OPINION

Revisiting the biological roles of PAI2 (SERPINB2) in cancer

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Abstract | Tumour expression of the urokinase plasminogen activator correlates with invasive capacity. Consequently, inhibition of this serine protease by physiological inhibitors should decrease invasion and metastasis. However, of the two main urokinase inhibitors, high tumour levels of the type 1 inhibitor actually promote tumour progression, whereas high levels of the type 2 inhibitor decrease tumour growth and metastasis. We propose that the basis of this apparently paradoxical action of two similar serine protease inhibitors lies in key structural differences controlling interactions with components of the extracellular matrix and endocytosis–signalling co-receptors.

Metastasis is intrinsically linked to the ability of tumour cells to escape the constraining extracellular matrix (ECM)[1]. The broad-spectrum serine protease plasmin facilitates this process by degrading components of the ECM[2]. Plasmin is generated by the plasminogen activation system, a tightly regulated network of protease activators, receptors and inhibitors (FIG. 1) that becomes deregulated during tumour progression[2]. Accordingly, components of this system are potent biomarkers for cancer progression and patient survival. Numerous studies have identified co-expression of the serine protease urokinase plasminogen activator (uPA, also known as PLAU) and one of its inhibitors, plasminogen activator inhibitor type 1 (PAI1, also known as SERPINE1, see BOX 1), as an independent marker of poor prognosis in many cancer types[3]. Significantly, uPA and PAI1 were recently included in the 2007 update of the American Society of Clinical Oncology recommendations for prognosis of node-negative breast cancer[4].

The link between PAI1 and poor patient prognosis may reflect dynamic interactions with ECM components and endocytosis–signalling co-receptors that ultimately promote tumour growth and metastasis, which are supplementary to its classical biochemical activity as a uPA inhibitor. Paradoxically, tumour-associated expression of another classical uPA inhibitor, plasminogen activator inhibitor type 2 (PAI2, SERPINB2), is associated with increased survival in patients with breast cancer[3,5], and recent novel data have highlighted key structural and functional differences between these serpins[6,7]. These differences suggest that PAI2 does not possess the additional functions attributed to PAI1 and acts predominantly as a protease inhibitor *in vivo*. In this Perspective, we incorporate these novel structural and functional data with a thorough review of the available prognostic data for PAI2 in multiple cancer types, and propose a hypothesis for the mechanism underlying differential prognosis of high PAI1 levels versus high PAI2 levels in cancer.

Cellular and tissue expression of PAI2

As receptor-bound plasmin is protected from inhibition by α_2-antiplasmin (also known as SERPINF2)[8], direct inhibition of uPA and tissue plasminogen activator (tPA, also known as PLAT) by PAI1 and PAI2 are key regulatory mechanisms of pericellular plasminogen activation (FIG. 1). In comparison with PAI2, the role of PAI1 in the plasminogen activation system has been studied in depth[9,10]. *In vivo*, PAI1 expression can be highly induced in both endothelial cells and activated platelets[9] and its role in inhibiting thrombolysis through the rapid inhibition of tPA is especially well documented[11]. PAI1 is also an established regulator of diverse plasmin-dependent and plasmin-independent physiological processes involving vascular remodelling and angiogenesis[12]. This includes effects on cell adhesion and migration through an interaction with the ECM protein vitronectin, and subsequent modulation of integrin–uPAR (PLAUR)–uPA interactions with the ECM[12]. In addition, inhibition of uPA by PAI1 induces secondary high-affinity interactions with the low-density lipoprotein receptor (LDLR) family of endocytosis receptors[13–17], with further effects on migration, adhesion and proliferation. These processes will be addressed in detail below.

PAI2 can be considered a stress protein as it is one of the most upregulated proteins of activated monocytes and macrophages and differentiating keratinocytes, and its expression is also highly inducible in fibroblasts and endothelial cells[10,18]. *PAI2* transcription is stimulated by a variety of inflammatory mediators, and by viral or bacterial infection[18], so biological roles in the regulation of inflammation and wound healing have been proposed[18]. However, attempts at defining the precise physiological functions of PAI2 have been somewhat confounded by its bi-topological existence in both a predominant cytosolic (47 kDa) form and an extracellular, glycosylated (60 kDa) form[10,19–21]. The reason for the intracellular accumulation of PAI2 is not entirely clear but it might be linked to an inefficient, mildly hydrophobic internal signal peptide[19,21–24], as increasing the hydrophobicity of the signal peptide results in enhanced PAI2 secretion[22].

The prevalence of the cytosolic form of PAI2 has fostered some debate in the field regarding potential extracellular and pericellular pathophysiological role(s), and more recent research has focused on the somewhat contentious intracellular functions of PAI2. Nevertheless, extracellular PAI2 does exist *in vivo* and mediates important serpin-related biological functions.

