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Review

Proprotein Convertases in Tumor Progression and Malignancy

Novel Targets in Cancer Therapy

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The mammalian subtilisin/kexin-like proprotein convertase (PC) family has been implicated in the activation of a wide spectrum of proteins. These proteins are usually synthesized as inactive precursors before their conversion to fully mature bioactive forms. A large majority of these active proteins such as matrix metalloproteases, growth factors, and adhesion molecules are crucial in the processes of cellular transformation, acquisition of the tumorigenic phenotype, and metastases formation. Inhibition of PCs significantly affects the malignant phenotype of various tumor cells. In addition to direct tumor cell proliferation and migration blockade, PC inhibitors can also be used to target tumor angiogenesis. In this Review article we discuss a number of recent findings on the clinical relevance of PCs in cancer patients, their implication in the regulation of multiple cellular functions that impact on the invasive/metastatic potential of cancer cells. Thus, PC inhibitors may constitute new promising agents for the treatment of multiple tumors and/or in adjuvant therapy to prevent recurrence. ***(Am J Pathol 2002, 160:1921–1935)***

To regulate biological activity, a wide variety of proteins are synthesized as inactive precursors that are subsequently converted to their mature active forms by proteolytic enzymes known as proprotein convertases (PCs). The PCs are usually activating proteases and have not been reported to inactivate polypeptides, a process usually performed by degradative enzymes. To date, eight mammalian members of subtilisin-related PCs have been identified including, furin, PC1/PC3, PC2, PC4, PACE4, PC5/PC6, PC7/LPC/PC8, and SKI-1/S1P.[1–7] PCs are multidomain serine proteinases consisting of a signal peptide followed by pro, catalytic, middle, and cytoplasmic domains. Homology is highest in the catalytic domains and lowest in the carboxyl-terminal domains. Furin, PC1, PC2, PC4, PACE4, PC5, and PC7 cleave precursor proteins at basic residues within the general motif (K/R)-$(X)_n$-(K/R)↓, where $n = 0$, 2, 4 or 6 and X is usually not Cys.[1–5] In contrast, the subtilisin kexin isozyme-1 (SKI-1) processes precursors at non-basic amino acids within the motif (R/K)-X-(L,V)-(L,T,K,F)↓.[1,6–9] Furin, PC5-B, PC7, and SKI-1 are the only members of the mammalian PCs with a transmembrane domain and cycle between the trans-Golgi network and the cell surface. These enzymes as well as PC5, PACE4, and PC4 are involved in the processing of proteins secreted via the constitutive pathway. In contrast, PC1 and PC2 are found within dense core secretory granules and process proteins secreted by the regulated secretory pathway.[1–9] Like many other proteases, PCs are synthesized as inactive zymogens with an N-terminal prosegment extension. This conserved region is autocatalytically removed during PCs/SKI-1 maturation,[1–7] by cleavage either at RXKR↓ (for PCs) or for SKI-1 at the motif RX(V,L)(K,F,L)↓. So far the only known substrates of the novel enzyme SKI-1/S1P[6,7] are: probrain-derived neurotrophic factor,[6] sterol regulatory element-binding proteins,[7] the endoplasmic reticulum-stress response transcription factor ATF6,[8] and the surface glycoprotein GP-C of Lassa virus.[9] Thus, PCs are responsible for processing of neuropeptides, receptors, growth factors (GFs), cell surface glycoproteins, and enzymes, whereas SKI-1 cleaves proproteins that control

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cholesterol and lipid metabolism, and are involved in neural protection and growth and in the endoplasmic reticulum-stress response pathway. After proteolysis by the convertases, usually the mature proteins/peptides are subject to several other modifications necessary to achieve full bioactivity. The most common being the removal of carboxy-terminal basic residues by carboxypeptidase E or D (CPE, CPD).[10]

PCs in Cancers and Clinical Relevance

Multiple approaches, eg, suppression of gene expression or enzyme inhibition, support the hypothesis that PCs play a role in the genesis and progression of different proliferative disorders, including cancer.[11–22] Although elevated expression of different PCs was reported for different human cancers and tumor cell lines,[23–37] the relative importance of various PCs in these cancers has not yet been clarified. Tumor expression of PCs can be studied at the protein level by techniques such as immunohistochemical staining and Western blot analysis, and at the mRNA level by reverse transcriptase-polymerase chain reaction, Northern blot analysis, RNase protection, or *in situ* hybridization. Table 1 summarizes the results of studies on the expression of various PCs in human cancers and tumor cell lines. Early studies revealed a high furin expression in advanced lung tumors.[23] Such an association has subsequently been confirmed in other malignancies such as breast,[24] head, and neck[25] cancers. Based on these studies furin expression in tumors may constitute a significant prognostic factor independent of other conventional clinicopathological ones. Other studies described a significant association between high expression of PC1 and PC2 in neuroendocrine tumors, suggesting their involvement in the malignancy of tumor cells with a neural and/or endocrine phenotype.[26–28] Although these studies showed a positive association between PC1 or PC2 expression and the extent of tumors, further research is required to elucidate the importance and the prognostic role of these enzymes in endocrine-related cancers.

Studies on the prognostic impact of PACE4 expression in tumors are less conclusive. We previously reported that PACE4 expression is significantly higher in breast tumors.[24] In studies reported by Bassi and colleagues[25] PACE4 expression was found to be up-regulated in human head and neck tumors and tumor cell lines. Using an animal model of human squamous cell carcinoma, the same group demonstrated that PACE4 expression is implicated in the process of tumor progression and invasiveness.[17] However, this may be tissue-dependent because analysis of lung solid tumors revealed that only half expressed PACE4 and therein its mRNA level was lower than that of furin.[23]

PC5 and PC7 have been examined for their prognostic relevance in only a few human cancers (Table 1).[24,29] These studies showed a positive association between PC7 expression and the extent of breast tumors of which PC5 was undetectable.[24] In contrast, analysis of various human colon cancer cells, revealed the expression of PC5.[29] The significance of these findings is at present not known.

In conclusion, what seems clear is that furin up-regulation is correlated with tumor progression and invasiveness. Further research is required to elucidate the prognostic role of the other PCs in various types of cancer.

PC Substrates in Tumor Growth and Metastasis

No report has yet appeared to indicate that the PCs could inactivate protein substrates or peptides *in trans*, even at high concentrations. The only exceptions are the inactivation of their inhibitory prosegment by an autocatalytic mechanism and the inactivation of the inhibitors proSAAS and 7B2 by their cognate enzymes PC1 and PC2, respectively. The exact role of the modulation of PC expression and/or activity in tumor development and metastasis remains unclear. Nevertheless, because PCs are directly responsible for the activation of critical proteins implicated in neoplasia, they may be targets of cancer therapy. Among these substrates, matrix metalloproteases (MMPs), GFs, and adhesion molecules through degradation of the extracellular matrix (ECM), modulation of cell growth, and/or migration are involved in tumor progression and metastasis (Table 2).

