Furin inhibition results in absent or decreased invasiveness and tumorigenicity of human cancer cells

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Pro-protein convertases such as furin are expressed in many human tumor lines and primary tumors. Furin processes stromelysin-3, membrane type 1 matrix metalloproteinase (MMPs) involved in tumor cell invasiveness, as well as growth factors such as transforming growth factor β1. Evaluation of furin expression in head and neck squamous cell carcinoma (HNSCC) cells exhibiting different invasive ability showed that furin overexpression correlated with their respective invasiveness. The use of a selective furin inhibitor, alpha 1-PDX (PDX) was studied in three furin-expressing invasive HNSCC cell lines. The effects of PDX transfection were evaluated *in vivo* and *in vitro* to determine changes in the malignant phenotype. Transfection of HNSCC cell lines with PDX resulted in significant decrease or absence of tumorigenicity after s.c. inoculation into severe combined immunodeficient mice. Likewise, *in vitro* invasiveness was reduced ≈50%. The *in vivo* invasion assay using tracheal xenotransplants showed even more drastic reductions of the invasive ability of PDX-transfected cells (up to an 80% decrease). PDX-transfected cells did not invade or penetrated less into the tracheal wall tissues than their vector alone-transfected counterparts. In addition, the former cells showed a remarkable decrease in MMP-2 processing and activity. After PDX transfection the cells were less efficient in processing the tumor progression-associated furin substrates transforming growth factor β1 and pro-membrane type 1-MMP. These findings indicate that furin inhibition is a feasible approach to attenuate and even abolish certain critical attributes of the advanced malignant phenotype. Thus, furin should be considered as a promising target for cancer therapy.

alpha1-PDX | squamous cell carcinoma | invasion | tumor progression

Furin and PACE4 are members of the protease family of pro-protein convertases (PCs) that activate precursor proteins by cleavage at the specific recognition sequence RXK/RR (1). These PCs are ubiquitously expressed and have been implicated in many physiological and pathological processes (1, 2). Some of their substrates are proteins that have been identified as having significant roles during the development of the advanced malignant phenotype. For example, stromelysin-3 and membrane type 1 matrix metalloproteinase (MT1-MMP), both MMPs containing the amino acid motif recognized by the cleaving enzyme, are known substrates of these PCs (3, 4). The acquisition of the invasive/metastatic phenotype is one of the crucial elements of tumor progression, and activated MMPs are instrumental in achieving this phenotype.

MMPs are not the only substrates of PCs. A group of growth factors and growth factor receptors such as transforming growth factor (TGF)-β1, insulin-like growth 2, and insulin-like growth-1R and adhesion molecules such as E-cadherin, show similar amino acid motifs and can thus be activated by furin and PACE4 (5–8).

In our laboratory we have shown that PACE4 is overexpressed in 50% of murine chemically induced spindle cell tumors, and that PACE4 transfected murine tumor cell lines exhibit a remarkable enhancement of their *in vivo* invasive ability (9).

Another PC ubiquitously expressed in human tissues is furin. It is widely expressed at very low levels in normal human tissues. Whereas PACE4 is more relevant to rodents, furin is the predominant PC in non-neuroendocrine human tissues, because furin, but not PACE4, is able to process human stromelysin-3 (3, 10, 11). In addition, high levels of furin mRNA were found in human tumors (12–14).

Furin inhibition seems to be a logical approach to inhibiting the activation of its substrates, many of which are essential components of the invasive/metastatic cascade (e.g. stromelysin-3 and MT-MMPs). Using site-directed mutagenesis, a variant alpha 1-antitrypsin (alpha 1-AT) was constructed that contains in its reactive site RXXR, the minimal sequence required for efficient processing by furin (15, 16). This alpha 1-AT variant (alpha 1-PDX or PDX) is more than 3,000-fold more effective than alpha 1-AT at inhibiting furin *in vitro*. Furthermore, the P4 Arg of PDX greatly attenuates the thrombin inhibitory properties of this serpin (>300-fold) thus increasing the selectivity of PDX for furin. Expression studies show that PDX blocks the processing of the furin substrate HIV-1 gp160 in transfected cells (15). Because furin also processes substrates involved in tumor progression, such as stromelysin-3, MT1-MMP, and TGF-β1, its inhibition by PDX could be a promising tool in modulating tumor growth, invasion, and metastasis. Hence, the objective of this project was to examine the ability of competitive inhibitors of PCs to inhibit tumor growth and invasiveness of head and neck squamous cell carcinoma (HNSCC) cell lines. Herein, we demonstrate that overexpressing PDX in three different malignant HNSCC cell lines effectively reduces the processing of MMP-2 and significantly reduced invasiveness and *in vivo* tumorigenicity.

Materials and Methods

Cell Lines. Cell lines derived from human HNSCC (SCC 9, SCC 12, SCC 15, A 253, and Detroit 562) and the human fibrosarcoma cell line HT1080 were obtained from the American Type Culture Collection. All cell lines were cultured in S-MEM (Sigma) with 10% FBS and 2 mM L-glutamine (17, 18).

Transfection of Mammalian Cell Lines. Cells were transfected with either pCI.neo (Promega, mammalian expression vector) or pCI.neo.PDX plasmid (full-length PDX cDNA cloned into the *Eco*RI–*Sal*I site of pci.Neo plasmid) (15). Transfections were

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 MMP; PC, pro-protein convertase; PDX, alpha-1 PDX; Scid, severe combined immunodeficient; TGF, transforming growth factor.

