Inhibition of Proprotein Convertases Enhances Cell Migration and Metastases Development of Human Colon Carcinoma Cells in a Rat Model

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Although proprotein convertases are involved in tumor development, nothing is known about their role in metastatic dissemination. To investigate the involvement of convertase inhibition, we used human colon carcinoma cells overexpressing $\alpha 1$ -antitrypsin Portland ($\alpha 1$ -PDX, PDX39P cells), a potent convertase inhibitor. We previously reported that these cells bear uncleaved integrin α subunits and display an altered attachment to vitronectin that is correlated with defects in the intracellular signaling pathways activated by $\alpha v\beta 5$ integrin ligation. In this study, we demonstrate that the inhibition of proprotein convertase activity either by overexpression of $\alpha 1$ -PDX or with the synthetic inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk) led to a significant increase in cell migration supported by the $\alpha v\beta 5$ integrin. A collagen gel invasion assay showed that PDX39P cells also displayed an invasive ability, contrary to control cells. Moreover, when injected to immunosuppressed newborn rats, PDX39P cells were highly invasive, as they induce 10 times more metastases than mock-transfected cells. In addition, the aggressiveness of PDX39P cells can be greatly reduced by a function-blocking monoclonal antibody (mAb) against the αv subunit. It thus seems that inhibition of proprotein convertases enhances the *in vivo* invasiveness of colon tumor cells likely due to an increase in cell migration mediated by αv integrins. (*Am J Pathol* 2004, 164:1925–1933)

The acquisition of cell motility and the capacity to invade basement matrix membranes and adjacent tissues play a

central role in the complex multi-step process of metastasis. Cell migration is the result of dynamic interactions between the cell, the extracellular matrix (ECM), and the cytoskeleton. Integrins connect the ECM proteins outside to the actin cytoskeleton within the cell, allowing the traction required for cell migration.^{1,2} Integrins also play a crucial role in signal transduction events that control anchorage-independent growth and survival of tumor cells (for reviews, see references^{3–5}). Therefore, these adhesion molecules play a pivotal role in tumor cell invasion and metastasis.^{5–7}

Integrins are heterodimeric proteins composed by the non-covalent association of α and β subunits. Some integrin α chains undergo a post-translational cleavage in their extracellular domain by furin and PC5A, two proprotein convertases (PCs) of the subtilisin/kexin family.^{8,9} The role of this cleavage in integrin function remains unclear. Arguably, this post-translational processing of the α chain is not required for ligand binding,^{10–12} but is essential for the signaling function of $\alpha 6\beta 1$ and $\alpha v\beta 5$ integrins.^{10,13} Moreover, recent data suggest that the $\alpha 6$ subunit cleavage might be linked to the differentiation state of lens cells.¹⁴

PCs are responsible for the biological activation of a variety of precursor proteins, including pro-hormones and their receptors and adhesion molecules. They are ubiquitously expressed and are involved in many physiological and pathological processes.¹⁵ The potential clinical and pharmacological role of the convertases fostered the development of several inhibitors. The most promising protein-based specific inhibitor of PCs is an $\alpha 1$ -antitrypsin variant known as $\alpha 1$ -antitrypsin Portland ($\alpha 1$ -PDX). $\alpha 1$ -PDX is a selective inhibitor of furin and, to a lesser extent, of PC5B¹⁶ and blocks the convertase-dependent processing of various precursors, including integrins.^{10,17–19}

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Recent reports have suggested a possible role for PCs in tumorigenesis (for reviews, see references^{15,20}). Thus, furin expression levels correlate with invasiveness in human head and neck tumors and cell lines.²¹ Moreover, further data show that tumors obtained after subcutaneous inoculation of furin-overexpressing cells are larger and develop earlier than the controls.²² Conversely, inhibition of PCs resulted in reduced *in vivo* tumorigenicity and *in vitro* cell proliferation.^{23–25}

We have recently reported that the absence of endoproteolytic cleavage of αv integrins, by overexpression of $\alpha 1$ -PDX in colonic cancer cells, has important consequences on signal transduction pathways leading to alterations in integrin function such as cell adhesion.¹⁰ PCs inhibition also leads to reduced cell proliferation *in vitro* and delayed tumor growth in nude mice inoculated with tumor cells.²³ However, herein we demonstrate that PCs inhibition moderately reduced subcutaneous tumor growth but dramatically enhanced metastasis in immunosuppressed newborn rats. This aggressive behavior is likely due to an important increase in αv integrin-dependent cell migration.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Cergy-Pontoise, France) and fetal calf serum (FCS) from BioWhittaker (Fontenay-sous-Bois, France). The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKK-cmk) was from Bachem (Voisins-le-Bretonneux, France). Protease inhibitors were from Sigma (St. Louis, MO). ³⁵S-methionine was provided by Amersham (Les Ulis, France). Laminin-1 and vitronectin were prepared according to Timpl et al²⁶ and Yatogho et al,²⁷ respectively.