Metalloproteinases

The destruction of the basement membrane and ECM is associated with tumor cell invasion and metastasis. ECM is a complex structure that consists of collagen, proteoglycans, and other protein such as fibronectin, vitronectin, and laminin. Secreted proteinases from malignant and stromal cells degrade many ECM components, facilitating the detachment of these cells and their invasiveness. The level of expression of these proteinases in tumor cells is associated with advanced-stage tumorigenesis and poor prognosis. ECM degradation is a complex process involving a cascade of proteolytic events in which the primary step likely implicates enzyme activation by the PCs. The latter were reported to process the following metalloproteinases: stromelysin-3 (str-3),[38,39] membrane-type MMPs (MT-MMPs),[40] the adamalysin metalloproteinases (ADAMs),[41] and the adamalysin metalloproteinases with thrombospondin motifs (ADAM-TS).[42,43] The expression of these metalloproteinases has been correlated with increased local aggressiveness, metastasis, and poor clinical outcome.[44–49]

Stromelysin-3 (Str-3)

Str-3 is a member of the MMP family of which expression has been correlated with both increased local aggressiveness and poor clinical outcome.[50] Activation of Str-3 by furin occurred intracellularly before secretion (Table 2).[38,39]

Table 1. Proprotein Convertase Expression in Human Cancers and Tumor Cell Lines

	Expressed PCs	Co-localized substrates	Techniques used	Ref.
Human tumors				
Pituitary adenomas	PC1, PC2	ACTH, chromogranin A	RT-PCR, *in situ* hybridization, immunostaining	2, 26
Head and neck tumors	Furin, PACE4	MT1-MMP	RT-PCR, Western blot	25
Breast tumors	PC1, Furin, PAECE4, PC7	–	RT-PCR, *in situ* hybridization	24
Lung tumors			Northern blot	23
Adenocarcinoma	Furin, PACE4	–		
Squamous cell lung carcinoma	Furin	–		
Small-cell lung carcinoma	Furin PC2	–		
Carcinoids			*In situ* hybridization, immunostaining	28
Bronchial carcinoids	PC1, PC2	–		
Rectal carcinoids	PC1	–		
Bile duct carcinoids	PC1	–		
Thyroid medullary carcinoma	PC1	–		
Tumor cell lines				
Pituitary adenomas				
HP75, Att-20	Furin, PC1, PC2, PACE4	Chromogranin A	RT-PCR, *in situ* hybridization, immunostaining	5, 27, 32
Medullary thyroid carcinoma				
rMTC 6–23	Furin, PC2	Neurotensin	Northern blot, Western blot	32
Head and neck carcinomas				
SCC9, SCC12, SCC13, SCC15	Furin, PACE4	MT1-MMP	RT-PCR, immunostaining, Western blot	25
SCC71, A253, det.262, FaDu				
Spindle cell carcinoma				
CC4B, CH72	PACE4	Stromelysin 3	RT-PCR, Western blot analysis, immunostaining	17
Glioblastoma				
Lln-18, U138MG, U87MG	Furin	TGF-β1,2	Northern blot, Western blot	22
D247MG, T98G, LN-229				
U373MG, LN-308, LN-428, U251MG				
Lung cancer cells				
H82, H345, H520, H209, H354, H510, H69, H146	PC1, PC2, furin	Neurotensin, GRP	Northern blot, Rnase protection assay	23, 37
Pancreas				
RIN5F, 027B2	PC1, PC2 furin	Cholecystokinin, proinsulin Somatostatin, proglucagon	Western blot, immunostaining	30, 34
Breast cancer				
MCF-7, ZR-75-1, T-470, BT-20	PC1, furin, PAECE4, PC7	Enkephalin, vasopressin	RT-PCR, Western blot	24, 35, 36
MDA-MB-157, MDA-MB-468				
MDA-MB-231				
Intestine				
STC-1	PC1, PC2	Cholecystokinin	Western blot	30
Colon cancer				
HCT8, LoVo, HT29	PC5	Neurotensin	RT-PCR, Western blot	29
LS174T, coloDM320, C119A				
Gonadal cancer				
H-500 rat Leydig tumor cells	Furin	PTHRP	Immunostaining	13

MT-MMPs

MT-MMPs, a new family of MMPs, are overexpressed in a wide variety of carcinomas, especially in the colon[44,45] and brain[51] and were reported to be involved in metastasis. Expression of these MMPs, particularly MT1-MMP was shown to process pro-MMP-2 thereby enhancing invasiveness both *in vitro* and *in vivo*.[40,52] MMP-2 is the enzyme that degrades collagen IV, a major type of collagen in basement membranes. MT1-MMP possesses two typical recognition motifs for PCs, namely ArgArgProArg[92] and ArgArg-LysArg[111] that were recently reported to be cleaved by furin-like enzymes at both sites (Table 2).[40]