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performed according to the manufacturer's instruction (Lipofectamine, GIBCO). Transfected cells were grown in S-MEM, 10% FBS, L-glutamine, and pen-strep, and selected in gentamicin (G418, 800 μg/ml) 2 days after transfection. Cells were propagated, and single clones were selected. Expression of PDX was evaluated by Western blot analysis.

Western and Northern Analysis. Cells were trypsinized, washed twice with PBS buffer, and resuspended in RIPA buffer (1× PBS/0.1% SDS/0.5% Na deoxycholate/1% Nonidet P-40) with the addition of 1 mM aprotinin, 100 mM PMSF, and 100 mM Na_3VO_3 and digested at 4°C for 30 min. The digests were centrifuged, and the supernatants were used as cell lysates. One hundred micrograms (for furin analysis) or 25 μg (PDX analysis) of cell lysates was subjected to electrophoresis in 8% SDS/PAGE and blotted onto a nitrocellulose membrane. Immunoblotting was performed either with mAb against the Cys-rich region of furin (MON-152, Alexis, San Diego) or with a rabbit α1-antitrypsin polyclonal antibody (Sigma). The membranes were washed and incubated for 30 min with horseradish peroxidase-labeled anti-mouse (furin) or anti-rabbit (PDX) IgG. Immunoreactive bands were visualized with the ECL system (Amersham Pharmacia).

Cells were grown to 80% confluency and incubated overnight in serum-free S-MEM for TGF-β1 analysis. Conditioned medium was collected and concentrated by filtration (Centriprep Microcon YM-10, Millipore). Twenty micrograms of proteins from the conditioned medium was fractionated by electrophoresis in 4–20% gradient SDS/PAGE under reductive conditions and transferred to a nitrocellulose membrane. The membranes were immunoblotted with affinity-purified goat anti-human latent-associated peptide LAP (TGF-β1) IgG (R & D Systems), and, after washing, with horseradish peroxidase anti-goat IgG. An enhanced chemiluminescence detection system (Amersham Pharmacia) was used for band detection. Analysis of TGF-β1 in cell lysates was performed as described for furin analysis.

For MT1-MMP detection, cells were plated in 24-well tissue culture plates to 80% confluency and washed. The cells were lysed by treatment with RIPA lysis buffer. The samples were loaded in a 10% SDS/PAGE and immunoblotted. A rabbit polyclonal antibody against the hinge region of human MT1-MMP (Chemicon) and a mAb directed against the hemopexin-like domain (clone 113–5B7, Chemicon) were used as primary antibodies. HT1080 cells were used as positive control.

PDX RNA expression was evaluated by Northern analysis in transfected cells before animal inoculation and in s.c. tumor excised 6–7 weeks after inoculation by using the complete PDX cDNA as probe (16).

Doubling Time. Cells were grown in 12-well plates and counted every other day. Log of cell numbers versus days was plotted to obtain the doubling time for each transfected cell line.

Zymography. A total of 1×10^6 cells were grown overnight in serum-free S-MEM containing 2 mM L-glutamine and pen-strep. The conditioned media were concentrated down to 200 μl by using Amicon centripreps (Fisher) and 20 μl of each sample was loaded on a 10% NOVEX (San Diego) precast zymogram (gelatin) gel. The gel was run, renatured, and developed according to the manufacturer's instruction. Gelatinase zymography standards were purchased from Chemicon.

***In Vitro* Invasion Assay.** The ability of cells to degrade extracellular matrix *in vitro* was assessed by using Biocoat Matrigel invasion chambers (Becton Dickinson) according to the manufacturer's instructions (19).

***In Vivo* Invasion Assay.** Tracheal transplants were prepared as described (19, 20). Cells (5×10^5) from each transfected cell line were inoculated into de-epithelialized rat trachea (Zivic–Miller). Six to 10 tracheas were used for each cell line. After cell inoculation, the tracheas were sealed and transplanted into the dorsal s.c. tissues of severe combined immunodeficient (Scid) mice. Tracheal transplants were removed surgically at 8 weeks, sectioned into 3-mm thick rings, and fixed in 10% formalin. After hematoxylin/eosin staining, the degree of invasion of the tracheal wall was determined by measuring the length of maximum penetration of the tumor cells into the tracheal wall. All microscopic images of cross sections of tracheal transplants were digitized at ×40. The lengths were determined by measuring the distance between the luminal center and the most distant point of tumor invasion. If the lumen was obliterated, the distance measured was between the geometric center of the tumor mass inside the tracheal lumen and the most distant point of tumor invasion either in or outside the tracheal wall. Each tracheal transplant was represented by 2–6 measurements corresponding to the number of cross sections containing tumor cells. A mean was calculated for each tracheal transplant and for each group of transfected cells. The results were expressed in μm of penetration depth.

***In Vivo* Tumorigenicity.** Either PDX- or vector alone-transfected cells (5×10^6) were injected into the s.c. tissues of Scid mice (five mice per cell line). Tumors were measured twice a week after the appearance of the first tumor for each pair of PDX- and vector alone-transfected transfected cells by using a Vernier caliper. Volumes (V) of the tumors were obtained by using the following equation: $V = [(L_1 + L_2)/2] \times L_1 \times L_2 \times 0.526$, where L_1 and L_2 are the length and width of the s.c. tumor (21).