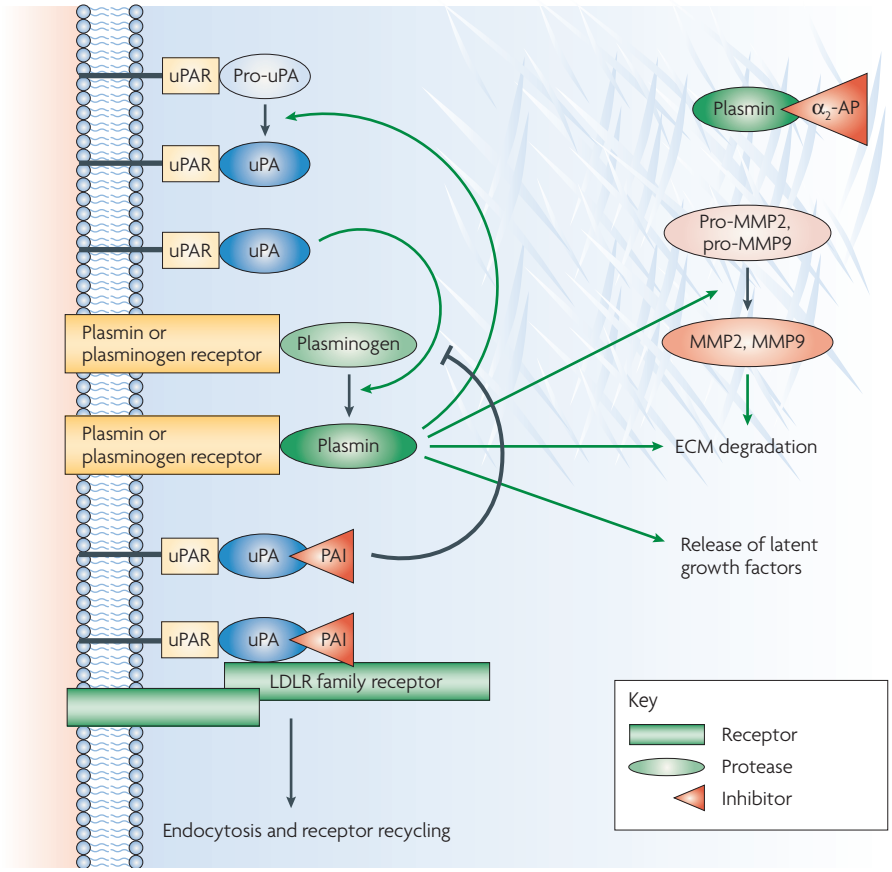

Figure 1 | **Proteolytic cascade regulating plasminogen activation at the cell surface.** Schematic representation of the classical role of urokinase plasminogen activator (uPA), showing the assembly and regulation of the plasminogen activation proteolytic cascade through interactions with various cell surface co-receptors and inhibitors. The serine protease uPA, bound to its specific cell surface receptor (uPAR), efficiently cleaves cell surface-bound plasminogen zymogen at the Arg580–Val581 amide bond, activating the broad-spectrum serine protease plasmin[150,151]. Multiple plasmin and plasminogen receptor proteins have been identified[150] and uPAR is anchored to the plasma membrane outer leaflet by a glycosyl phosphatidylinositol moiety. In a feed-forward loop, activation of uPAR-bound pro-uPA to two-chain uPA through plasmin-mediated proteolytic cleavage facilitates further activation of additional co-localized plasminogen to plasmin. As receptor-bound plasmin is refractory to inhibition by its circulating inhibitor α_2-antiplasmin (α_2-AP), this cyclical positive feedback mechanism is highly effective in amplifying plasmin production[150,152]. Plasmin promotes tissue degradation and remodelling of the local extracellular environment directly, by degrading extracellular matrix (ECM) molecules and activating or releasing latent growth factors[150,151]. Plasmin also potentially activates a limited subset of pro-matrix metalloproteinases (pro-MMPs) such as pro-MMP2 and pro-MMP9, although other activation mechanisms might be more relevant *in vivo*[153].The proteolytic activity of both soluble and receptor-bound uPA is efficiently inhibited by plasminogen activator inhibitors type 1 and 2 (PAI1 and PAI2)[10,154,155]. Following uPA inhibition and formation of uPA-PAI complexes, uPAR-bound uPA–PAI associates with low-density lipoprotein receptor (LDLR) proteins, leading to endocytosis degradation of uPA-PAI complexes, and partial recycling of unoccupied uPAR to the cell surface[155]. Not shown: plasminogen is also activated by plasma kallikrein[156] and tissue-type plasminogen activator (tPA)[10]. The activation of tPA is potentiated by co-binding to fibrin and several cell surface receptors and binding moieties[10,157,158].

Extracellular roles for PAI2 and the serpin inhibitory mechanism. Under physiological conditions PAI2 is not usually detectable in human plasma, except during pregnancy when trophoblasts produce high levels of PAI2 (REFS 10,25). As decreased plasma levels of PAI2 correlate with intrauterine growth retardation and preeclampsia in humans, a role for PAI2 in human placental maintenance and fetal development has been suggested[25]. However, PAI2 is not required for normal murine development, survival or fertility[26], although a phenotype for adipose tissue development in *Pai2*$^{-/-}$ mice was recently reported[27]. Dougherty *et al.*[26] suggested that, as *Pai2* mRNA is only detected at significant levels in the murine placenta late in gestation[28], the lack of obvious developmental phenotypes in *Pai2*$^{-/-}$ mice does not preclude a role for PAI2 in human development. Unfortunately, studies investigating spontaneous or xenograft tumour growth and metastasis in *Pai2*$^{-/-}$ mice have not been performed to date, but such experiments would yield invaluable data on the role of PAI2 in these processes.