Table 2. Sequence of the Cleavage Sites of Precursor Proteins

Site(s) of processing		P6	P5	P4	P3	P2	P1 ↓	P′1	P′2	NCBI, accession
Matrix metalloproteinases										
STR-1:		V	M	R	K	P	R	C	G	XM 058067
STR-2:		V	M	R	K	P	R	C	G	AAH02591
STR-3:	1-	S	L	R	P	P	R	C	G	P24347
	2-	R	N	R	Q	K	R	F	V	P24347
MT-1MMP	1-	A	M	R	R	P	R	C	G	P50281
	2-	N	V	R	R	K	R	Y	A	P50281
MT-2 MMP	1-	W	M	K	R	P	R	C	G	P51511
	2-	R	R	R	R	K	R	Y	A	P51511
MT-3 MMP	1-	W	M	K	K	P	R	C	G	P51512
	2-	H	I	R	R	K	R	Y	A	P51512
MT-4 MMP	1-	L	M	K	T	P	R	C	S	Q9ULZ9
	2-	Q	A	R	R	R	R	Q	A	Q9ULZ9
MT-5 MMP	1-	W	M	K	K	P	R	C	G	Q9Y5R2
	2-	R	R	R	N	K	R	Y	A	Q9Y5R2
MMP-1:		V	M	K	Q	P	R	C	G	P03956
MMP-2:		T	M	R	K	P	R	C	G	P08253
MMP-8:		M	M	K	K	P	R	C	G	XP006273
MMP-9:		A	M	R	T	P	R	C	G	XP029934
MMP-13:		V	M	K	K	P	R	C	G	XP040746
ADAM1		P	P	R	S	R	K	P	D	AAA74920
ADAM8		P	S	R	E	T	R	Y	V	XP005675
ADAM9		L	L	R	R	R	R	A	V	NP003807
ADAM10		L	L	R	K	K	R	T	T	XP007741
ADAM12		A	R	R	H	K	R	E	T	XP005838
ADAM15		H	I	R	R	R	R	D	V	Q13444
ADAM17		V	H	R	V	K	R	R	A	P78536
ADAMTS-1		S	I	R	K	K	R	F	V	Q9UHI8
ADAMTS-2	1-	G	V	R	T	R	R	A	A	P79331
	2-	R	R	R	M	R	R	H	A	P79331
ADAMTS-3		T	M	R	R	R	R	H	A	O15072
ADAMTS-4		P	R	R	A	K	R	F	A	XP042446
ADAMTS-5/11		W	R	R	R	R	R	S	I	Q9UNA0
ADAMTS-13		R	Q	R	Q	R	R	A	A	CAC83682
Integrins										
Integrin αIIb		H	K	R	D	R	R	Q	I	P08514
Integrin α3		P	Q	R	R	R	R	Q	L	XP008432
Integrin α4		H	V	I	S	K	R	S	T	XP039011
Integrin α5		H	H	Q	Q	K	R	E	A	AAH08786
Integrin α6		N	S	R	K	K	R	E	I	NP000201
Integrin α7		R	D	R	R	R	R	E	L	Q13683
Integrin α8		H	L	V	R	K	R	D	V	AAA93514
Integrin α^E		T	A	R	Q	R	R	A	L	XP008508
Integrin αv		H	L	I	T	K	R	D	L	XP002379
Growth factor										
TGF-β1		S	S	R	H	R	R	A	L	XP_008912
Lefty protein		R	S	R	G	K	R	F	S	O00292
Pancreatic polypeptide		P	R	Y	G	K	R	H	K	P01298
Gastrin		A	S	H	H	R	R	Q	L	P01350
Insulin	1-	T	P	K	T	R	R	E	A	XP028180
	2-	G	S	L	Q	K	R	G	I	XP028180
IGF-1		P	A	K	S	A	R	S	V	P01343
IGF-2		P	A	K	S	E	R	D	V	XP028189
PDGF-A		P	I	R	R	K	R	S	I	NP002598
PDGF-B		L	A	R	G	R	R	S	L	NP148937
PDGF-C		F	G	R	K	S	R	V	V	NP057289
PDGF-D		H	D	R	K	S	K	V	D	AAK56136
VEGF-C		H	S	I	I	R	R	S	L	P49767
VEGF-D		Y	S	I	I	R	R	S	I	NP_004460
FGF-23		P	R	R	H	T	R	S	A	Q9GZV9
EGF	1-	H	H	Y	S	V	R	N	S	
	2-	K	W	W	E	L	R	H	A	P01133
Endothelin-1		L	R	R	S	K	R	C	S	P05305
PTHRP		S	R	R	L	K	R	A	V	P12272
Parathyroid hormone		K	S	V	K	K	R	S	V	XP031173
Neurotrophin-3		T	S	R	R	K	R	Y	A	P20783
Neurotrophin-4		N	R	S	R	R	G	V	S	A42687

(*Table continues*)

Most of the indicated PC-like sites have been proven in cellular and/or *in vitro* experiments. However, others are only predicted.

Table 2. *Continued*

Site(s) of processing	P6	P5	P4	P3	P2	P1 ↓	P′1	P′2	NCBI, accession
β-NGF	T	H	R	S	K	R	S	S	XP002122
BDNF	S	M	R	V	R	R	H	S	XP006027
APRIL	R	S	R	K	R	R	A	V	O75888
BAFF	N	S	R	N	K	R	A	V	Q9Y275
HB-EGF	R	D	R	K	V	R	D	L	Q99075
HGF	K	T	K	Q	L	R	V	V	XP052260
Growth factor receptors									
Insulin receptor	P	S	R	K	R	R	S	L	XP048347
IGF-1 receptor	P	E	R	K	R	R	D	V	IGHUR1
HGF receptor	E	K	R	K	K	R	S	T	P08581
Others									
Ldl-related protein	S	N	R	H	R	R	Q	I	Q07954
Leptin receptor	Q	V	R	G	K	R	L	D	P48357
Notch-1-receptor	S	R	K	R	R	R	Q	H	AAG33848

Adamalysin Metalloproteinases

ADAMs are a family of membrane-associated multidomain zinc-dependent metalloproteinases with high sequence homology and domain organization, similar to the snake venom metalloproteases of the adamalysin subfamily.[46] Sixteen of the 30 ADAM proteins identified to date are predicted to be catalytically active, based on the presence of a conserved zinc-binding sequence (HEXXH) in the protease domain, whereas the other members are not likely to be active proteases. The prodomains of several ADAM proteins such as ADAM12 are constitutively cleaved by a furin-type PCs as they progress through the secretory pathway (Table 2).[41] This family was reported to play a role in diverse biological processes such as fertilization, myogenesis, neurogenesis, and cell surface proteolysis and shedding of different proteins.[41,47,53–59] Of the proteins shed by the ADAMs, are cytokines and GFs such as transforming growth factor-α (TGF-α), epidermal growth factor (EGF), heparin-binding EGF, tumor necrosis factor-α (TNF-α), c-Kit-ligand-1 (KL-1), colony-stimulating factor-1 (CSF-1), and Fas-ligand (Fas-L), receptors such as TNF receptor-I (TNFR1, p60 TNFR), TNF receptor-II (TNFR2, p80 TNFR), p75 nerve growth factor receptor (p75NGFR), interleukin-6 receptor (IL-6R), thyroid-stimulating hormone receptor, adhesion molecules such as L-selectin, and others proteins such as protein tyrosine phosphatase σ (PTPσ), protein tyrosine phosphatase LAR (LAR), amyloid precursor protein, and angiotensin-converting enzyme.[53,56] Certain released molecules can be cleaved by more than one enzyme, and some enzymes can cleave more than one substrate. For example, cleavage of TNF-α can be mediated by ADAM17 (TACE) and ADAM10, and α-secretase activity for amyloid precursor protein has been attributed also to ADAM17,[54] ADAM9,[57] and ADAM10.[59] It is not known how these protease(s) select their substrate because reliable consensus cleavage sites have not been identified. In addition to their proteolytic function, some members of the ADAM family such as ADAM15 and ADAM2 can support integrin binding via their disintegrin domain.[58,60,61] It is increasingly recognized that ADAMs represent a novel group of membrane proteases that are important for cellular interactions under physiological and pathophysiological conditions including cancer. Recently, several ADAM family members were described to be dramatically up-regulated in many tumor cells. This includes cells derived from a range of hematological malignancies[47] and breast, prostate, lung, and colon cancer.[42,48,62] Therefore, it is of particular interest that the member of the ADAM family reported to shed cell-associated neural adhesion molecules such as L1 may be relevant to promote cell migration and invasion.[62,63] Interestingly, in these cells the putative tumor suppressor gene MDC (ADAM11) was expressed at a very low level.[62,63]