Results

Furin Expression and PDX Transfection. Furin expression was studied in five HNSCCs. The levels of furin expression were correlated with the *in vivo* invasiveness of these cells previously determined in our laboratory by using the tracheal xenotransplant invasion assay (17, 18). The least invasive cell line, SCC 12 (18), showed marginal expression of this protein. Conversely, the highest levels of furin expression were observed in the very invasive cell lines A 253 and Detroit 562 (17, 18). SCC 9 and SCC 15, characterized by moderate invasiveness (17), had intermediate levels of furin expression between the two other sets of cell lines (Fig. 1*A*).

Three HNSCC cell lines (SCC 15, A253, and Detroit 562) expressing moderate to high levels of furin protein were selected for stable transfection with PDX cDNA. After checking for the efficiency of the transfection procedure by Western blot analysis (Fig. 1*B*), one clone tranfected with vector alone and one with PDX exhibiting high levels of protein expression, were selected for further characterization. The three PDX clones and their corresponding vector-alone controls did not show any significant difference in doubling time (data not shown).

Inhibition of Furin Substrate Activation and MMP Expression. The ability of cells to cleave pro-TGF-β1 was used to evaluate the inhibition of furin activity. The expression of this growth factor at low levels was seen in all cell lines both in cell lysates and supernatants. TGF-β1 cleavage is reported to take place in the trans-Golgi network, where furin is located and then transported to the extracellular millieu (22). The products of this enzymatic cleavage are less abundant inside the cells than in the extracellular compartment (23). Accordingly, only the band corresponding to the pro-TGF-β1 was observed in cell lysates, and a similar relative increase in signal was noted in all of the PDX versus the vector alone-transfected cells (Fig. 1*C*). When supernatants were analyzed, accumulation of the pro-TGF-β1 (50 kDa correspond-

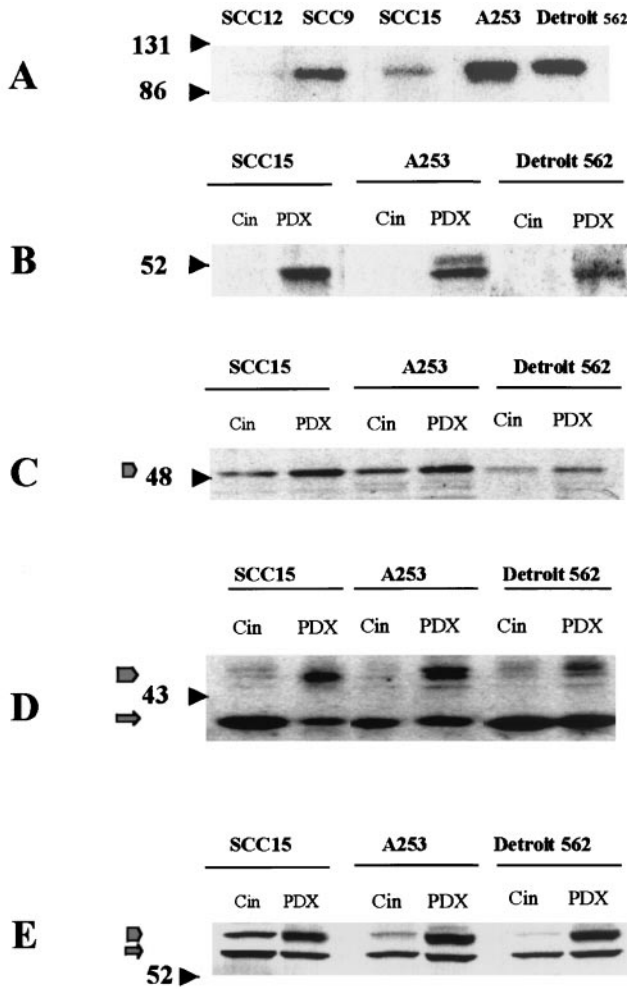

Fig. 1. (*A*) Western blot analysis of furin expression in HNSCC. Moderate to high levels of furin were seen in four cell lines. Detroit 562 and A 253 are aggressive cell lines that showed high levels of invasiveness *in vivo*, whereas SCC 15 and SCC 9 are moderately invasive. The SCC 12 cell line is characterized by slow growth and minimal invasiveness and has practically no furin expression. (*B*) Western analysis of PDX expression in transfected cells. The vector alone (Cin)-transfected cells are negative and all PDX-transfected cells show intense expression (PDX). Although SCC 15 and Detroit 562 expressed one form of the protein, A 253 showed two bands corresponding to ≈46 and 50 kDa, that may represent nonglycosylated (46 kDa) and glycosylated (50 kDa) forms. (*C*) Western analysis of TGF-β1 in cell lysates. Note the relatively higher accumulation of the intracellular pro form (pentagon-shaped arrow) in PDX transfectants (PDX) (compared with the lower expression of Cin vector-alone transfectants). (*D*) Western analysis of TGF-β1 expression and processing in HNSCC lines transfected with PDX in the conditioned medium. PDX transfectants have reduced levels of the cleavage product (40 kDa, full arrow) and accumulation of the nonprocessed, 50-kDa protein (pentagon-shaped arrow). (*E*) Western analysis of MT1-MMP showing the processed form (60 kDa, full arrow) in the vector alone-transfected cells (Cin) and the proform (63 kDa, pentagon-shaped arrow) in the PDX-transfected cells (PDX).