PAI2 is also detectable in other human bodily fluids, including gingival fluid[29], saliva[30], peritoneal fluid[31] and infectious pleural effusions[32]. Furthermore, the ratio of intracellular:extracellular PAI2 can be altered by various factors *in vitro*[33,34]. These findings suggest that the secretion of PAI2 is a highly regulated event that is not solely controlled by an inefficient secretion signal. Additionally, non-glycosylated PAI2 has been observed in plasma taken from pregnant women, amniotic fluid and cord blood, and in the conditioned medium of U-937 cells exposed to phorbol ester[10]. As phorbol esters induce PAI2 expression and the presence of cytosolic proteins in the extracellular environment is often predicated by cell death, it has been suggested that cell death (tissue necrosis or apoptosis) may be one route enabling non-glycosylated PAI2 to reach the extracellular environment[10]. There is also evidence of non-glycosylated PAI2 secretion by viable primary human monocytes through an ER–Golgi-independent pathway[33]. Hence, the normally low circulating levels of PAI2 in the blood are not necessarily reflective of locally secreted PAI2 levels in tissues. Finally, PAI2 is approximately 10- and 100-fold slower than PAI1 at inhibiting uPA and tPA, respectively, *in vitro*[35], yet tPA–PAI2 complexes have been detected in both saliva[30] and gingival crevicular fluid[36], and uPA–PAI2 complexes have been detected in human gestational tissues[37]. These observations provide clear evidence of uPA and tPA inhibition by PAI2 *in vivo*.

Extracellular PAI2 inhibits uPA through the unique serpin 'suicide' trapping mechanism. Serpins form covalent complexes with their target proteases, distinct from the classical 'lock and key' mechanism used by other small-molecule protease inhibitors. The reactive centre loop (RCL) of the serpin acts as a bait for the protease active site but, before completion of the proteolysis reaction can occur, cleavage of the RCL induces a large conformational change in the serpin. This so-called stressed (S)-to-relaxed (R) transition is crucial to the inhibitory activity of serpins, involving insertion of the RCL into the body of the serpin molecule (as an extra strand of β-sheet A) and a dramatic increase

in the stability of the molecule. Elegant structural studies (such as REF. 38) have been performed on this transition showing that the protease, which is still covalently bound to the RCL, moves some 70 Å to the opposite pole of the serpin molecule during the S-to-R transition. This effectively crushes the protease, distorting the active site and preventing hydrolysis of the acyl enzyme intermediate, effectively trapping the protease in a stable serpin–protease complex (such as uPA–PAI2). Further, the structural transitions associated with the inhibitory action of serpins form the basis for selective recognition by cellular receptors such as by members of the low-density lipoprotein receptor (LDLR) family of endocytosis receptors, with important implications for functions in cell signalling and migration (see below and FIGS 2, 3).

Potential functions of intracellular PAI2. Several novel functions of the intracellular form of PAI2 have been proposed[18], which appear to be independent of serpin function. Interactions with a variety of cytosolic proteins have been implicated, including retinoblastoma protein (RB)[39], interferon regulatory factor 3 (IRF3) (REF. 40), proteasome subunit β type 1 (PSMB1) (REF. 41), pre-mRNA processing factor 8 (PRPF8) (REF. 42), annexins (I, II, IV and V)[43], and fusion kinase ZNF198 (also known as ZMYM2)–FGFR1 (REF. 44). Furthermore, expression of PAI2 has been observed in the nucleus[39,45,46], where it is thought to interact with RB, preventing RB degradation[39], in addition to modulating its own expression[46]. An emerging theme in these studies is resistance to apoptosis (induced, for example, by tumour necrosis factor α (TNFα)) following overexpression of PAI2 (REFS 44,47–50). These effects appear, however, to be cell type- or context-dependent as PAI2 knockdown in monocytes had no effect on apoptosis induced by serum withdrawal, hydrogen peroxide or a monoclonal antibody to CD95 (also known as FAS) (REF. 51). Additionally, a recent study reported that, whereas TNFα stimulation increased PAI2 expression in HT-1080 and Isreco-1 cells, overexpression of PAI2 in these and other cells lines conferred no protection against TNFα-induced apoptosis[52]. Importantly, this study used lentiviral mediated delivery of PAI2 to maintain heterogeneity of PAI2-overexpressing cell lines and thereby avoid any potential clonal bias introduced by selection of transfected cells. A role for intracellular PAI2 in the regulation of papillomavirus replication and cytopathic effect has also been reported[39]. These effects were linked to the ability of PAI2 to inhibit papillomavirus-induced degradation of RB and so maintain RB levels[39]. However, in addition to affording no protection from apoptosis, lentiviral mediated overexpression of PAI2 (REF. 52) affected RB levels in only one of the three cell lines tested and this effect was independent of its protease-inhibitory activity.

Due to these conflicting results, the exact function of intracellular PAI2 remains unclear. Given the observations of non-glycosylated PAI2 in the extracellular milieu, it is possible that intracellular non-glycosylated PAI2 is released under inflammatory or other conditions that result in acute cell death or damage. This, and/or conditions that enhance locally secreted glycosylated PAI2 in tissues, could thereby limit pericellular and extracellular proteolysis during tissue remodelling processes.

PAI1 versus PAI2 biology

Differential vitronectin binding. At supraphysiological levels, PAI1 interacts with the ECM component vitronectin, and completely blocks the interaction of vitronectin with uPAR and integrins[53]. However, at physiological levels of PAI1 a more dynamic process takes place, in which PAI1 acts as a 'molecular switch', switching its affinity between vitronectin and endocytosis receptors following uPA inhibition[54]. Vitronectin binding also stabilises the active (S) conformation of PAI1 (BOX 1), preventing it from adopting a latent, non-inhibitory conformation[55]. Consequently, inhibition of uPA by vitronectin-bound PAI1 stimulates directed cell migration partially through facilitation of an interaction between vitronectin and co-localized uPAR–integrins[12] (FIG. 2a). The ability of PAI1 to direct vitronectin-dependent cell adhesion and migration is not emulated by PAI2 as it does not bind to vitronectin[56] (FIG. 2b). Additionally, despite high (but still physiological) PAI1 levels in metastatic breast tumours, uPA activity is still detectable[57] and it is available for inhibition and/or targeting by exogenous inhibitors such as recombinant PAI2 (REFS 58–62). So, although PAI1 and PAI2 have similar inhibitory biochemical properties, these additional interactions of PAI1 in the pericellular environment may have a large influence on its actual inhibitory capability. Thus, in the context of the tumour microenvironment, it is likely that secreted or released PAI2 may be the *bona fide* uPA inhibitor, a hypothesis supported by other researchers[63–68].