Adamalysin Metalloproteinases with Thrombospondin Motifs

ADAM-TSs are a new member of the ADAM family containing thrombospondin-type motifs. They consist of multiple domains of proteins common to the ADAM family, including pro-, metalloprotease-like, and disintegrin-like. The first member of this family, called ADAM-TS1, was originally cloned from a colon adenocarcinoma cell line.[42] Based on its capacity to form a covalent complex with α2-macroglobulin, recent studies demonstrated that ADAM-TS1 protein is proteolytically active (Table 2).[43] In addition, the maturation of ADAM-TS1 precursor is impaired in the furin-deficient cell line, LoVo, and the processing ability of the cells is restored by the co-expression of the furin cDNA.[64] The only members of the ADAM-TS family with established substrates are ADAM-TS2 (procollagen-N-proteinase) and ADAM-TS4 and -TS11 (aggrecanases-1 and -2, respectively).[42,64,65] These proteinases cleave aggrecan at one or more of five specific sites in the aggrecan core protein.[42,64,65] Recently, brain-enriched hyaluronan binding (BEHAB)/brevican, a brain-specific ECM protein was reported to be processed by ADAM-TS4. The processed form of BEHAB/brevican is dramatically increased in human gliomas, a notoriously invasive tumor.[49] The rat 9L gliosarcoma cell line, which does not express BEHAB/brevican and forms noninvasive tumors when grown as intracranial grafts, can form invasive tumors when it is transfected with a 5′ cDNA fragment of BEHAB/brevican, but not when transfected with the full-length cDNA.[49] Interestingly, the expression of ADAM-TS4 is induced during

endothelial cells undergoing differentiation into tube-like structures suggesting its implication in angiogenesis.[66] These observations link ADAM-TS family members to invasion and the blocking the activation of ADAM-TSs by PC inhibitors may provide a novel therapeutic strategy.

Adhesion Molecules

Cell adhesion molecules (CAMs) are cell-surface proteins that mediate cell-cell and/or cell-ECM interactions. They control cellular traffic, transmigration through the endothelium, homing in and localization to various target organs during inflammation, and tumor cell colonization.[67,68] Most of the CAMs characterized so far fall into three categories of proteins: the immunoglobulin (Ig), integrin, or selectin families.

The Immunoglobulin Family

The Ig family includes intercellular adhesion molecules ICAM-1, ICAM-2, and ICAM-3, vascular CAM-1 (VCAM-1), and mucosal addressin CAM-1 (MadCAM-1), none of which are believed to be substrates for PC processing. However, the convertases seem to be required for their expression and probably function. Indeed, the expression of ICAM-1 and VCAM-1 on endothelial cells is induced by various cytokines and GFs such as interferon-γ, interleukin-1 (IL-1), TNF-α, insulin-like growth factor (IGF-1), and endothelins.[69–76] Some of these precursors were reported to be directly processed by PCs such as IGF-1 and endothelins (Table 2).[77–79] Others are indirectly regulated by the PCs through the cleavage of their cognate enzymes, for example TNF-α converting enzyme (TACE)[80] and ADAM10[56] that process pro-TNF-α are themselves activated by PCs.[59,80,81]

The Integrin Family

Its now well established that integrins are implicated in tumor progression and metastasis.[82,83] Integrins are heterodimeric $\alpha\beta$ transmembrane receptors that bind ligands such as the ICAMs, VCAMs, and several ECM components. The extracellular domains of both subunits are required for ligand binding whereas the cytoplasmic tails interact with the cytoskeleton, induce changes in cell shape and motility, and transduce growth and survival signals.[84,85] In addition, activation of integrins was reported to mediate MMPs and urokinase activity of many tumor cells, including melanoma and colon carcinoma.[86–88] Although β-subunits are not cleaved by PCs, a total of 9 of 18 known α-subunits possess a potential PC-cleavage site, with α3, α4, α5, α6, and αv proven to be PC5 and furin substrates (Table 2).[89,90] Recently, Berthet and colleagues[91] reported that the endoproteolytic cleavage of αv integrin is important for the signal transduction pathway mediating cell adhesion. Blockade of $\alpha v\beta 5$ cleavage resulted in a decreased phosphorylation of focal adhesion kinase (FAK) and paxillin, two important molecules involved in cell adhesion.

The Selectin Family

The selectin family members, L-selectin, P-selectin, and E-selectin are involved in the adhesion of leukocytes to activate the endothelium. Adhesion is initiated by weak interactions that produce a characteristic rolling motion of leukocytes on the endothelial surface. This rapid on-off attachment is necessary for activation and engagement of integrins and their counterreceptors on the leukocytes and endothelial cells, respectively. The integrin-mediated, high-affinity binding is in turn required for leukocyte arrest and extravasation.[67] On the basis of *in vitro* studies it is postulated that similar cell-cell interactions may also occur between circulating malignant cells and the vascular endothelium during tumor dissemination.[92–94] In general, the selectins bind sialylated, glycosylated, or sulfated glycans on glycoproteins, glycolipids, or proteoglycans.[95] The tetrasaccharides sialyl-Lewis^x (sLew^x) and sialyl-Lewis^a (sLew^a) are recognized by all three selectins. *In vitro* adhesion studies showed that human colorectal, pancreatic, and gastric carcinoma cells use sLew^x and related carbohydrate determinants to adhere to TNF-α-inducible E-selectin on cultured vascular endothelial cells.[93,96–98] *In vivo* studies in turn showed that inhibition of liver metastasis of the highly metastatic human pancreas adenocarcinoma (PCI) cells could be blocked with antibodies to sLew^a and that lung colonization by colon carcinoma HT29 cells could be blocked by a soluble E-selectin fusion protein.[97,98] Under normal physiological conditions, vascular endothelial cells express low constitutive levels of E-selectin. Several cytokines and GFs such as IL-1, TNF-α, vascular endothelial growth factor, IGF-1, and endothelins induce the expression of E-selectin.[75,77,99,100] Many of these molecules directly or indirectly require PC activity (Table 2). Thus, although like VCAMs and ICAMs, selectins are not processed by PCs, the latter may indirectly control their expression via the activation of some of the above inducers. Recently, we demonstrated that E-selectin expression on sinusoids was described to be one of the early molecular events involved in metastasis.[101] The arrest of tumor cells in the hepatic circulation causes a cascade of events, which start with a rapid release of IL-1 and TNF-α (and other mediators). In turn, these stimulate the expression of E-selectin on hepatic endothelial cells, resulting in an enhanced tumor cell adhesion and subsequent metastases.[101]

GFs and Their Receptors (GFRs)

The availability of GFs is critical for malignant transformation and metastasis. These molecules mediate cell entry into, and progression through, the cell cycle. They are divided into two main categories namely, competence factors such as platelet-derived growth factor, vascular endothelial growth factor, and basic fibroblast growth factor that enable cells to enter the G_1 phase and progression factors such as IGF-1 that are required for progression from G_1 into S phase and ultimately resulting in cell division.[102–105] Many of these proteins as synthesized as proproteins that are processed and activated by