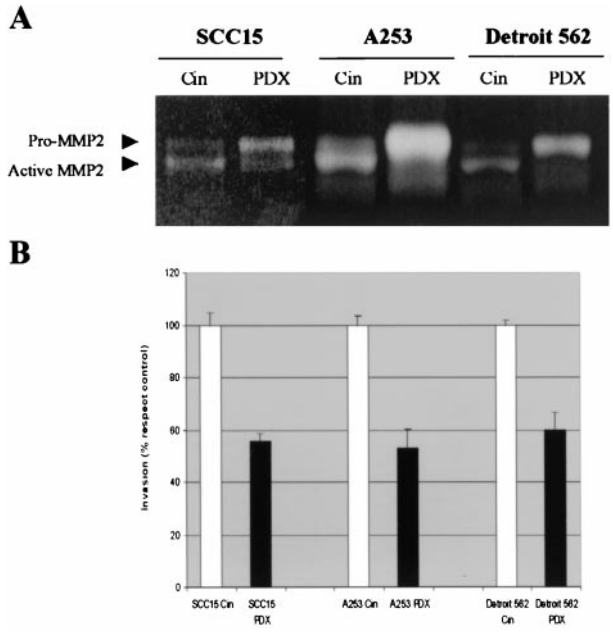

Fig. 2. (*A*) Gelatinase zymogram of transfected cells. Note that the three vector alone–transfected cell lines (Cin) exhibit a clear pattern of double bands corresponding to a 72-kDa proform (pro-MMP2) and a 64-kDa processed form (MMP-2), whereas the PDX-transfected cells show an accumulation of the pro form and a minimal or absent processed form. (*B*) Histogram of the *in vitro* invasion assays performed with PDX transfectants from three HNSCC lines and their respective vector alone-transfected cells. Results are expressed as percent of their respective vector alone transfectant. Each bar represents a mean of three different experiments. A total of 18 filters (three per clone) and 9,207 cells were counted (≈1,000–1,600 cells per transfectant clone). One-sided *t* test indicated statistically significant differences ($P < 0.005$).

ing to the intact precursor protein) was observed in the PDX-transfected cells, whereas the vector alone-transfected cells exhibited a relative increase of the cleaved pro-region (40-kDa band) (Fig. 1*D*). Thus, PDX-transfected cells showed a decreased processing of endogenous TGF-β1.

MT1-MMP processing was studied by Western analysis in cell lysates using two different antibodies. When using the antibody raised against the hinge region of the protein, cell lines A 253 and Detroit 562 showed a doublet representing the putative pro and processed forms of this enzyme. In the PDX-transfected cells the band corresponding to the pro-form (63 kDa) was remarkably more prominent than the lower 60-kDa band, whereas in the vector alone-transfected cells the band corresponding to the mature form was practically the only one detected. SCC 15 transfected with vector alone showed the two bands corresponding to the mature MT1-MMP (60 kDa) and the pro form (63 kDa). In the PDX transfectant an increase in the slower migrating band was observed, indicating that MT1-MMP processing was partially inhibited (Fig. 1*E*). These results were confirmed by using mAb that recognizes the hemopexin-like domain (data not shown).

Zymography was performed as a tool to evaluate the ability of the transfectants to degrade collagen IV. As depicted in Fig. 2*A*, the three PDX-transfected cell clones exhibited decreased activation of MMP-2. This was evidenced by the accumulation of the pro form and the decrease in the levels of the processed form as compared with the control vector alone-transfected cells. SCC 15 showed low MMP-2 levels that correlated well to its relatively low invasiveness. Higher expression of this MMP was observed in the other two more aggressive cell lines, A 253 and Detroit 562. Despite the enhanced expression of MMP-2 in these aggressive cell lines, MMP-2 processing was almost completely prevented after transfection with PDX.

***In Vitro* and *in Vivo* Invasion.** The selected clones from the three PDX-transfected HNSCC cell lines showed a statistically significant decrease in the *in vitro* invasive ability when compared with their respective vector alone-transfected counterparts (Fig. 2*B*). Overall, the PDX-transfected cells exhibited approximately half the invasive ability of the control cells.

