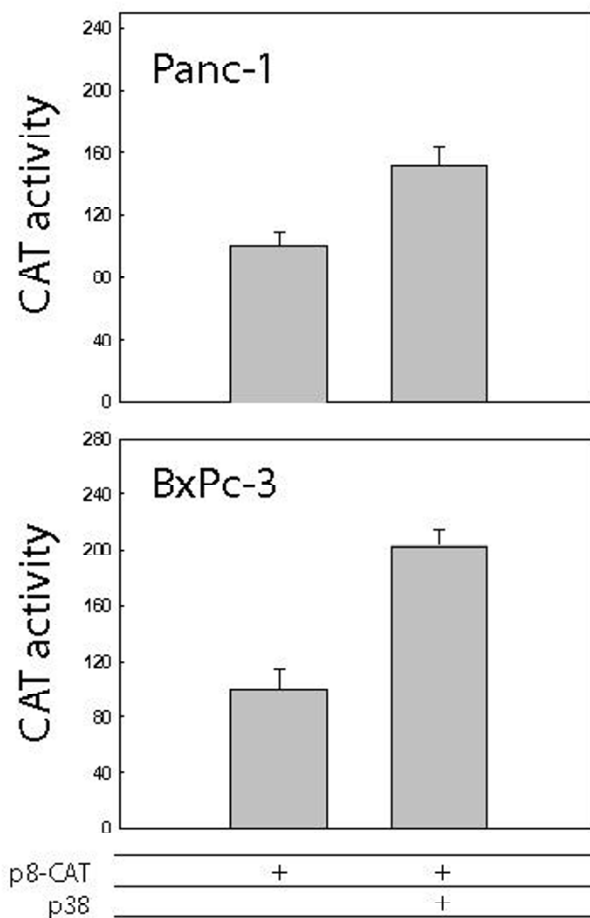


Figure 10
p8 transcription is induced by the p38 pathway. Panc-1 and BxPc-3 were cultivated in 30 mm diameter culture dishes for 24 hours, then transiently transfected with 0.5 μ g of p8-CAT and 0.5 μ g of pCMV/ β gal plasmids. The expression plasmid pCEFL HA p38 encoding the wild-type p38 (0.5 μ g) was co-transfected with p8-CAT and pCMV/ β gal plasmids as indicated. Cell extracts were prepared 24 hours after transfection and CAT and β -galactosidase activities were measured. CAT activity was normalized to β -galactosidase activity. Experiments were carried out in triplicate and repeated two times.

for this study because they both express high levels of p8 (Figure 1) and because they present with different mechanisms of transformation and genetic backgrounds, Panc-1 being wild-type for Smad4/DPC4 but mutated for K-ras and BxPc-3 mutated for Smad4/DPC4 and wild-type for K-ras [18,19]. This work presents evidences that p8 inhibits the growth rate of pancreatic cancer-derived cells and that the intracellular pathways promoting cell growth

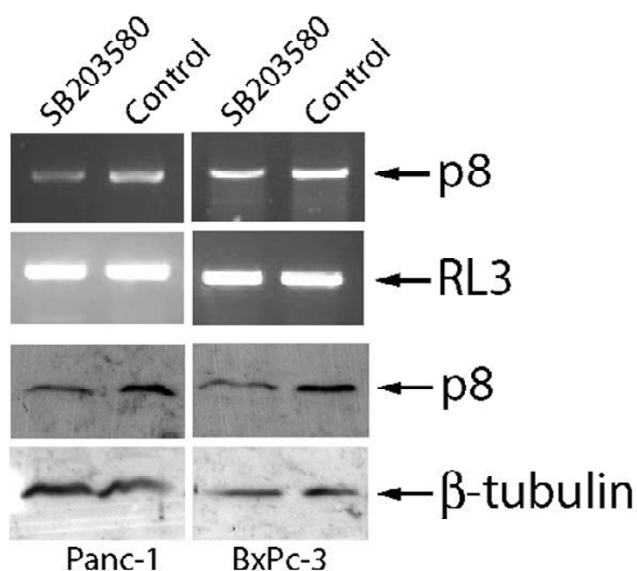


Figure 11
The SB503280 p38 inhibitor reduces p8 expression in Panc-1 and BxPc-3 cells. Panc-1 and BxPc-3 cells were treated with 10 μ M of SB203580 for 12 hours and total RNA and protein were prepared and p8 mRNA and p8 protein expression were measured by RT-PCR and Western blot respectively as described in Material and Methods. RL3 mRNA expression was used as control in RT-PCR experiments and β -tubulin was used as a housekeeping control protein in Western blots.

down-regulate p8 expression whereas those promoting growth arrest up-regulate its expression. Together, these results suggest that p8 is downstream of some cell growth regulators and therefore regulation of p8 expression or its activity could be used as a target for treating pancreatic cancer.