PC-like enzymes (Table 2). Parallel increased expression of both GFs and PCs may result in tumor growth. Of the GFs shown to be processed by PCs include IGF-1, endothelins, nerve growth factor, PTH, and TGF-β (Table 2).[77,78,106–108] Others, are suspected to be PC substrates based on the presence of the RXXR$\downarrow$ motif, eg, FGF23 (Table 2)[109] that is reported to be involved in tumor-induced osteomalacia. Many GFs mediate their effects through receptor tyrosine kinases (RTKs) that transmit signals to the nucleus through an intricate network of adapter and signaling molecules.[104] Receptor activation through ligand binding generally induces receptor dimerization and autophosphorylation through a *trans* mechanism.[104,110] Transphosphorylation at specific tyrosine residues in turn generates binding sites that are recognized by proteins involved in cellular signaling. Several proteins that are phosphorylated through GF-associated kinase activity were identified as downstream mediators of receptor-associated signal transduction include: phospholipase Grb,[111] Cγ (PLCγ),[112] GTPase-activating protein,[113] phosphatidylinositol 3-kinase (PI-3 k),[114] insulin receptor substrate (IRS), and homology and collagen (Src).[115,116] RTKs are implicated in malignant transformation and tumor progression.[117,118] Overexpression or mutations/insertions in GF receptors (GFRs) that result in constitutively high levels of proteins, or active kinases are documented in many tumors.[119,120] Frequently this is accompanied by constitutive high expression of the respective ligands providing an autocrine mechanism for growth autonomy.[121] Several GFRs contain a consensus PC-cleavage site, and are critical for tumor growth and metastases, eg, the HGF receptor (HGF-R),[122] insulin receptor,[123–125] and IGF-1 receptor.[20,126] Hwang and colleagues,[125] reported that cleavage of insulin receptor is essential in the signal transduction of insulin. In agreement with this observation, we recently showed that the uncleaved IGF-1 receptor is unable to undergo the critical steps for IGF-1-mediated growth and survival of tumor cells, ie, IGF-1-sensitive autophosphorylation and IRS-1 phosphorylation.[20] However, Komada and colleagues[122] previously reported that both cleaved and uncleaved HGF receptors can bind HGF and mediate their autophosphorylation and cell growth. This unusual result indicates that proteolytic processing of this receptor may not be essential for some of the tested signal transduction pathways of HGF. However, to better define the role of PC-mediated cleavage of this receptor, more extensive analysis is required.

PC Inhibitors

The potential clinical and pharmacological role of the convertases has fostered the development of both peptide- and protein-based PC inhibitors.[1–5] The most promising protein-based specific inhibitors of PCs are a α1-anti-trypsin variant known as α1-anti-trypsin Portland or α1-PDX[127,128] and the individual PC prosegment-based inhibitors.[129,130]

The α_1-Anti-Trypsin Variant, α_1-Anti-Trypsin Portland (α_1-PDX)

Like furin substrates, inhibitors of furin also require the interaction of enzyme subsites with the basic residues of the substrate. Previously, the trypsin inhibitor and the third domain of turkey ovomucoid have been reported to be inhibitors for furin.[131,132] However, their equilibrium constant was representative of a moderate, rather than a potent, inhibitor. Inhibition of furin in the subnanomolar range was accomplished by bioengineering the α_1-anti-trypsin variant, α_1-PDX (α_1-anti-trypsin Portland).[127] Kinetic analysis shows that a portion of bound α_1-PDX operates as a suicide inhibitor.[128] Once bound to furin's active site, α1-PDX can either undergo proteolysis by furin or form a kinetically trapped sodium dodecyl sulfate-stable complex with the enzyme.[128,133] In an attempt to produce a specific furin inhibitor others researcher mutated the bait region of the general protease inhibitor α_2-macroglobulin (RV*GFYESD*VM[690] into RV*RSKRSL*VM[690]).[134] This variant was reported to inhibit processing of furin substrates, eg, human immunodeficiency virus type-1 glycoprotein gp160, von Willebrand factor, and TGF-β1 was inhibited in COS-1 cells.[134] The ovalbumin-type serpin human proteinase inhibitor-8, containing two instances of the minimal furin recognition sequence (VVRNS*RCSR*M[343]), has also been shown to inhibit furin in a rapid, tight binding manner that is characteristic of physiological serpin-proteinase interactions.[135] However, the cytosolic localization of inhibition inhibitor-8 requires the addition of a signal peptide before it could inhibit furin *in vivo*, and even then its *ex vivo* inhibitory property is yet to be proven. More recently Cameron and colleagues[136] reported that although the hexa-D-arginine is a potent and relatively specific furin inhibitor, the reduced ability of this highly charged peptide to cross the cell membrane for *in vivo* therapeutics is problematic. Based on these studies, to date the most promising protein-based specific inhibitor of PCs is the α1-anti-trypsin variant, α1-PDX. This serpin was first showed to be a potent inhibitor of furin-mediated cleavage of HIV gp160,[127,137] but subsequently demonstrated to also inhibit all PCs involved in processing within the constitutive secretory pathway.[138–140] Recent studies showed that endogenous inhibition of precursor convertases by α_1-PDX reduces the production of the amyloid precursor α-secretase product amyloid precursor protein-α[59–141] and blocks the activation of the pore-forming toxin proaerolysin,[142] the maturation of the surface glycoproteins of infectious pathogens,[127,128,137] the proteolytic activation of bone morphogenetic protein-4 (BMP-4),[143] the cleavage of Notch,[144] insulin-like growth factor-1 receptor (IGF-1R),[20] and TGF-β.[145,146] The potential therapeutic value of PC inhibitors was recently reinforced by a report showing that exogenous addition of α1-PDX potentially inhibits the furin-dependent processing of human cytomegalovirus envelope glycoprotein gB, thus reducing the titer of infectious hCMV more effectively than currently used anti-herpetic agents.[128] As uptake of α_1-PDX into the cell could not be detected in cell lines lacking the enzyme, it was suggested that the

reported furin inhibition by the external application of α1-PDX occurs because furin is localized to the trans-Golgi network and cycles to the cell surface, where it could meet α1-PDX, and back via endosomal compartments.[128] A similar mechanism was attributed for the prevention of *Pseudomonas* exotoxin A activation when extracellular α_1-PDX was applied to A7 melanoma cells.[128] These studies collectively suggest that inhibition of PCs localized in the interior of the cell can be initiated at the extracellular cell surface by PC binding to α1-PDX and uptake, resulting in inhibition of pathological disease processes including cancer.

α1-PDX and Tumor Cell Malignant Phenotypes

The *in vitro* and *in vivo* studies recently reported by our group and others demonstrated that the serpin α1-PDX could be a powerful tool for tumorigenesis inhibition.[21,20] In *ex vivo* studies, using human leukemia Jurkat T and colon carcinoma HT29 cell lines and mouse pituitary tumor AtT20 cells, we found that stable expression of α1-PDX provoked dramatic changes in several malignant phenotypes and metastatic potential. This includes cellular growth, survival, invasiveness, and tumorigenesis.