Box 1 | **Nomenclature and structure of serpin genes and proteins.**

The serpins are a large, broadly distributed family of structurally similar but functionally diverse proteins, with over 1,500 members in many phyla (including animals, plants, bacteria and virus). A comprehensive review and phylogenetic analysis of the serpin gene family led to the identification of 16 clades (A–P) and the construction of a systematic nomenclature that is now becoming more widely used[148].

Most serpins function as inhibitors of serine proteases but some have activity against cysteine proteases and there are rare examples of non-inhibitory functions including hormone transport, molecular chaperone activity and chromatin condensation. The demonstrated physiological roles of serpins are diverse and include regulation of fibrinolysis, apoptosis, tumour suppression, inflammation, development and blood pressure. Numerous examples of mutation or altered expression of serpins have been described with various pathological consequences (so-called 'serpinopathies'), including emphysema, hypertension, thrombosis, liver disease, metastasis and dementia. A comprehensive database of serpin mutations is available at the Structural Medicine Laboratory at the Cambridge Institute for Medical Research (see URL in Further information).

The structural biology of serpins is quite unique (FIG. 3) and has been studied intensely (over 70 solved structures in the Research Collaboratory for Structural Bioinformatics Protein Data Bank). The native structure of serpins (Smart: SM00093, Pfam: PF00079) is highly conserved (consisting of three β-sheets and 7–9 α-helices) and instead of folding into the most stable conformation, serpins fold into a metastable state that has been likened to a form of 'molecular mousetrap'. In this state, the flexible reactive centre loop is extended as a kind of 'bait' for the target protease. Many of the pathological serpin mutations have been shown to render the inhibitors inactive by causing misfolding or polymerization of mutant proteins (for a detailed review see REF. 55). For a more detailed overview of serpin biology, refer to REF. 149 and the Whisstock Laboratory serpin page.

For a detailed description of the serpin inhibitory mechanism refer to REFS 38,55 and this movie of the serpin inhibitory mechanism from the Structural Medicine Laboratory at the Cambridge Institute for Medical Research.