Rat mAb 69.6.5 against human αv integrin subunit was produced in our laboratory.²⁸ Mouse monoclonal antibodies (mAbs) VNR139 (anti- αv subunit) and P1F6 (anti- $\alpha v \beta 5$) and polyclonal antibody anti-HGF receptor were from Chemicon (Temecula, CA). Mouse C3VLA3 antibody against $\alpha 3$ integrin subunit was purchased from Immunotech (Marseille, France). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham.

Cell Migration

Human IGROV1 and HT29-D4 cells, derived from human ovarian and colonic adenocarcinomas, respectively, were cultured as described.^{10,29} Control (empty vector, PDX0) and stably $\alpha 1$ -PDX-transfected (PDX39P) HT29-D4 cells were described as previously reported.¹⁰

In vitro haptotaxis assays were performed using modified Boyden chambers (Transwell, Costar, Cambridge, MA), as previously described.³⁰ In some experiments, cells were cultured for 48 hours in the presence of increasing concentrations of the furin inhibitor dec-RVKK-cmk. Cells were then metabolically labeled with 1 μ Ci/ml

³⁵S-methionine for 2 hours and tested for migration in the presence of phorbol 12-myristate 13-acetate (PMA). Following incubation at 37°C, cells on the upper surface of the membrane were wiped with a cotton swab. Radioactivity of the migrated cells in the lower surface was determined in a liquid scintillation counter.

Collagen Invasion Assay

Collagen invasion assays were performed as previously described.³¹ Type I collagen was dissolved in bicarbonate buffer-containing DMEM neutralized with 1 mol/L NaOH. Samples (1.2 ml) were poured into a 6-well plate and incubated overnight at 37°C for gelation. Cells were harvested using Moscona and trypsin/EDTA buffers, and seeded on top of the collagen gels. After incubation for 24 hours at 37°C in the presence or absence of 10 nmol/L PMA, the depth of cell migration inside the collagen was measured using an inverted microscope. Invasive and superficial cells were counted in 12 predetermined fields of 0.157 mm². The invasion index is the percentage of cells invading the gel over the total number of cells. DHD-FIB and MCF7/AZ cells were used as positive and negative controls of cell invasion, respectively.

In Vivo Tumor Induction

The Wistar newborn rats (six per cell line) received a ventral subcutaneous injection of 10⁶ cells suspended in 100 μ l phosphate-buffered saline (PBS). All rats were subsequently immunosuppressed by dorsal subcutaneous injections of anti-thymocyte serum³² at days 0, 2, 7, 14 and maintained in a specific pathogen-free environment throughout the experiment. Three weeks later, animals were sacrificed and the tumors and lungs were excised and processed for further analyses. The number of metastases of each lung was macroscopically and microscopically determined by three experimenters.

Reverse-Transcription/Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from tumors, metastases, and cultured cells using Trizol (Gibco). cDNAs were amplified by PCR, as already described,²³ using the following oligonucleotides:

oligonucleotides (sense) oligonucleotides (anti-sense)
 $\alpha 1$ -PDX TCTTCTTCCTGCCTGATGAGG TAATACGAC-
TCACTATAGGG

GAPDH TGGAAATCCCATCACCCTCT GTCTTCTGG-
GTGGCAGTGAT

The transcript of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was taken as reference.