The Role of PCs in Cell Growth

The availability of GFs is critical for malignant transformation and metastasis. The anti-proliferate effect of α1-PDX on tumor cells is probably because of the inhibition of the processing of various proteins, including GFs and/or their corresponding receptors.[77,147–150] Previously, it was postulated that the critical nature of the furin processing of various precursors may explain the anti-proliferative effect of furin blockade on H-500 rat Leydig tumor cells,[12] the pancreatic β-cell line MIN6,[16] and the gastric mucus cells.[15] Kayo and colleagues[16] showed that conditioned medium derived from furin-overexpressing MIN6 cells stimulated the growth of their parental control cells, whereas the medium from cells expressing α1-PDX resulted in a lower growth rate. These results suggest that high furin expression stimulates growth through an autocrine/paracrine mechanism. In agreement with this hypothesis our group recently demonstrated the importance of the processing of IGF-1 receptor in the mediation of IGF-1-mediated tumor cell proliferation.[20] A functional IGF-1R is known to be required for cell growth of various transformed cells. Overexpression and/or constitutive activation of IGF-1R in a variety of cell types leads to ligand-dependent growth in serum-free medium and to the establishment of a transformed phenotype such as the ability to form colonies in soft agar and tumors in mice.[118,151] Similarly, studies reported on TGF-β1 processing by PCs revealed that inhibition of TGF-β1 in glioblastoma and in head neck squamous cell carcinoma (HNSCC) cell lines by α1-PDX could be a promising tool in modulating tumor growth and immunotherapy.[21,22]

The Role of PCs in Cell Survival

Similarly to PCs role in growth, PC cell-survival function is also substantial. Thus, the expression of α1-PDX in tumor cells exaggerates the apoptotic phenotype (DNA degradation and propidium iodide-positive cells) after serum deprivation, as we reported for HT-29 and Jurkat leukemia cells.[20] The role of PC in cellular survival can be explained by their role in the maturation of various proteins known to be anti-apoptotic mediators participating in various autocrine/paracrine mechanisms. A likely scenario may involve one or more secreted ligands and/or their receptors, all of which require processing by PC-like enzymes. Examples include IGF-1 and IGF-2 that are synthesized and secreted by these cells[152] and processed by furin.[148,149] Because overexpression of α1-PDX in tumor cells inhibited the processing of IGF-1R and its furin-processed ligands, IGF-1 and IGF-2, it is liable to abrogate their autocrine/paracrine protective effects. The protective effect of many proteins is apparently dependent on their ability to induce a cascade of events leading to downstream effector pathway phosphorylation, including FAK,[153,154] PI-3K1,[155] and IRS-1.[156] After phosphorylation these molecules mediated their anti-apoptotic effect through the activation of several negative death regulators such as Bcl-2 or inhibition of IL-converting enzyme-like caspases.[153–157] Interestingly, expression of α1-PDX in tumor cells resulted in a reduction of basal tyrosine phosphorylation of FAK, PI-3K, and IRS-1.[20] Exogenous addition of FAK, PI-3K, and IRS-1 activators such as IGF-1, failed to increase tyrosine phosphorylation of these molecules in tumor cells and to rescue the cells from apoptosis. This suggests a blockade in the transmission of the autocrine/paracrine anti-apoptotic signals in these cells.

The Role of PCs in Cell Invasion

PCs role in *in vitro* invasion is now well documented. Inhibition of PCs in different tumor cells resulted in a significant reduction in their invasiveness.[20,25] This reduction is because of processing blockade of proteins directly involved in the mechanism of invasion such as MMPs or to proteins such as GFs and/or integrins reported to induce the expression of ECM-degrading protein including urokinase and MMPs.[20,25,38,39,40–43] Recently, the inhibitory effect of α1-PDX on *in vivo* invasion was reported by Bassi and colleagues.[21] They used an *in vivo* invasion assay based on the penetration of tumor cells into the tracheal wall. When transplanted in the tracheas the control-transfected cells penetrated deeply into the tracheal wall and reached the adventitia and the surrounding peritracheal tissues. Whereas, the penetration of α1-PDX-transfected cells in the tracheal wall was markedly decreased and never reached the outer surface of the trachea. They estimated this *in vivo* invasion reduction to be ~70 to 80%. We also found that α1-PDX decreased the invasiveness of colon carcinoma HT-29[20] in which the processing of MT1-MMP was inhibited (Figure 1). In these cells the mRNA level of plasminogen activator urokinase-type plasminogen activator (uPA)

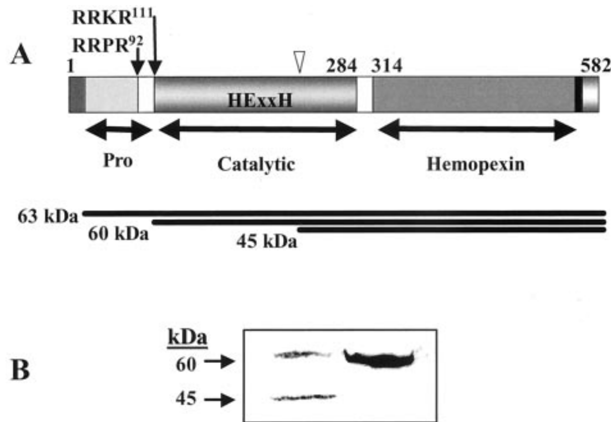

Figure 1. Pro-MT1-MMP processing. **A:** Schematic representation of the structural aspects of the human pro-MT1-MMP (63 kd) and its PC-processing sites at the RRPR$^{92}\downarrow$ and RRKR$^{111}\downarrow$ The mature enzyme (63 kd) can be further autocatalytically cleaved (itrio) into a 45-kd C-terminal form. Shown are the pro-, catalytic (with the HExxH signature), and hemopexin-like domains. **B:** Western blots of the cell lysates obtained from the colon carcinoma cell line HT-29 transfected with pIRES2-EGFP vector alone (HT-29) or a recombinant vector containing cDNA of α1-PDX (HT-29/PDX) using mouse anti-human MT1-MMP monoclonal antibody (mAb 3319) recognizing the catalytic domain.

and tissue-type plasminogen activator (tPA), the urokinase-type plasminogen activator receptor (uPAR), and the uPA inhibitor plasminogen activator inhibitor-1 (PAI-1), molecules involved in invasion processes and believed not to be processed by PCs, was significantly reduced. This data demonstrated both the direct and the indirect involvement of PCs in tumor cell invasion.