Silencing p8 expression was able to strongly promote cell growth in both cell types, Panc-1 and BxPc-3, suggesting that p8 may act downstream of the ras- or Smad4/DPC4-dependent ways. Also, we found that stimulating cell growth by the complex combination of growth factors contained in fetal calf serum down-regulated expression of p8 whereas, on the contrary, treating the cells with TGF β -1, which promotes cell cycle arrest, stimulates p8 expression. Therefore, p8 gene expression seems to be regulated in opposite directions by mechanisms promoting cell growth or cell cycle arrest. It is interesting to note that while p8 expression is under the control of cell growth regulatory pathways such as Ras \rightarrow Raf \rightarrow MEK \rightarrow ERK, JNK, p38 and TGF β -1, p8 can affect cell cycle progression,

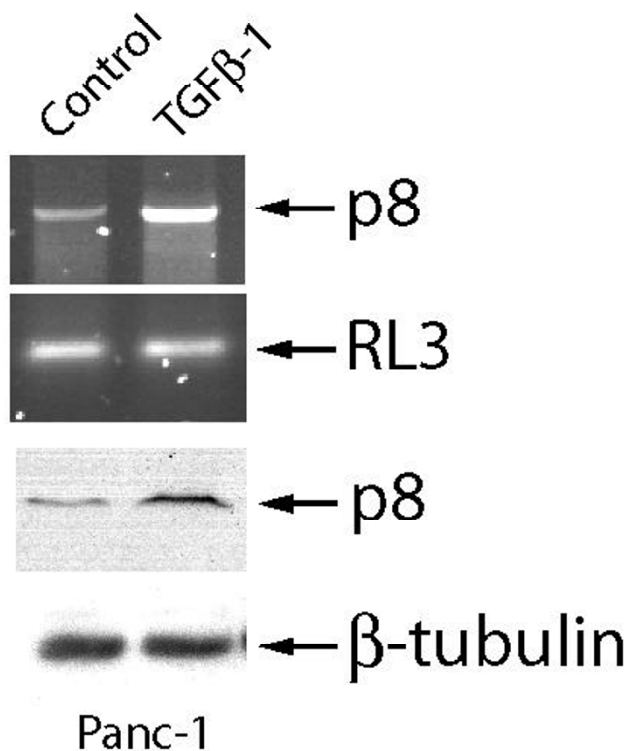


Figure 12
TGF β -1 increases p8 expression in Panc-1 cells. Panc-1 cells were treated with TGF β -1 (5 ng/ml) for 12 hours under standard conditions. Total RNA and protein were prepared and p8 mRNA and p8 protein expressions were measured by RT-PCR and Western blot respectively as described in Material and Methods. RL3 mRNA expression was used as a control in RT-PCR experiments and β -tubulin as a housekeeping control protein in Western blots.

suggesting that p8 is a target for factors regulating pancreatic cell growth.

A mechanism by which p8 could regulate cell cycle progression in embryonic fibroblasts was previously proposed [11]. In fact, p8 seems to take action upstream from cyclin-dependent kinases because the intracellular levels and activities of Cdk2 and Cdk4 are decreased when p8 is expressed. Concomitantly, the cyclin-dependent kinase inhibitor p27 is expressed at a low level in p8-deficient cells which may explain the increased activity of Cdk2 and Cdk4. The mechanism by which p8 regulates the intracellular level of those proteins remains to be determined. However, because p8 is a transcriptional cofactor, it is possible that regulation of expression of these molecules takes place, at least in part, at the transcription level.