Immunoprecipitation and Immunoblot

PDX0 and PDX39P tumors were cut in small pieces and ground with an UltraTurax in 20 mmol/L Tris-HCl (pH 8), 200 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X100

(RIPA buffer) containing a mixture of protease inhibitors (1 mmol/L PMSF, 500 U/ml aprotinin, 1 μ g/ml leupeptin, 1 μ mol/L pepstatin, 1 mmol/L iodoacetamide, and 1 mmol/L o-phenanthroline). Cells were directly solubilized with the buffer. In some experiments, lysates were clarified by centrifugation and incubated with 5 μ g rat mAb 69.6.5 overnight at 4°C and then with protein G-agarose for 45 minutes. Pellets were washed three times with RIPA buffer, three times with RIPA buffer/500 mmol/L NaCl, and once with PBS. Cell lysates or immunoprecipitated proteins were resolved by SDS-PAGE and blotted onto a nitrocellulose sheet. Membranes were blocked in PBS/5% nonfat milk/0.2% Tween 20 and probed overnight at 4°C with anti- α v mAb VNR139 (1:1000) or anti-HGF receptor (1:500). Blots were then revealed by chemiluminescence using HRP-conjugated secondary antibodies.

Results

Proprotein Convertases Inhibition Increases Cell Migration

We previously reported that cells expressing high levels of the PCs inhibitor α 1-PDX (PDX39P cells) showed a reduced attachment to vitronectin.¹⁰ To determine whether α 1-PDX expression also affects cell migration we used haptotaxis assays toward attractive proteins in modified Boyden chambers. As matrix proteins, we used vitronectin, the sole ligand for the integrin α v β 5 whose cleavage appears to be important for its function, and laminin-1 as a cleavage-independent substrate.¹⁰ As already reported for the parental cell line,³⁰ both ECM proteins allow protein kinase C (PKC)-dependent migration of transfected cells (Figure 1). On stimulation with the phorbol ester PMA, the ability of PDX39P cell to migrate toward vitronectin was enhanced by more than threefold when compared to mock-transfected PDX0 cells (Figure 1A) or to the parental HT29-D4 cells (not shown). Moreover, ligand concentration allowing maximal migration was slightly higher for PDX39P (8 μ g/ml) than for PDX0 (2 μ g/ml). On the contrary, laminin-1 supported cell migration with the same efficiency for all cell types.

Integrins are the major adhesion receptors, but integral membrane proteoglycans are also present at the cell surface and can bind ECM components. However, in our case, migration to vitronectin was dependent on α v β 5 integrins. Indeed, as shown on Figure 1B, the function-blocking mAb P1F6, against the α v β 5 integrin, prevented cell motility of both PDX0 and PDX39P cells toward vitronectin, while a mAb blocking the α 3 subunit failed to affect cell migration.

To ascertain that the difference in behavior was not due to the selection process of transfected cells, PDX0 cells were treated for 48 hours with increasing concentrations of the furin inhibitor dec-RVKR-cmk before performing the migration assay. As illustrated in Figure 2A, migration of PDX0 cells to vitronectin increased with inhibitor concentration. The same result was obtained with laminin-5 which is another ECM protein recognized by

Figure 1. Effect of α 1-PDX expression on PMA-induced cell migration. **A:** Cell motility toward vitronectin or laminin-1 was determined using modified Boyden chambers. The bottom surface of porous membrane was pre-coated with the indicated concentrations of purified ECM protein. PDX0 (open diamonds) and PDX39P (closed circles) cells were seeded into the upper reservoir with 10 nmol/L PMA and allowed to migrate toward the lower reservoir for 5 hours at 37°C. Cells that migrated to the underside of the chamber were stained with Coomassie blue and enumerated. **B:** Cell migration toward 2.5 μ g/ml vitronectin was performed as described above in the absence or presence of 10 μ g/ml of function-blocking anti- α v β 5 (P1F6) or anti- α 3 antibodies. Data are means $\pm$ SD of two independent experiments performed in triplicate.

cleavable α integrin subunits in PDX cells (unpublished results). Immunoblot confirmed that dec-RVKR-cmk indeed impaired, almost completely, the cleavage of α v subunit on PDX0 cells (Figure 2B). The α v-containing integrins have been involved in ovarian tumor progression. We therefore repeated the experiment with the ovarian cell line IGROV1 whose migration to vitronectin is α v-dependent.²⁹ As shown in Figure 2C, incubation of IGROV1 cells with dec-RVKR-cmk also increased cell migration to vitronectin.