The Role PCs in Tumorigenesis and Angiogenesis

The anti-tumor effects of PC inhibition was initially reported by our group. Expression of α1-PDX in colon carcinoma HT-29 tumor cells delayed the appearance, the incidence, and the vascularization of palpable tumors.[20] Subsequently, the study reported by Bassi and colleagues[21] have confirmed the inhibition of tumor growth by PC inhibition and found that the levels of furin mRNA and protein expression correlate with the aggressiveness of tumor cell lines derived from head and neck and lung cancers. These studies demonstrated that the inhibitory effect of α1-PDX on tumor growth and invasiveness in *in vivo* systems may be underestimated because of the significant loss in α1-PDX expression in the subcutaneous tumors during their growth. However, it is not clear whether the observed progressively lower levels of α1-PDX mRNA in tumors[21] is because of a specific loss of the cDNA from tumor cells. Future studies should address this issue by comparing the levels of α1-PDX to those of a specific marker co-expressed with this serpin, eg, green fluorescent protein. This may eliminate the trivial explanation of a dilution effect of the original tumor cells by host cells. However, similar observations were reported by Leitlein and colleagues,[22] on the loss of α1-PDX expression *ex vivo*. These authors claim that α1-PDX expression was not very stable in glioma cell lines and was lost within a few passages *in vitro*. Angiogenesis is a significant prognostic factor in various cancers, but the factors that control angiogenesis *in vivo* are not well defined. Usually tumor angiogenesis is mediated by tu-

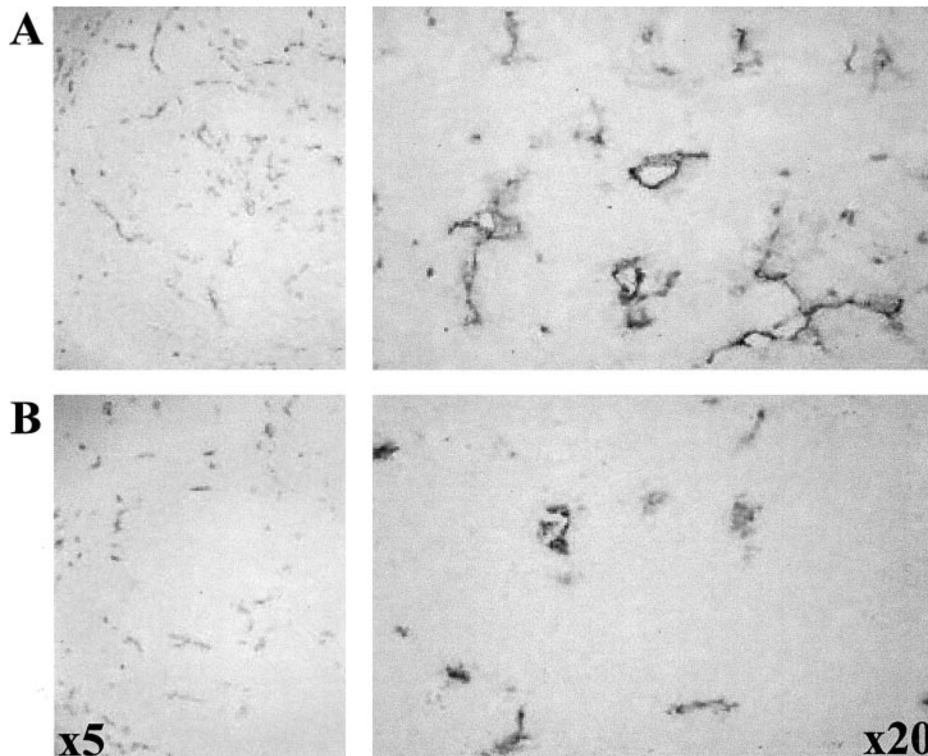

Figure 2. Extent of angiogenesis determined by immunostaining for CD31. The immunohistochemical analysis were performed on tissue obtained from control tumors (HT-29) or tumors developed from HT-29 cells expressing α1-PDX c-DNA (HT-29/PDX).

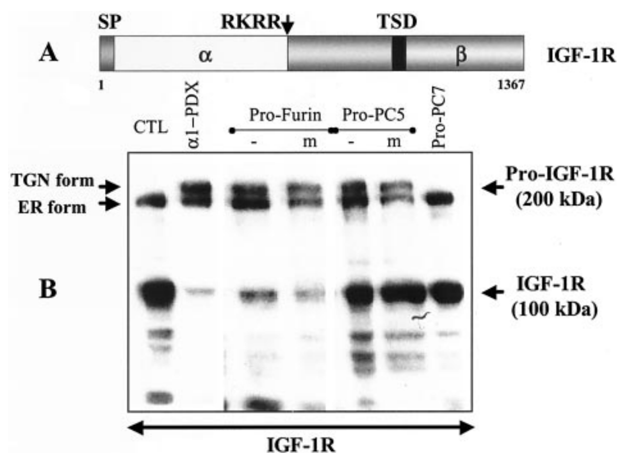

Figure 3. Inhibition of the proteolytic processing of IGF-1R by PC prosegments. **A:** Schematic representation of the primary structure of human IGF-1R. The positions of its signal peptide (SP), PC-processing site (RKRR), and transmembrane domain (TMD) as well as the α- and β-subunits are shown. **B:** IGF-1R processing was analyzed by Western blotting of cell lysates obtained from transiently transfected HK293 cells with IGR-1R cDNA together with either the pIRES2-EGFP vector alone (control, CTL) or a recombinant vector containing either profurin, pro-PC5, or pro-PC7 cDNA. Cells expressing α1-PDX are shown for comparison. Note that the inhibitors specifically inhibit the processing of the trans Golgi network form of pro-IGF-1R (210 kd) and not the endoplasmic reticulum-form (200 kd) into the 100-kd β-chain. Also note that the prosegment inhibitor mutated at its secondary cleavage site (m) (148) is as effective as the wild-type inhibitor and that pro-PC7 does not inhibit processing.

mor-secreted angiogenic GFs that interact with their surface receptors expressed on endothelial cells. Multiple angiogenic proteins are known, including vascular endothelial growth factor and its four isoforms (121, 165, 189, and 206 amino acids), TGF-β1, pleiotrophin, acidic and basic fibroblast growth factor. Immunohistochemical analysis of CD31 antigen expression, a marker of endothelial cells revealed a reduced tumor vascularization of tumors developed from tumor cells expressing α1-PDX (Figure 2).[20] This suggests the importance of the PCs in tumor vessel formation through direct/indirect activation of various angiogenic proteins.