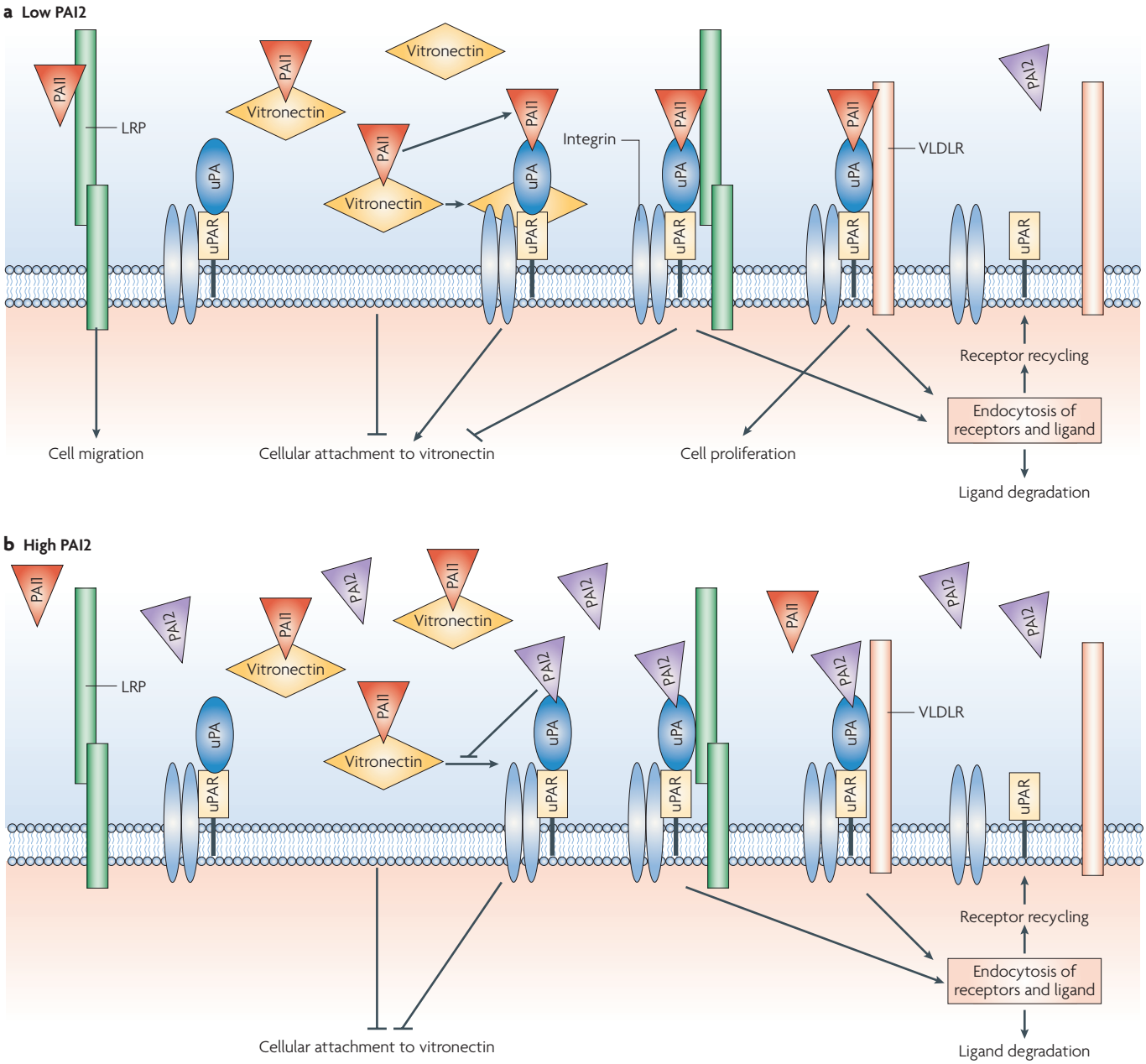

Figure 2 | **The proposed mechanism of improved patient prognosis associated with high plasminogen activator inhibitor type 2 (PAI2) expression.** Differing cell surface interactions might explain the disparity between PAI1 and PAI2 in cancer prognosis. **a** | In tumours with low PAI2 levels, PAI1 contributes to poor patient prognosis through the stimulation of tumour vascularization, growth and metastasis. This is achieved through various complex interactions that increase both cell proliferation and migration. PAI1 bound to vitronectin prevents cellular attachment through urokinase plasminogen activator receptor (uPAR) and integrins. However, on uPA inhibition, PAI1 loses its affinity for vitronectin, freeing up vitronectin for binding by the now co-localized uPAR and integrins, initiating the rounds of cell attachment and de-attachment required for efficient cell migration[12]. Following uPA inhibition, uPA–PAI1 binds with high affinity to members of the low-density lipoprotein (LDL) receptor family, stimulating endocytosis, degradation of uPA-PAI1, and partial recycling of the receptors. However, this interaction also generates other cell type- and receptor-specific responses. The interaction of uPA–PAI1 with LDL-related protein (LRP) causes a decrease in extracellular signal-regulated kinase (Erk) phosphorylation and cell migration[94], although it may also cause a loss in cell adhesion due to the removal of integrins from the plasma membrane[13]. The interaction of uPA–PAI1 with very-low-density lipoprotein receptor (VLDLR) stimulates sustained Erk phosphorylation and increases cell proliferation[17]. Additionally, PAI1 can bind directly to LRP, inducing activation of the Jak–Stat pathway, leading to increased cell motility[99]. **b** | High PAI2 levels in tumours might contribute to good patient outcome solely through inhibition of uPA, which ultimately reduces invasive capacity by preventing plasmin-mediated extracellular matrix degradation and growth factor activation (FIG. 1). Although uPA–PAI2 is cleared from the cell surface through interactions with both LRP and VLDLR, these are of lower affinity than those of uPA–PAI1 owing to the lack of a complete LDLR binding motif in PAI2 (REF. 7). Therefore, unlike uPA–PAI1, endocytosis of uPA–PAI2 through VLDLR does not induce signalling events leading to cell proliferation[7]. Additionally, PAI2 does not bind directly to LRP[6], therefore it is unable to induce cell migration through binding of this receptor. High PAI2 levels also potentially compete with vitronectin-bound PAI1 for uPA binding, preventing the removal of PAI1 from vitronectin, and therefore decreasing vitronectin-dependent cell migration. Some interactions not directly involving PAI1 or PAI2 have been omitted for the sake of clarity.

Structural differences affecting interactions with endocytosis receptors. Following inhibition of uPA at the cell surface, uPA–PAI1 complexes are internalized by interactions with at least three members of the LDLR family of endocytosis receptors: LDL-related protein (LRP)[6,69,70], very-low-density lipoprotein receptor (VLDLR)[7,71–73] and LRP2 (REF. 74). Internalization of uPA–PAI2 complexes by LRP[6] and VLDLR[7] has been demonstrated, but PAI2 endocytosis by LRP2 has not yet been addressed. Importantly, unlike PAI1 (REF. 75), PAI2 is unable to bind directly to these endocytosis receptors[6,7] (FIG. 2b). Consequently, uPA–PAI2 binds with lower affinity than uPA–PAI1 to both LRP and VLDLR as determined by surface plasmon resonance[6,7], a method of direct, real-time measurement of protein–protein interactions. Comparison of structural characteristics of PAI1 and PAI2 in their relaxed conformations provides a clear explanation for the differential binding of PAI1 and PAI2 to VLDLR and LRP[7] (FIG. 3). Structural studies have previously identified positively charged residues within helix D of PAI1 that contribute significantly to the high-affinity binding of the uPA–PAI1 complex with LDLR family members[76–79]. Accordingly, these residues conform with the proposed common binding motif for high-affinity LDLR family ligands of two basic residues separated by 2–5 residues and amino-terminally flanked by hydrophobic residues[80]. Interestingly, this motif is not conserved within the helix D of PAI2 (REF. 7) (FIG. 3e), explaining the lower affinity of uPA–PAI2 for this receptor family. Although these biochemical differences may seem trivial, the biological consequences of this differential receptor binding are quite striking.