Taken together, the results described above clearly demonstrate that inhibition of PCs leads to an enhanced cell migration. Migration of cells used in this study requires activation of PKC, either by PMA or by growth factors.^{30,33} Hepatocyte growth factor (HGF) is produced by various cell types, including tumor-bordering myofibroblasts, and functions locally as a cell migration inducer. As illustrated in Figure 3A, HGF promoted cell migration to vitronectin more efficiently than PMA. It is worth noting that, as observed on PMA stimulation, PDX39P cells are more motile than PDX0 in response to a physiological inducer such as HGF. This is not due to a higher expression of the HGF receptor, as both cells expressed

Figure 2. PCs inhibition leads to increased cell migration. **A:** PDX0 cells were treated with various concentrations of the furin inhibitor dec-RVKR-cmk for 48 hours at 37°C. After ³⁵S-methionine labeling, motility of PDX39P and PDX0 cells toward vitronectin was measured in modified Boyden chambers in the presence of 10 nmol/L PMA. **B:** Proteins from PDX0 cells treated (+) or not (-) for 48 hours with 100 μmol/L dec-RVKR-cmk were solubilized and resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions. After blotting, the nitrocellulose membrane was probed with the anti-αv antibody VNR139. Uncleaved (αv^{NC}) and cleaved (αv^C) αv integrin subunits are emphasized. **C:** IGROV1 cells were treated with various concentrations of dec-RVKR-cmk for 48 hours at 37°C. Migration assays were performed, as described in Figure 1, using 8 μg/ml vitronectin. Results (± SD), expressed as the percentage of untreated cells (control), are from a representative experiment of three performed in duplicate.

similar amounts of receptor (Figure 3B). Interestingly, this receptor, which is a PCs substrate,³⁴ was cleaved in PDX39P cells, indicating that other PCs than PC5A or furin may be involved in its endoproteolytic processing. This also demonstrates that the enhanced effect of HGF on cell migration is not due to a defect in receptor maturation.

It has also been reported that PKC controls cell motility by regulating the expression of the plasminogen activator uPA. When exposed to uPA, PDX39P cells migrate more readily than control cells toward vitronectin (Figure 3A). It thus appears that whatever the stimulus used, the α1-PDX-expressing cells displayed an increase in cell migration compared to control cells.

Figure 3. HGF and uPA induce cell migration. **A:** Migration of ³⁵S-methionine-labeled PDX0 (filled bars) and PDX39P (open bars) cells was measured in modified Boyden chambers in the presence of PMA, HGF, or uPA. Results (± SD) are from a representative experiment of two performed in triplicate. **B:** Proteins from PDX cells were resolved by SDS-polyacrylamide gel electrophoresis under reducing (R) or non-reducing (NR) conditions. After blotting, the nitrocellulose membrane was probed with an antibody against the β chain of the human HGF receptor.

Expression of α1-PDX Increases the Invasive Potential

The invasive potential of PDX cells was assessed *in vitro* in gels of type I collagen, the main component of ECM. Results were quantified and expressed as percentage of invasive cells in the collagen layer. As shown in Figure 4, stimulation of HT29-D4 and PDX0 cells by PMA did not alter their invasive potential. On the contrary, invasion of collagen gels by PDX39P cells was dramatically enhanced on PMA treatment. These results show that expression of the PCs inhibitor α1-PDX leads to a marked increase in *in vitro* invasion when cells are stimulated by PMA, as observed above for cell migration.

Proprotein Convertases Inhibition Increases Metastases Formation

To determine whether increased mobility and invasiveness of PDX39P cells could lead to an enhanced *in vivo* aggressiveness, we analyzed the ability of cells to develop metastases in an experimental animal model widely used for digestive cancers.^{35,36} PDX0 and PDX39P cells were inoculated into two groups of immunosuppressed newborn rats, and tumors and lungs were excised for further analyses after 21 days. Although similar in appearance (Figure 5A), PDX39P-induced tumors were 30% smaller compared to PDX0-induced tumors (Figure 5B). As seen in Figure 5C, rats inoculated with PDX0 cells

Figure 4. Effect of $\alpha 1$ -PDX expression on collagen gel invasion. Cells were allowed to invade type I collagen gels in the absence (open bars) or the presence (filled bars) of PMA for 24 hours at 37°C. The invasion index is the percentage of cells invading the gel. Results ($\pm$ SD) are from a representative experiment of three performed in triplicate.

developed a few lung metastases. Conversely, lungs from PDX39P cell-inoculated rats were colonized by a very high number of large metastases, leading to a dramatic increase in lung volume. This was confirmed by metastases quantification (Figure 5D). The aggressiveness of PDX39P cells led to the death of 17% of animals inoculated with these cells before the experiment completion. The autopsy of dead animals revealed a massive colonization of lung tissues by tumor cells (Figure 5E). This is in accordance with the fact that micrographs of PDX39P-induced tumors showed morphological evidence of higher local invasiveness and infiltrative pattern (invasion of adjacent tissues, vascular invasion, and tumor cell emboli) when compared to PDX0-induced tumors (data not shown).