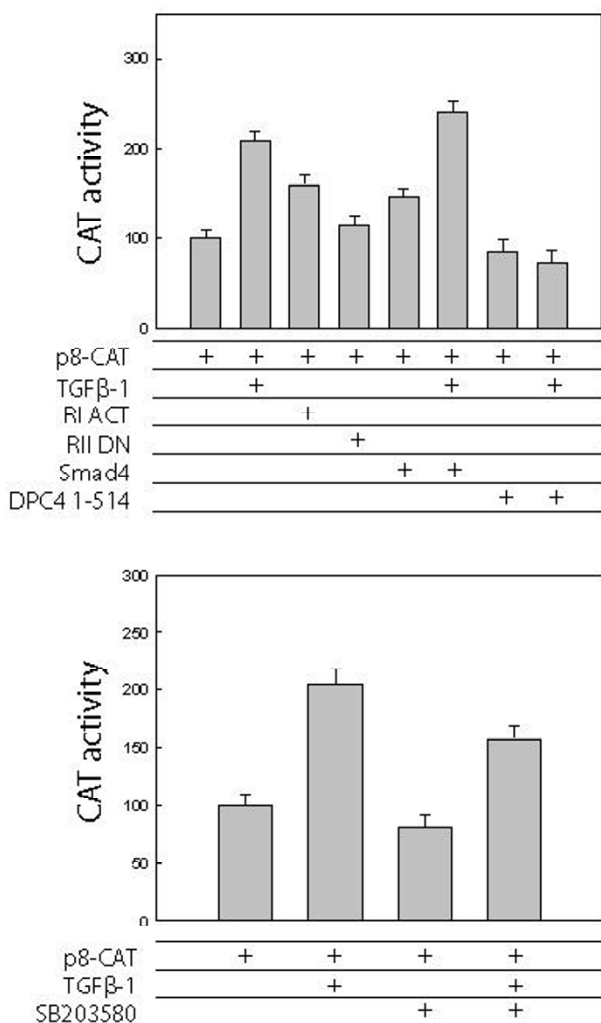


Figure 13

p8 transcription is activated by TGFβ-1 through Smad4 in Panc-1 cells. Panc-1 cells were cultivated in 30 mm diameter culture dishes for 24 hours and then transiently transfected with 0.5 μg of p8-CAT and 0.5 μg of pCMV/βgal plasmids. Plasmids encoding constitutively activated type I TGFβ receptor (RI ACT), dominant negative type II TGFβ receptor (RII DN), dominant negative of Smad4 (DPC4 1-514 a.a.) and the Smad4 wild type (0.5 μg) were co-transfected with p8-CAT and pCMV/βgal plasmids in the presence or not of TGFβ-1 (5 ng/ml) or the p38 inhibitor SB203580 as indicated. Cell extracts were prepared 24 hours after transfection and CAT and β-galactosidase activities were measured. CAT activity was normalized to β-galactosidase activity. Experiments were carried out in triplicate and repeated two or three times.

Interestingly, expression of p8 mRNA seems to be regulated in a cell type- and stimulus-specific manner since, for example, p38 can induce p8 expression in response to

stress in fibroblasts [3] but not in renal mesangial cells treated with endothelin [13]. In pancreatic cancer-derived cells p38 seems to play a major role since it is involved in p8 activation as judged by transient transfection assays and using a specific p38 inhibitor (Figures 10 and 11). In addition, p38 is also involved in TGFβ-1-induced p8 expression because about 40% of the TGFβ-1 effect was abolished when p38 activity was specifically blocked (Figure 13). On the other hand, ERK and JNK are inducers of p8 expression in mesangial cells treated with endothelin, but not involved in the activation of p8 in response to stress in fibroblasts [3], and even repressors in pancreatic cells (Figures 5, 6, 7, 8 and 9). Finally, PI3 kinase is an inducer of p8 expression in both endothelin-mediated p8 activation in mesangial cells [13] and pancreatic cells (data not shown).

Based on these observations, overexpression of p8 could be considered a possible goal for treating pancreatic tumours, in order to limit their growth. However, we previously reported that p8 repression would prevent ras^{V12}/E1A transformed fibroblasts from evolving as tumours in nude mice [16]. This apparent contradiction needs to be resolved before considering p8 as a target for treating cancer progression.