PAI2 does not mediate cell signalling. As uPAR is a glycophosphatidylinositol-anchored protein, with no transmembrane region, signalling events initiated by uPAR are mediated by integrins and co-receptors (such as epidermal growth factor receptor (EGFR) and FPR2) that interact with uPAR or the uPAR signalling complex[17,81–83]. The binding of uPA to uPAR induces a variety of cell type-specific responses, including the activation of $p56^{HCK}$ and $p59^{HCK}$ (REF. 84), the Jak–Stat pathway[85,86], focal adhesion kinase (PTK2)[87–89], protein kinase Cε[90], casein kinase 2 (REF. 85) and extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2, also known as mitogen-activated protein kinase 3 (MAPK3) and MAPK1, respectively)[91–93].

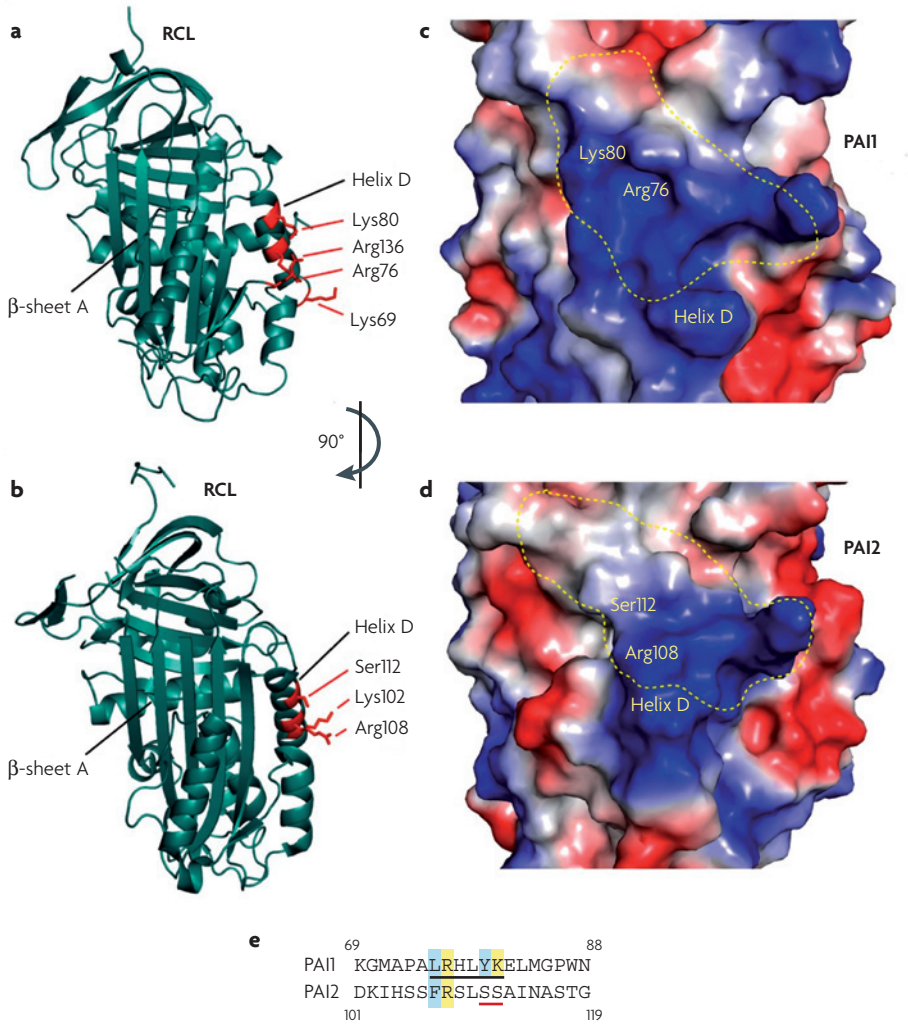

Figure 3 | **Structural comparison of plasminogen activator inhibitor type 1 (PAI1) and PAI2 receptor binding interfaces showing position of key receptor binding residues.** Comparison of structural characteristics of PAI1 (REF. 159) and PAI2 (REF. 160) in their relaxed conformations (that is, mimicking the conformation in urokinase plasminogen activator (uPA)–serpin complexes). Arg76, Lys80 and Lys88 within and adjacent to helix D, along with Arg118 and/or Lys122, mediate binding of uPA–PAI1 to low-density lipoprotein-related protein (LRP) and very-low-density lipoprotein receptor (VLDLR)[77,78], with Arg76 forming part of a cryptic high-affinity binding site for LRP that is exposed by complex formation with uPA[79]. These residues conform with the proposed common binding motif for LRP ligands[80] but this motif is not conserved in PAI2. The corresponding residue to Arg76 in PAI1 is conserved in PAI2 (Arg108) but the residue corresponding to Lys80 is replaced by Ser112 in PAI2 and the adjacent hydrophobic residue is not conserved. Further, there are clear differences in the surface topography and overall electrostatic charge between PAI1 and PAI2. **a**, **b** | Ribbon diagram showing secondary structure and key binding residues around α-helix D of PAI1 and PAI2. **c**, **d** | Surface representation showing regions of positive electrostatic potential in blue, regions of negative potential in red and neutral regions in white. **e** | Alignment of helix D amino acid sequence from PAI1 and PAI2. The putative minimal binding motif[80] in PAI1 is underlined with basic and hydrophobic residues highlighted in yellow and blue, respectively. RCL, reactive centre loop. Figure modified, with permission, from REF. 7 © Biochemical Society (2007).