To ascertain that PDX39P cells still expressed $\alpha 1$ -PDX after inoculation into rats we used two approaches. First, expression of the $\alpha 1$ -PDX gene was assessed by semi-quantitative RT-PCR analysis in inoculated cells, as well as in several randomly chosen tumors and their corresponding metastases. The antisense oligomer was chosen from the vector promoter sequence to distinguish from endogenous $\alpha 1$ -antitrypsin. All but one of the PDX39P tumors tested were positive, indicating that expression of $\alpha 1$ -PDX persisted during tumor growth (Figure 6A). More interestingly, all metastases developed by rats inoculated with PDX39P cells were $\alpha 1$ -PDX positive. Second, we checked for the cleavage status of αv integrins in cells, tumors, and metastases by Western blot using a mAb specific for the human subunit. As illustrated in Figure 6B, only cell lysates from PDX39P tumors and their corresponding metastases displayed uncleaved αv subunits, suggesting that the expression level of $\alpha 1$ -PDX was sufficient to impede, at least partially, integrin processing.

Figure 5. $\alpha 1$ -PDX expression increases metastases formation. **A:** Macroscopic appearance of PDX39P- (a and b) and PDX0-induced tumors (c and d) 3 weeks after subcutaneous inoculation of cells in immunosuppressed newborn rats. **B:** Tumor volume was evaluated according to Stragand method. Data are mean values $\pm$ SD from two experiments (total of 12 rats per cell line). **C:** Macroscopic appearance of the lungs from PDX39P- (a and b) and PDX0-inoculated rats (c and d) 3 weeks after injection of tumor cells. **D:** Quantification of the number of lung metastases in PDX39P- and control cells-inoculated animals was done on each lung by three experimenters. Data represent mean values $\pm$ SD from two experiments (total of 12 rats per cell line). **E:** Masson trichrome staining of paraffin-embedded section. Micrograph (magnification, $\times 200$) shows voluminous metastases (M) with little healthy tissue. Pneumocytes (arrows) are pushed back beneath the pressure of metastatic cells.

Aggressiveness of PDX39P Cells Is αv -Dependent

The malignancy of tumor cells is essentially due to their invasive and metastatic potential, which needs integrin-dependent cell migration. To check the role of αv integrins in tumor cell aggressiveness, inoculation of cells into rats was performed in association with a function-blocking anti- αv mAb (69.6.5). The presence of the anti-integrin antibody led to a strong reduction in the number of lung metastases in PDX39P-inoculated rats (Figure 7; compare with Figure 5). Note that the mAb used in this experiment has been raised in rat and only blocked human αv integrins from tumor cells and not rat integrins from stromal or vascular cells. This experiment shows that the activity of αv integrins present on the tumor cell surface is essential for the invasiveness of colon carcinoma cells.

Discussion

PCs represent interesting potential targets for the development of new therapeutic agents. However, the efficient

Figure 6. α 1-PDX expression in PDX39P-induced tumors and metastases. **A:** Expression of the α 1-PDX gene was assessed by semi-quantitative RT-PCR analysis of total RNA extracted from inoculated cells (C), and from randomly chosen tumors (T) and their corresponding metastases (M). A gene product with the expected size is emphasized by an arrow. Bp, bp. **B:** The α integrin subunit, extracted from cells (C), tumors (T) or metastases (M), was immunoprecipitated with mAb 69.6.5 and resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions. After Western blot, the nitrocellulose membrane was probed with the anti- α v antibody VN139. Uncleaved (α v^{NC}) and cleaved (α v^C) α v integrin subunits are emphasized. Analysis was performed on five (PDX0) or six (PDX39P) tumors and at least one of their corresponding metastasis. For the sake of clarity, only one sample of each category is shown.

development and clinical application of PCs inhibitors require a better understanding of their molecular mechanisms *in vitro* and their impact on tumor growth and metastasis in various animal models before testing their efficacy in human disease. We, and others, previously observed that PCs inhibition leads to delayed tumor development and reduced tumor volume in nude or immunodeficient mice.^{22–25} However, herein we used immunosuppressed newborn rats to test the metastatic dissemination. Results obtained with this animal model show that the role of PCs in tumor development and progression is far from being as simple as suggested by previous observations.