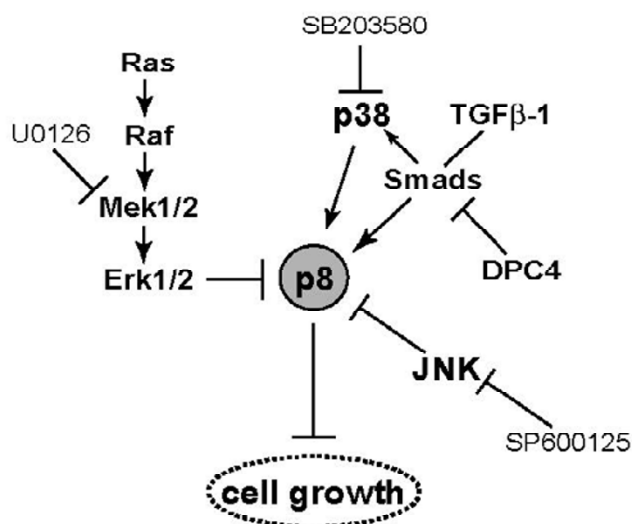
Conclusions

In conclusion (see Figure 14), we report in this paper that inhibition of p8 expression by an anti-sense strategy increases the growth rate of both Panc-1 and BxPc-3 pancreatic cancer-derived cells. Moreover, ERK- and JNK-mediated pathways down-regulate p8 expression, whereas p38 and TGFβ-1 pathways induce p8 expression. Also, cell growth triggered by expression of a RAS mutated protein or by 10% fetal calf serum induces down-regulation of p8 expression. Together, these results indicate that p8 is an intracellular cell growth inhibitor and that it is oppositely regulated by growth-promoting or growth-inhibiting factors in pancreatic cancer-derived cells.

Material and Methods

Cell lines and cell culture conditions

The human pancreatic cancer cell lines Panc-1 and BxPc-3 were a kind gift of Dr C. Susini (INSERM U.531, Toulouse) and A. Hajri (IRCAD, Strasbourg) respectively. Panc-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicilin G and 100 μg/ml streptomycin. BxPc-3 were cultivated in RPMI 1640 medium in the presence of 2 mM L-glutamine, 4.5 g/L glucose, 10 mM Hepes, 1.0 mM sodium pyruvate, 10% fetal calf serum and 100 IU/ml penicilin G and 100 μg/ml streptomycin. Human recombinant TGFβ-1 was obtained from Sigma, and specific SB203580, U0126 and

**Figure 14**

Schematic representation of the mechanisms regulating p8 expression in pancreatic cancer-derived cells.

SP600125 inhibitors were from Calbiochem and utilized at 10 μ M.

Expression plasmids

Expression plasmids encoding p38 (pCEFL HA p38), Erk2 (pcDNAIII HA ERK2), JNK (pcDNAIII HA JNK), the wild-type Raf (pcDNA RAF BXB) and the Raf dominant negative (pcDNA RAF 301 K375W) were obtained from O Coso (University of Buenos Aires). Plasmids encoding the constitutively activated type I TGF β receptor (RI ACT), the dominant negative type II TGF β receptor (RII DN) and the Smad4 dominant negative (DPC4 1-514 a.a.) were obtained from R Urrutia (Mayo Clinic, Rochester) and the wild type Smad4 was from C Heldin (Ludwig Institute, Uppsala).

Pancreatic p8-deficient cells

To silence p8 expression in pancreatic cells, we infected these cells with a retrovirus expressing human p8 in the antisense orientation. The retroviral vector was constructed as follows: human p8 cDNA was subcloned in HindIII and XhoI restriction sites of the pLPC plasmid (obtained from S. Lowe) in the antisense orientation. Amphotrope human p8 expressing retrovirus was then generated by transient transfection using Phoenix amphotrope packaging cells. Viral supernatant was used to infect Panc-1 and BxPc-3 pancreatic cells and the population of p8-silenced cells was isolated by selection in presence of puromycin (1 μ g/ml). As control, cells were infected with the pLPC empty vector.

p8 expression in arrested and growing cells

One million of Panc-1 or BxPc-3 cells were cultivated on 10-cm Petri plates in standard conditions (with 10% FCS). After 48 h, culture media were changed for fresh media with FCS restricted to 0.1%, in order to stop growth. After 24 hours of growth arrest, culture medium was replaced either by medium with 10% fetal calf serum to resume cell growth or, as control, by medium with 0.1% fetal calf serum. Twenty four hours later cells were recovered and RNA and protein extracted.

p8 expression in TGF β -1-treated Panc-1 cells

One million of Panc-1 cells were cultivated in 10-cm culture dishes for 48 hours under standard conditions before TGF β -1 treatment. Human recombinant TGF β -1 (5 ng/ml) was added to cells, without changing the culture medium, and cells were collected 12 hours later for RNA and protein preparation.