The interaction of components of the plasminogen activation system with members of the LDLR family can indirectly affect signalling activity by regulating levels of uPA–uPAR on the cell surface[94] and also by directly transmitting signals through adaptor proteins attached to the cytoplasmic domains of the LDLRs[95,96]. On MCF-7 breast cancer cells, the ligation of uPA to uPAR stimulates transient Erk phosphorylation and vitronectin-dependent cell migration[17,97]. The inhibition of uPA by PAI1 sustains the phosphorylation of Erk, stimulating enhanced cell proliferation[7,17].

These events are facilitated by an interaction with VLDLR (FIG. 2a) and mediated via the high-affinity binding site within PAI1 (REF. 17) (FIG. 3), through an ill-defined mechanism that possibly involves an interaction with β3 integrin[17] and transactivation of EGFR[98]. PAI1 is also capable of stimulating cell migration independently of uPA, tPA and vitronectin, as the direct interaction between PAI1 and LRP increases motility through activation of the Jak–Stat pathway[99] (FIG. 2a).

The absence of an LDLR binding site within PAI2 precludes binding of uPA–PAI2 to VLDLR with sufficient affinity to induce sustained mitogenic cell signalling events in MCF-7 cells[7] (FIG. 2b). Furthermore, PAI2 is not able to bind LRP independently of uPA[6] and is therefore unlikely to activate the Jak–Stat pathway and stimulate the cell migration that is mediated by direct binding of PAI1 to LRP[99]. Together, these data suggest that PAI2 may be able to inhibit and clear cell surface uPA, and therefore inhibit plasmin formation *in vivo*, without initiating the cell signalling events and subsequent increased metastatic potential that are associated with PAI1 (FIG. 2). Indeed, an anti-proliferative effect mediated by the protease inhibitory capacity of extracellular PAI2 has been observed with the THP-1 monocyte cell line, although the mechanism underlying this effect was not determined[100]. Direct *in vivo* experimental evidence of these effects would provide a simple explanation for the disparate relationships observed between PAI1 and PAI2 expression and disease outcome in various cancers.

Prognostic significance of PAI2

Experimental tumour model systems. The contribution of PAI2 to improved patient outcome by decreasing tumour growth and metastasis is supported by several experimental tumour models. For example, PAI2 has been shown to modulate xenograft metastasis in rodent models using uPA-expressing cell lines transfected with a PAI2 expression vector[101–103]. Both intracellular and extracellular expression of PAI2 was observed in these cells, along with the complete inhibition of cell surface uPA and significantly decreased ECM degradation *in vitro*[101,102]. In all cases, xenograft tumours were formed in the presence of PAI2, but were consistently surrounded by a dense collagenous capsule, and metastases were reduced or completely absent. In separate studies, intraperitoneal or intratumoural injection of recombinant PAI2 also resulted in decreased tumour size[10]. The comparable physiological outcomes obtained by administration of exogenous PAI2 or transfection of implanted tumour cells with *PAI2* cDNA suggest that the inhibition of extracellular uPA activity is the mechanism underlying this reduction in tumour size and metastasis. Additionally, there are multiple *in vitro* studies that correlate anti-tumorigenic phenomena (such as the expression of tumour suppressor genes, anti-angiogenic factors or infection with a tumour-suppressing E1A adenovirus) with an increase in PAI2 expression[104–107], or pro-tumorigenic stimuli (such as oncogene expression or treatment with phorbol esters) with a subsequent decrease in PAI2 levels[108,109].

Overview of clinicopathological evidence. Concurrent increased protein expression of uPA and PAI1 is a powerful marker of poor prognosis in many different types of solid tumour[3,110–112]. For patients with breast cancer, uPA together with PAI1 is predictive of outcome independent of the classical prognostic factors and outperforms other biological markers such as oestrogen receptors, ERBB2 (also known as HER-2), p53 and cathepsin D[113]. In this context, and in light of experimental evidence for PAI2-mediated inhibition of tumour growth and metastasis, the prognostic relevance of PAI2 expression is of significant interest. To this end, we have collated the findings of all published data investigating the prognostic value of PAI2 expression, which encompasses 50 separate studies covering 15 tumour types (TABLE 1 and Supplementary information S1 (table)). Of those studies that analysed tumour samples against matched normal tissue, all found that PAI2 expression was increased in the tumour over normal tissue, as was expression of uPA, uPAR, PAI1 and occasionally tPA (though the role of tPA in cancer is less clear than that of uPA). It is important to note that the arbitrarily assigned levels of 'high expression' for PAI2 are consistently much lower than those defined for PAI1 (PAI1, mean = 32.2 ± 32.1 ng/mg (n = 7 studies), median = 9.0 ± 6.3 ng/mg (n = 5 studies); PAI2, mean = 7.4 ± 9.6 ng/mg (n = 9 studies), median = 2.5 ± 1.1 ng/mg (n = 8 studies))[5,64,65,114–125], suggesting that a small increase in PAI1 expression may be able to overwhelm the effects of a concurrent increase in PAI2 levels. However, it must also be noted that a proportion of PAI1 might be in the inactive, latent form and that these values reflect antigen levels (as measured by enzyme-linked immunosorbent assay), which might or might not relate to protease inhibitory capacity.