PC Prosegment-Based Inhibitors

To date the only naturally occurring intracellular PC inhibitor found in the constitutive secretory pathway are PC's own propeptides or prosegments,[2,6,158] and in the case of PC1 its C-terminal domain.[159] The activities of the regulated secretory pathway convertases, PC1 and PC2, are however regulated by selective and specific inhibitors known as pro-SAAS[160,161] and 7B2,[162] respectively. For many proteins, the prosegment serves as an intramolecular chaperone that is essential for their correct folding,[163] or/and transport and secretion.[164] In the case of many proteolytic enzymes such as cathepsins, carboxypeptidases, papain, and subtilases, this prosegment was reported to be a very potent inhibitor that is highly specific for its associated protease. In these enzyme systems the prosegment is still an effective inhibitor even after initial proteolysis and in most cases an additional cleavage within the prosegment to release it and thereby to fully activate the enzyme.[158,165] In accordance with the notion that PCs are initially produced as zymogens and proteolytically activated, Anderson and colleagues[129] demonstrated that the prosegment of furin

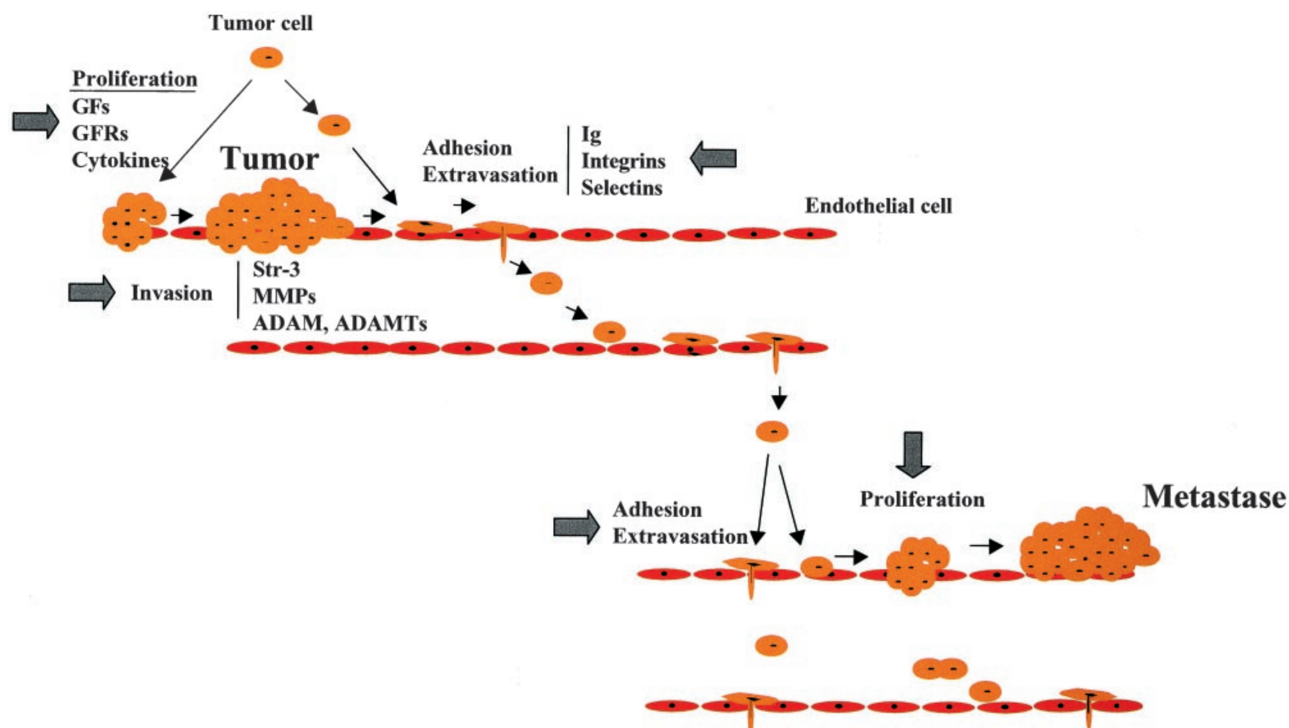

Figure 4. Schematic representation of cascade events implicating PCs for tumor growth and metastasis. By activating GFs, cytokines, and their receptors (GFRs), PCs control tumor cell proliferation and growth. By activating or inducing adhesion molecules convertases modulate adhesion, invasion, and migration of tumor cells and subsequently metastases formation. **Arrows** indicate the potential sites for PCs inhibitors.

when used as a fusion protein to glutathione *S*-transferase exhibits a potent *in vitro* inhibitory activity on furin.[165] We have also shown that the prosegment of furin and PC7 purified from bacterial culture media, are very potent inhibitors of their respective enzymes. Additionally, *in vitro* studies demonstrated the synthetic peptides derived from the prosegments of PC1, PC7, and furin are potent inhibitors of their enzymes.[129,130,166,167] Based on these data, we surmised that, aside from α1-PDX, the PC prosegments may be applied in cancer therapy, as they were in Alzheimer's disease β-secretase analysis.[168] Indeed these prosegments were shown to inhibit intracellularly the processing of GF precursors such as nerve growth factor and brain-derived neurotrophic factor,[129] both of which have been linked to neuronal cancer and metastasis.[169] In addition, we recently demonstrated that the cellular processing of IGF-1R, known to be crucial for the malignant phenotype of tumor cells,[20,118,151] is effectively blocked by α1-PDX[20] as well as by the prosegments of furin and PC5 but not by that of PC7 (Figure 3). Although some data exist to show that α1-PDX does not significantly inhibit thrombin,[127] these are not exhaustive and the ability of α1-PDX to inhibit other classes of proteases is yet to be fully elucidated. In addition, because prosegment inhibitors are not always completely selective for their cognate enzymes and can inhibit other PCs,[129] it will be necessary to modify their structure to improve their selectivity without compromising their potency (N. Nour and N. G. Seidah, in preparation).

Concluding Remarks

Regulation of cellular proliferation, differentiation, and adhesion are complex processes in which different biological systems interact. Disruption of these processes is a hallmark of malignant transformation resulting in tumor progression and acquisition of a metastatic potential. The key to successfully developing efficient clinical strategies against cancers is to target a protein known to be essential in the growth of tumors and to validate that the disruption of this target selectively blocks the growth of the transformed cells. In this Review we have described recent progress made in establishing a novel approach to inhibiting tumor growth and tumor cell malignant phenotypes. We have outlined PC inhibition as a strategy to simultaneously disrupt the function of numerous proteins involved in the acquisition of the invasive/metastatic potential of cancer cells (Figure 4). The potential of this approach has been firmly established by the use of PC inhibitors (α1-PDX and PC-prosegments) targeting the activity of the PCs. The cumulative data available established that α1-PDX is not only able to block the interaction of GFs and their receptors such as IGF-1/IGF-1R, and the activation of several MMPs but also to inhibit tumor cell proliferation and invasion (Figure 4). This potent effect further translates into an ability to slow down the growth of human tumors in a nude mouse model through an anti-proliferative and anti-angiogenic mechanism (Figure 2).[20,21]

These studies represent the first example of a potentially new approach to controlling tumor cell growth and behavior through the inhibition of precursor processing. Although the use of general PC inhibitors may be advantageous, in some cases it may be necessary to target only one member of the PC family. Therefore, one of the important future developments would be to find and express PC inhibitors specific for each member of the family. This is feasible, as was demonstrated for PC1 (proSAAS)[160,161,170] and PC2 (7B2).[162,171] These could be used alone or in combination to target specific tumors. In the long term, these inhibitors may provide a rationale for testing this family of compounds as anti-metastatic agents or in conjunction with standard therapy in clinical settings. Thus, the anticipated results will improve our knowledge of the role of convertases in proliferative diseases, and lead to the design of potent and selective convertase-inhibiting reagents and novel pharmacological strategies.

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