Figure 7. Effect of anti- α v integrin mAb on metastases formation. **A:** PDX0 and PDX39P cells were injected subcutaneously into immunosuppressed newborn rats (six per cell line) along with 50 μ g of the function-blocking rat anti- α v mAb 69.6.5. The antibody was then injected along the anti-thymocyte serum at days 2, 7, and 14. Photographs represent lungs excised from animals on day 21 after injection of cells. **B:** The number of lung metastases was macroscopically and microscopically quantified by three independent experimenters. Values represent means $\pm$ SD.

As previously reported for nude mouse,²³ the expression of the convertase inhibitor α 1-PDX slightly decreased the tumorigenicity of human colonic PDX39P cells in immunosuppressed newborn rats. The reduced size of subcutaneous tumors in response to α 1-PDX expression is likely due to differences in cell proliferation, as already reported in *in vitro* studies.^{22–24} By histological staining with an antibody against Ki67, a nuclear protein expressed in proliferating cells, we confirmed that *in situ* cell proliferation was higher in PDX0- than in PDX39P-induced tumors (data not shown).

However, despite their lower growth rate and the smaller tumors they produced, α 1-PDX-expressing cells exhibit a very aggressive behavior when injected to immunosuppressed rats. Indeed, PDX39P-induced tumors showed morphological evidence of higher local invasiveness and infiltrative pattern and produced about 10 times more metastases than PDX0-induced tumors.

Among other processes, metastatic spread requires ECM remodeling, a reduction in cell adhesion, and an increase in cell motility, conditions fulfilled by PDX39P cells. The invasive behavior of PDX39P cells observed *in vivo* is associated with an increased cell migration toward vitronectin and with the invasion of collagen gels *in vitro*, which may be explained by alterations in cytoskeleton remodeling reported in a prior study.³⁷ The motile and invasive behavior likely involves integrin α v β 5, the sole receptor for vitronectin in HT29-D4²⁸ because a function-blocking mAb against the α v subunit was efficient to prevent both *in vivo* invasion and *in vitro* cell motility (see Figures 1B and 7). Besides, the enhanced motility is clearly due to PCs inhibition (and not to the selection of one or a few clones of PDX39P during passaging) because incubation of PDX0 cells with a synthetic furin inhibitor, which prevents integrin cleavage, led to increased migration. Furthermore, PCs inhibition also enhanced motility of the ovarian cell line IGROV1 whose migration to vitronectin is α v-dependent.²⁹ It also should be emphasized that the increased motile behavior of α 1-PDX-expressing cells was observed no matter which migration inductor we tested (PMA, HGF, or uPA).

All α integrin subunits expressed at the cell surface in PDX39P cells lack endoproteolytic cleavage. This leads to a poor attachment of PDX39P cells to substrates recognized exclusively by cleavable α subunits and to a defect in the FAK signaling pathway.¹⁰ It is notable that, despite the reduced adhesion they allow, these same ECM proteins (ie, vitronectin and laminin-5) support the increased motility of PDX39P cells. It is worth noting that the inhibitor expression persisted on inoculation of PDX39P cells into immunosuppressed rats, as α 1-PDX mRNA was present in PDX39P-induced tumors and metastases. Moreover, the expression level of the inhibitor was high enough to partially impede α v subunit cleavage. This suggests that the absence of endoproteolytic cleavage of the α v integrin subunit may be responsible for the observed increase in cell migration and aggressiveness. In support of this hypothesis, we observed that the blockage of α v subunit processing, by site-directed mutagenesis of the cleavage site, led to an invasive behavior on collagen on transfection in α v-deficient mel-

anoma cells M21L. This work will be described in detail elsewhere (V. Berthet, manuscript in preparation).

As can be seen in Figure 1 and already reported,³⁰ cell migration could only be observed in a narrow range of vitronectin concentration. Such biphasic curves in cell migration have also been reported for other cell lines.^{29,38} As cell migration requires the formation of new attachments at the cell front and the break of attachments at the rear, maximum cell speed is predicted to occur at an intermediate ratio of cell-substratum adhesiveness to intracellular contractile force.³⁹ Low ligand concentrations (or integrin activity) decrease the probability of formation of stable attachments by lamellipodes, while high ligand concentrations (or integrin activity) probably hinder cell migration by obstructing the release of adhesions at the rear. The reduced adhesion due to the absence of proteolytic processing of αv subunit¹⁰ could thus, at least partly, explain the increased motility of PDX39P cells.