A remarkable decrease in invasiveness of PDX-transfected

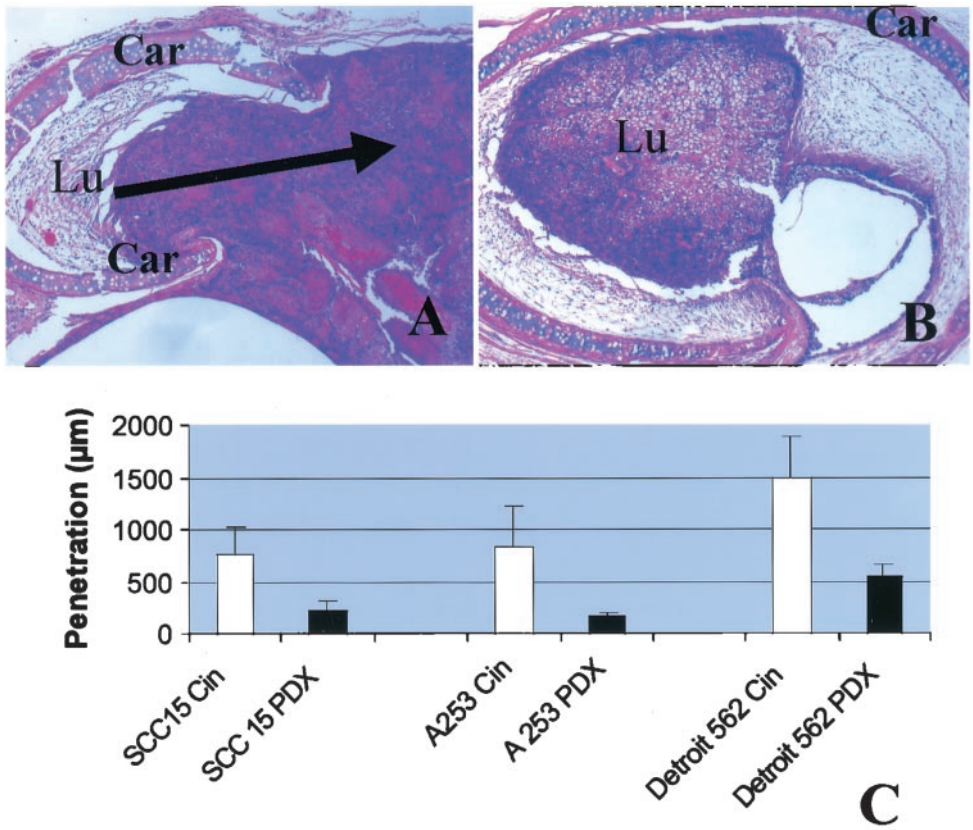

Fig. 3. *In vivo* invasion assay. (*A*) Micrograph of a tracheal transplant cross section showing the growth pattern of vector alone-transfected SCC 15 cells. Note that the cells originally placed in the lumen (Lu) have grown inside the tracheal wall and have entered the peritracheal space in the direction of the arrow. Cells invade through the area of least resistance, i.e., the *pars membranacea* between the opening of the tracheal cartilage ring (Car) as noted by the arrow. (*B*) Micrograph of a tracheal transplant cross section containing PDX-transfected SCC 15 cells. Note that the cells remain in the luminal (Lu) and interior area of the trachea. (*C*) Histogram showing the level of penetration of transfected HNSCC cells. Note a marked reduction in the depth of penetration of the PDX-transfected cells into the tracheal wall as compared with their respective vector alone-transfected counterparts (Cin cells). A total of 6–10 tracheal transplants per cell line (total: 24 transplants) were used in two different experiments. One-sided *t* test indicated statistically significant differences ($P < 0.05$). (Magnification: *A* and *B,* hematoxylin and eosin stain ×30.)

cells was noted by using an *in vivo* invasion assay based on the penetration of tumor cells into the tracheal wall. Most transplanted tracheas containing the vector alone-transfected cells penetrated deep into the tracheal wall through the *pars membranacea*, reaching the adventitia and the surrounding peritracheal tissues (Fig. 3*A*). The PDX-transfected cells showed a clear decrease in the depth of penetration of the tracheal wall. All xenografted tracheas showed the cells remaining inside the luminal area or in an immediately subluminal position in the lamina propria, never reaching the outer surface of the trachea (Fig. 3*B*). Quantitative evaluation of the penetration depth of tumor cells into the tracheal wall indicated that PDX-transfected cells exhibited a decreased invasiveness with respect to their vector alone-transfected counterparts of 70–80% (Fig. 3*C*).

s.c. Tumorigenicity. Transfection of SCC cell lines with PDX resulted in significant decrease or in some cases absence of tumor growth. The most striking results were obtained with PDX-transfected SCC 15 cells that showed no growth during the first 35 days. This finding contrasted with the gradual increase in size of the vector alone-transfected SCC 15 Cin cells that reached a mean volume of 75 mm^3 at 5 weeks (Fig. 4*A*). Tumors appeared late, i.e., at 38 days after inoculation of SCC 15 PDX cells, and remained significantly smaller than the controls. A similar pattern was seen with A 253 cells transfected with PDX (Fig. 4*B*). The differences between the PDX and the vector-alone cells were not as marked as in the case of SCC 15. Detroit 562 cells transfected with PDX were able to grow and produced s.c. tumors 2–3 weeks after transplantation. Nevertheless, these tumors were 30–50% smaller than the tumors produced after inoculation of the vector alone-transfected counterpart (Detroit 562 Cin cells) (Fig. 4*C*). The latency period to tumor formation was somewhat shorter for the A 253 PDX and Detroit 562 PDX cells than for the SCC 15 PDX-transfected cells, but was always longer that their respective vector-alone controls. Densitometric analysis of Northern blots showed that PDX expression of s.c. tumors excised 6–7 weeks after cell inoculation was 60–90% lower than that of the inoculated transfected cells (Fig. 5).