BxPc-3 ras^{V12}-expressing cells

pLPC-ras^{V12} and pLPC plasmids were obtained from S. Lowe. Phoenix amphotrope packaging cells (10^6) were plated in a 6-well plate, incubated for 24 hours, then transfected with PEI with 5 μ g of retroviral plasmid. After 48 hours, the medium containing virus was filtered (0.45 μ m filter, Millipore) to obtain the viral supernatant. Target BxPc-3 were plated at 2×10^5 cells per 35-mm dish and incubated overnight. For infections, the culture medium was replaced by an appropriate mix of the viral supernatant and culture medium (V/V), supplemented with 4 μ g/ml polybrene (Sigma), and cells were incubated at 37°C. BxPc-3 ras^{V12}-expressing cells were selected with puromycin (1 μ g/ml). Cells infected with the pLPC empty vector were used as control.

Western-blot analyses

One hundred μ g of total protein extracted from cells was separated with standard procedures on 15.0% SDS-PAGE using the Mini Protean System (Bio-Rad) and transferred to a nitrocellulose membrane (Sigma). The intracellular level of p8 was estimated by Western blot using a polyclonal antibody (1:1000) raised against human p8 [4].

Growth curves

One hundred thousand cells per well were plated in a series of 35-mm culture dishes. The cell number was estimated daily in triplicate, during 1 to 5 days, in a haemocytometer. Within experiments, each point was determined at least two times.

Cell transfection and gene reporter assays

Panc-1 and BxPc-3 (10^5) were cultivated in 30 mm diameter culture dishes for 24 hours then transiently transfected with 0.5 μ g of p8-CAT reporter plasmid and 0.5 μ g of pCMV/ β gal plasmid (to control transfection

efficiency) using the Fugene reagent in accordance with the manufacturer's protocol (Roche Molecular Biochemicals). The p8-CAT plasmid is the previously reported p-1471/+37p8-CAT promoter construct [5]. Reporter activities were measured as previously described [5]. Briefly, cell extracts were prepared with the reporter lysis buffer (Promega) 24 hours after transfection and CAT activity was determined by the phase extraction procedure [32] and β -galactosidase assay was performed essentially as described in Sambrook et al. [33]. CAT activity was normalized to β -galactosidase activity. Experiments were carried out in triplicate and repeated at least two times. Expression plasmids (0.5 μ g) were co-transfected with p8-CAT and pCMV/ β gal plasmids as indicated.

RT-PCR analysis

RNA was extracted using the Trizol (Life Technologies) procedure. Total RNA (1 μ g) was analyzed by RT-PCR with the SuperScript™ One-step RT-PCR System and the Platinum Taq kit (Life Technologies). RT-PCR was performed using different numbers of cycles to verify that the conditions chosen were within the linear range. The mRNA coding for p8 was specifically amplified with sense (5' GAAGAGAGGCAGGGAAGACA 3') and antisense (5' CTGCCGTGCGTGTCTATTTA 3') primers, in positions 72 and 643 of the cDNA (accession # NM_012385), respectively. As control, the transcript coding for the ribosomal protein RL3 was specifically amplified for 22 cycles with sense (5'GAAAGAAGTCGTGGAGGCTG3') and antisense (5'ATCTCATCTGCCCAAACAC3') primers, in positions 216 and 637 of the cDNA, respectively.

Author's contributions

CM prepared cells and retrovirus, carried out RNA purification, RT-PCR, Western blots, and cell growth experiments, NL carried out CAT assays, SV participated in the design of the study and analysis of data, JLI participated in the analysis of data and wrote the manuscript. All authors read and approved the final manuscript.

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