The molecular mechanism by which the endoproteolytic cleavage of αv subunit affects cell migration and aggressiveness is not precisely understood. Potential hypotheses include alterations in the cellular proteins associated with or regulated by αv integrins. Thus, the unconventional processing of αv by membrane type 1 matrix metalloproteinase (MT1-MMP), and the subsequent generation of the modified $\alpha v\beta 3$ integrin, results in enhanced functional activity of the integrin.^{40,41} Moreover, further studies indicate that cells displaying the unconventional $\alpha v\beta 3$ integrin and cells treated with the furin inhibitor dec-RVKR-cmk show an increased attachment to type I collagen.⁴² These results are consistent with furin-processed αv controlling the cross-talk between $\alpha v\beta 3$ and $\alpha 2\beta 1$ integrins. Along with our own results, this clearly demonstrates that the cleavage of αv subunit and the way it is cleaved can drastically influence the integrin function and consequently the behavior of malignant cells.

In addition, it is worth noting that both PDX0 and PDX39P cells synthesize vitronectin *in vivo* and that this protein was mostly extracellular in PDX39P-induced tumors, while it remains largely cytoplasmic in PDX0-induced tumors (not shown). Although alternative explanations are possible, we can postulate that extracellular vitronectin may facilitate the invasion process of PDX39P cells. It has been shown that vitronectin is produced, on cleavage by furin, under two functionally different forms⁴³ and that intact vitronectin induces matrix metalloproteinase expression and enhanced melanoma cells migration and invasion.⁴⁴ Accordingly, although this question has not been addressed in this study, it is possible that the expression of $\alpha 1$ -PDX would impinge on the vitronectin cleavage and facilitate cell invasiveness *in vivo*.

Correlation between changes in the function or in the repertoire of integrins and alterations in cell motility or invasiveness has been observed both *in vitro* and in physiopathological diseases.^{45,46} For example, FG carcinoma cells which express the $\alpha v\beta 5$ integrin bind to, but do not migrate on vitronectin, while variants expressing the $\alpha v\beta 3$ integrin were able to migrate on vitronectin via an $\alpha v\beta 3$ -dependent mechanism.⁴⁷ However, addition of epidermal growth factor (EGF) to parental $\alpha v\beta 3$ -deficient cells

provided a PKC-dependent signal that allowed FG cells to migrate on vitronectin via an $\alpha v\beta 5$ -dependent mechanism.⁴⁸ This example also shows that interactions with microenvironment (the presence of EGF in this case) may be critical for tumor cell behavior.

The tumor microenvironment modulates growth, spreading, and migration of xenografted cancer cells by secreted growth factors.³⁻⁵ We thus observed that coculture with rat myofibroblasts significantly increased PDX39P proliferation. Migration, adhesion, and spreading were also altered by myofibroblasts-secreted factors, including HGF and uPA (see Figure 3 and data not shown). Furthermore, it was shown that the convertase expression confers an estrogen dependency for proliferation, both *in vitro* and *in vivo*, on human breast cancer cells.⁴⁹ Differences in stromal microenvironment might thus explain the discrepancies between the present *in vivo* results and invasion assays using tracheal xenotransplants.^{22,25}

Given the role of PCs in biological activation of inactive precursors, their inhibition could be an interesting clinical strategy against cancers by simultaneously disrupting the function of numerous proteins involved in tumor cell invasion and tumor progression.^{15,20} However, the effect of $\alpha 1$ -PDX on certain convertase substrates, like adhesion molecules, MMPs or other proteases, that could potentially contribute to tumorigenesis should be taken into account when considering PC inhibitors as therapeutic tools for a variety of diseases, including cancer. Thus, inhibition of cadherin cleavage results in decreased intercellular adhesive strength,⁵⁰ a situation observed in many tumors. Our present data clearly show that inhibition of PCs can also result in a dramatic increase of tumor cells aggressiveness. Whether this enhanced malignancy is specific to colonic tumor cells remains to be determined.

Thus, it appears that inhibiting PCs actively contribute to the malignant phenotype of human colonic cancer cells when injected to immunosuppressed rats. Our observations collectively suggest that the enhanced *in vivo* invasiveness of $\alpha 1$ -PDX-expressing cells might be linked to the increased motility, supported by αv -containing integrins, although other molecules might also be involved.

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