Discussion

PCs play an important role in tumor development and progression (2). PACE-4 is overexpressed in aggressive murine skin tumors and SCC cell lines (9). Another PC, furin, is expressed at low levels in several normal human tissues. Conversely, furin is overexpressed in malignancies of the lung and breast (12–14) as well as in rat Leydig tumor cells (24).

The fact that substrates processed by furin and PACE4 include a group of notorious molecules involved directly or indirectly in tumor growth and progression supports the importance of PCs in the pathogenesis of cancer. Among these substrates, stromelysin-3, MT1-MMP, TGF-β1, and insulin-like growth factor 2 have been extensively studied, and their processing by furin and/or PACE-4 has been well described (3–5, 7). These studies have relied very heavily on the use of PC transfection or double

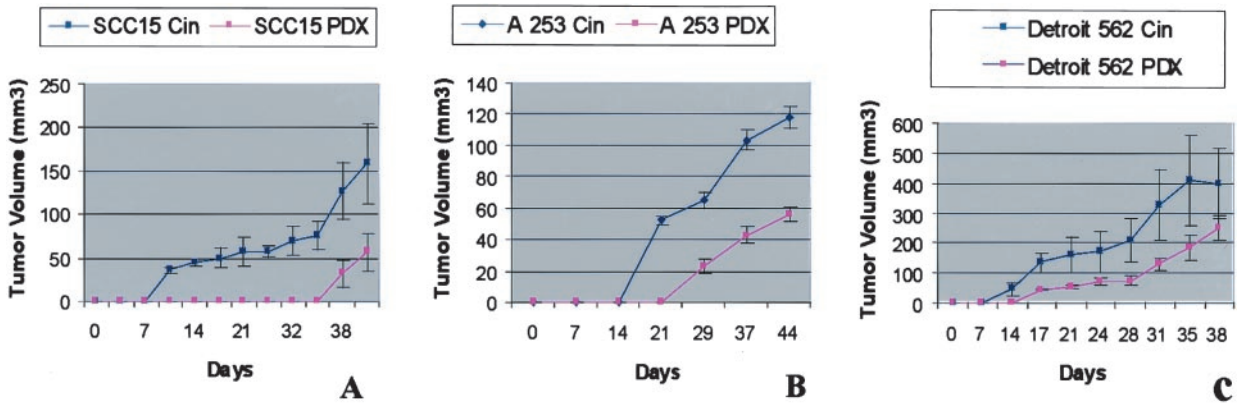

Fig. 4. s.c. tumorigenicity of transfectant cells after inoculation in the dorsal s.c. tissue of Scid mice. SCC 15 cells (*A*) showed the most striking difference in growth pattern, whereas the PDX-transfected cells produced no or very small tumors and the vector alone-transfected cells gave rise to relatively large tumors. The latency time to tumor detection was longer than that of the other PDX-transfected cells. The other two cell lines, A253 (*B*) and Detroit 562 (*C*), showed a decreased tumor growth pattern after PDX transfection. Note that there was a 2- to 3-fold difference between the volume attained by the tumors from vector alone-transfected and PDX-transfected cells. Each point represents the mean and standard error of measurements performed on 4–5 tumors per cell line. Rhombus = vector alone (Cin)-transfected cells; squares = PDX-transfected cells. ANOVA and log rank test indicated statistically significant differences in growth rate and time of tumor onset, respectively ($P < 0.05$).

transfection of PCs and their substrates. This approach has permitted researchers to focus on the mechanistic bases of PCs as cancer-associated processing enzymes. In the past, we have been able to use a similar approach to demonstrate the acquisition or enhancement of tumor invasiveness by transfecting with full-length PACE4 cDNA (9).

The human HNSCC cell lines SCC 15, Detroit 562, and A 253 are known to exhibit an invasive growth pattern when xenotransplanted into athymic nude mice (17). In this article we describe that in contrast to the less invasive cell lines, such as SCC 12 (18), these three HNSCC cell lines express moderate to high levels of furin protein. Because our studies show a positive correlation between tumor cell invasiveness and PC levels in murine and human squamous carcinoma cells, we proceeded to explore the possibility of inhibiting tumor growth and invasiveness by overexpressing the furin inhibitor PDX. Previous studies using this agent have shown that its overexpression downregulates the processing of several furin substrates (15, 16).

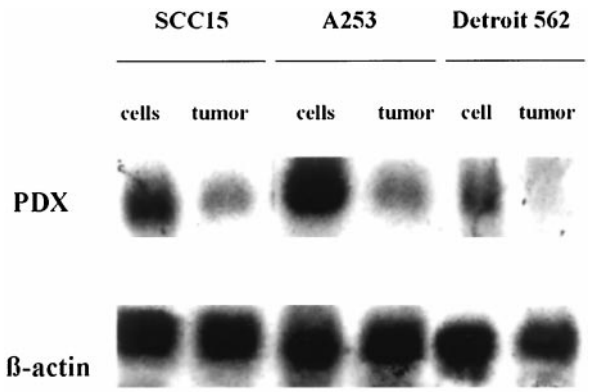

Fig. 5. Northern blot analysis of the expression of PDX in the original cell inoculates (Cells) and the respective tumor that developed s.c. 6–7 weeks after inoculation into Scid mice (Tumor). (*Upper*) PDX expression. (*Lower*) Reprobing of the same samples with a β-actin cDNA probe. Note the significant decrease of PDX mRNA expression in the lanes labeled Tumor when compared to the respective original cell line inoculum. Densitometric evaluation of the normalized PDX expression (with respect to β-actin expression) indicated the following percentages of PDX decrease in tumors compared to the inoculated cell line: SCC 15, 67%; A 253, 60%; and Detroit 562, 90%.

Herein, we have shown that overexpression of the furin inhibitor PDX is able to decrease and even abolish the invasive ability of three different malignant HNSCC cell lines. It is of interest to note that invasiveness was markedly decreased both *in vivo* and *in vitro*. In the *in vitro* invasion assays, PDX was able to decrease invasiveness by ≈50% whereas in the *in vivo* assay the reduction, as measured by the penetration of tumor cells into the tracheal wall, was 3- to 5-fold. This difference is possibly because of the more complex *in vivo* situation in which the inhibition of furin could have a more complex effect. In this case, PDX would inhibit the processing of substrates expressed by tumor cells and also would impinge on the processing of several furin substrates produced by stromal cells that are not present in the *in vitro* assay. This could be the case for stromelysin-3, MT1-MMP, and TGF-β, well known furin substrates that also are expressed by stromal cells of squamous cell carcinomas and other epithelial tumors (26–28). The detection of furin-substrate processing, such as that of TGF-β1 and MT1-MMP, is difficult to achieve in HNSCC cell cultures because of the low levels of endogenous protein expression in these cell lines. Most reports on furin-mediated processing are based on transfected cell systems in which either the substrate or both substrate and PC have been transfected to allow for easier detection of the artificially overexpressed proteins (3–5, 7). Nevertheless, in our cell system we could detect TGF-β1 expression in tumor cells and found that PDX has an inhibitory effect on its processing. This inhibition was noted because of an accumulation of the pro form of TGF-β1 in PDX-transfected cells, a fact that could not be observed in the vector alone-transfected cells. TGF-β1 is known for its role in activating MMP-2 in normal and cancer cells (29, 30) and for enhancing invasion and metastasis in several human and experimental cancer models (31–34).

Another furin substrate that has a paramount role in the processing of MMP-2 is MT1-MMP. The first step in Pro-MMP2 activation, that was clearly seen in our zymograms, is the conversion of the 72-kDa inactive protein to a processed 64-kDa form (35). This process is controlled by MT1-MMP (4), the main furin substrate that catalyzes this conversion. The expression of MT1-MMP was observed in the transfected cells by Western analysis. Despite its low expression levels, PDX-transfected cells exhibited a remarkable reduction in the lower band corresponding to the processed form of MT1-MMP and accumulation of the unprocessed form. Although other furin substrates, such as

TGF-β1, also contribute to the decrease in invasiveness seen in our PDX-transfected cells, the impairment in MT1-MMP processing and its direct consequence, the diminished MMP-2 activation, is likely the main factor behind the drastic PDX effects on tumor cell invasiveness. This event is particularly relevant to A 253 and Detroit 562 cells, the most aggressive of our series, in which the processing of MT1-MMP was drastically diminished. It is probable that in these more aggressive cell lines, other furin-independent mechanisms of activation of MT1-MMP are operating. Nevertheless, the reduction in pro-MMP-2 processing and *in vitro* and *in vivo* invasiveness was prominent in all PDX-transfected cells lines. The decrease of invasiveness also correlated very well with the remarkable decrease in the *in vivo* s.c. tumorigenicity observed after inoculation of all HNSCC cell lines transfected with PDX. It should be noted that although the three cell lines responded well to PDX overexpression, SCC 15 cells seemed to be most sensitive to the effects of PDX, perhaps because these cells are originally less invasive and are thus slightly more prone to the inhibitory effects of PDX than the more invasive cell lines. In addition, the inhibitory effect of PDX on tumor growth and invasiveness in our *in vivo* systems probably is underestimated because we observed a marked loss in PDX expression in the s.c. tumors. This finding suggests that a gradual loss of the transfected gene takes place *in vivo* and probably is the cause of the relatively accelerated growth of the s.c. tumors during the last weeks of the experiment.

Taken together this information indicates that PDX is a promising agent that is capable of modulating and even abolishing one of the most pernicious features of malignancy, i.e., tumor cell invasiveness. Based on these observations, targeting furin is a potential additional modality for cancer therapy.

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1. Molloy, S. S., Anderson, E. D., Jean, F. & Thomas, G. (1999) *Trends Cell. Biol.* **9,** 28–35.
2. Bassi, D. E., Mahloogi, H. & Klein-Szanto, A. (2000) *Mol. Carcinog.* **28,** 63–69.
3. Pei, D. & Weiss, S. J. (1995) *Nature (London)* **375,** 244–247.
4. Yana, I. & Weiss, S. J. (2000) *Mol. Biol. Cell* **7,** 2387–2401.
5. Dubois, C. M., Blanchette, F., Laprise, M. H., Leduc, R., Grondin, F. & Seidah, N. G. (2001) *Am. J. Pathol.* **158,** 305–316.
6. Posthaus, H., Dubois, C. M., Lapise, M. H., Grondin, F., Suter, M. M. & Muller, E. (1998) *FEBS Lett.* **438,** 306–310.
7. Duguay, S. J., Jin, Y., Stein, J., Duguay, A. N., Gardner, P. & Steiner, D. F. (1998) *J. Biol. Chem.* **273,** 18443–18451.
8. Lehmann, M., Andre, F., Bellan, C., Remacle-Bonnet, M., Garrouste, F., Parat, F., Lissitsky, J. C., Marvaldi, J. & Pommier, G. (1998) *Endocrinology* **139,** 3763–3771.
9. Hubbard, F. C., Goodrow, T., Liu, S. C., Brilliant, M. H., Basset, P., Mains, R. E. & Klein-Szanto, A. (1997) *Cancer Res.* **57,** 5226–5231.
10. Johnson, R. C., Darlington, D. N., Hand, T. A., Bloomquist, B. T. & Mains, R. E. (1994) *Endocrinology* **135,** 1178–1185.
11. Santavicca, M., Noel, A., Anglike, H., Stoll, I., Segain, J. P., Anglard, P., Chretien, M., Seidah, N. & Basset, P. (1996) *Biochem. J.* **315,** 953–958.
12. Schalken, J. A., Roebroek, A., Oomen, P., Wagenaar, S. S., Debruyne, F. M., Bloemers, H. P. & Van de Ven, W. J. (1987) *J. Clin. Invest.* **80,** 1545–1549.
13. Mbikay, M., Sirois, F., Yao, J., Seidah, N. G. & Chretien, M. (1997) *Br. J. Cancer* **75,** 1509–1514.
14. Cheng, M., Watson, P. H., Paterson, J. A., Seidah, N., Chretien, M. & Shiu R. (1997) *Int. J. Cancer* **71,** 966–971.
15. Anderson, E. D., Thomas, L., Hayflick, J. S. & Thomas, G. (1993) *J. Biol. Chem.* **268,** 24887–24891.
16. Jean, F., Thomas, L., Molloy, S. S., Liu, G., Jarvis, M. A., Nelson, J. A. & Thomas, G. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2864–2869. (First Published February 18, 2000, 10.1073/pnas.050504297)
17. Caamano, J., Zhang, S. Y., Rosvold, E. A., Bauer, B. & Klein-Szanto, A. J. P. (1993) *Am. J. Pathol.* **142,** 1131–1140.
18. Zhang, S. Y., Liu, S. C., Johnson, D. G. & Klein-Szanto, A. J. P. (2000) *Cancer Res.* **60,** 5972–5976.
19. Momiki, S., Caamano, J., Iizasa, T., Nakajima, M., Yamaguchi, Y. & Klein-Szanto, A. J. P. (1991) *Invasion Metastasis* **11,** 66–75.
20. Klein-Szanto, A. J., Iizasa, T., Momiki, S., Garcia-Palazzo, I., Caamano, J., Metcalf, R., Welsh, J. & Harris, C. C. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6693–6697.
21. Dionne, C. A., Camoratto, A. M., Jani, J. P., Emerson, E., Neff, N., Vaught, J. L., Murakata, C., Djakiew, D., Lamb, J., Bova, S., *et al.* (1998) *Clin. Cancer Res.* **4,** 1887–1898.
22. Gentry, L. E., Lioubin, M. N., Purchio, A. F. & Marquardt, H. (1988) *Mol. Cell. Biol.* **8,** 4162–4168.
23. Laprise, M. H., Grondin, F. & Dubois, C. M. (1998) *Biotechnol. Bioeng.* **58,** 85–91.
24. Liu, B., Amizuka, N., Goltzman, D. & Rabbani, S. A. (1995) *Int. J. Cancer* **63,** 276–281.
25. Asch, P. H., Basset, P., Roos, M., Grosshans, E., Bellocq, J. P. & Cribier, B. (1999) *Am. J. Dermatopathol.* **21,** 146–150.
26. Okada, A., Bellocq, J. P., Rouyer, N., Chenard, M. P., Rio, M. C., Chambon, P. & Basset, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2730–2734.
27. Mizoi, T., Ohtani, H., Miyazono, K., Miyazawa, M., Matsuno, S. & Nagura, H. (1993) *Cancer Res.* **53,** 183–190.
28. Gerdes, M. J., Larsen, M., McBride, L., Dang, T. D., Lu, B. & Rowley, D. R. (1998) *J. Histochem. Cytochem.* **46,** 379–388.
29. Miralles, F., Battelino, T., Czernichow, P. & Scharfmann, R. (1998) *J. Cell Biol.* **143,** 827–836.
30. Sehgal, I. & Thompson, T. C. (1999) *Mol. Biol. Cell* **10,** 407–416.
31. Akhurst, R. J. & Balmain, A. (1999) *J. Pathol.* **187,** 82–90.
32. Hasegawa, Y, Takanashi, S., Kanehira, Y., Tsushima, T., Imai, T. & Okumura, K. (2001) *Cancer* **91,** 964–971.
33. Pasche, B. (2001) *J. Cell Physiol.* **186,** 153–168.
34. McEarchern, J. A., Kobie, J. J., Mack, V., Wu, R. S., Meade-Tollin, L., Arteaga, C. L., Dumont, N., Besselsen, D., Seftor, E., Hendrix, M. J., *et al.* (2001) *Int. J. Cancer* **91,** 76–82.
35. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A. & Goldberg, G. I. (1995) *J. Biol. Chem.* **270,** 5331–5338.