Whether these observed increases in uPA, uPAR and PAI1 expression are predominantly due to specific polymorphisms or tumour-specific effects of various growth factors has not been determined[126–129]. To our knowledge no tumour-specific polymorphisms causing changes in expression of PAI2 have been identified. As PAI2 expression is strongly upregulated by many inflammatory and/or stress-related mediators[10], increases in tumour-associated PAI2 might reflect a host response to a rapidly growing and/or invasive tumour and not necessarily increased expression by tumour cells. Indeed, where analysed, PAI2 (as well as uPA, uPAR and PAI1) within tumour sections is often localized to tumour-associated stromal cells such as fibroblasts, macrophages and endothelial cells[63,66,130–139] (TABLE 1 and Supplementary information S1 (table)). In some cases differential cell type expression of PAI1 and PAI2 may potentially contribute to the opposing prognoses associated with these two serpins. For example, in one lung cancer study, PAI2 expression was restricted to the fibroblasts and correlated with the absence of lymph node involvement, whereas uPA and PAI1 in the tumour cells and fibroblasts correlated with lymph node involvement[139]. Additionally, PAI2 expression in oesophageal squamous cell carcinoma-associated fibroblasts correlated with increased patient survival time[137]. However, in a separate bladder cancer study, no association between stromal PAI2 and patient outcome was found, although only a small proportion of samples contained PAI2-positive stroma[132].

Breast cancer. Breast cancer is the most frequently studied cancer type in which the prognostic value of PAI2 expression has been assessed. Strikingly, all of the studies published (TABLE 1) demonstrate a significant association between PAI2 expression and prognosis. Specifically, relatively high tumour-associated PAI2 expression is linked with prolonged survival, decreased metastasis or decreased tumour size. Conversely, relatively low PAI2 expression was associated with the opposite effect. Two studies that found high PAI2 expression to be favourable also found that low PAI2 expression was associated with a favourable outcome[64,140], although these findings may actually reflect the concomitant low expression of uPA and PAI1 in these tumours. Another study suggested that high PAI2 expression was associated with increased sensitivity to tamoxifen treatment, in contrast

to uPA–PAI1 expression[122]. However, in this study, no link was found between oestrogen receptor and uPA, PAI1 or PAI2 expression, so the mechanism of this modulation in tamoxifen resistance is unknown.

Importantly, multivariate analysis from several studies revealed further subgroups of tumours in which the combination of high PAI1 and low PAI2 had increased significance for poor prognosis and *vice versa*[5,65,115,141,142]. Furthermore, in a study of 2,780 patients, high PAI2 expression was an indicator of positive prognosis only in primary invasive tumours that also expressed uPA and PAI1, and was independent of all other clinicopathological parameters[5]. This study is also corroborated by others in breast[5,65], head and neck[137,143], oral[63] and lung[144] cancer that demonstrate the importance of uPA expression for the significance of PAI2 expression (Supplementary information S1 (table)). These findings are supported by experimental evidence described above for a role for PAI2 in the inhibition of tumour-associated uPA *in vivo*.

Other cancer types. The results of the relatively few studies conducted into the prognostic value and functional role of PAI2 expression in other cancer types (head and neck, oral, colorectal, gastric, lung and pancreatic carcinomas) are not as clear as those for breast cancer; however, the general trend is towards a positive or neutral outcome associated with PAI2 expression (Supplementary information S1 (table)). Interestingly, all three studies conducted into endometrial cancer concluded that increased PAI2 expression was associated with increased disease recurrence, local invasion or more aggressive tumour stage. These differences might reflect functional disparity in the biochemistry of progression and metastasis of other tumour types. Ovarian cancer provides an illustration of this concept, where the ability of colony stimulating factor 1 (CSF1) to induce secretion of PAI2 has been investigated with respect to the poor prognosis associated with the high levels of soluble PAI2 in ascites, and the good prognosis associated with high levels of cell-associated (intracellular) PAI2 (REFS 34,130). As CSF1 is also known to upregulate the expression of both uPA and PAI1 (REF. 131) and stimulate tumour cell invasion in a uPA-dependent manner[145], it seems likely that this effect of CSF1, and not the presence of high levels of secreted PAI2, is responsible for the poor outcome. Indeed, in these studies, high PAI1 levels were significantly associated with

Table 1 | **PAI2 expression and breast cancer prognosis**

Sample size	Detection system	Prognostic impact	Ref.
80	ELISA	PAI2 expression was higher in carcinomas without lymph node involvement, in contrast to PAI1, which was higher in carcinomas with node involvement	125
314	ELISA	Low levels of PAI2 correlated with shorter disease-free survival in overall population, menopausal women and node-negative patients Poor prognosis was associated with a subgroup with high PAI1 and low PAI2 in the overall population and node-negative patients Concurrently high uPA and low PAI2 was also indicative of poor prognosis in menopausal women	65
1,012	ELISA	No association between PAI2 expression and prognosis in the overall population Concurrently high uPA and PAI2 expression was associated with prolonged relapse-free survival, metastasis-free survival and overall survival	116
50	RT-PCR	Low PAI2 expression was associated with lymph node involvement and correlated with high uPA, uPAR and PAI1 levels	142
170	ELISA, ICC	PAI2 expression was higher in carcinoma tissue than benign tissue High levels of PAI2 expression were associated with prolonged disease-free survival and overall survival PAI2 expressed in both stromal and tumour cells	134
73	ISH, IHC	*PAI2* mRNA and protein expressed by tumour cells, fibroblasts, macrophages and lymphocytes PAI2- (and PAI1)-positive cells predominantly located at the periphery of the invasive front of the tumour The presence of PAI2-positive cancer cells was associated with prolonged overall survival PAI2 expression in fibroblasts was not associated with overall survival	66
499	ELISA	Correlation between PAI2 expression and increased clinical tumour size, MSBR tumour grade and progesterone receptor expression High or low PAI2 expression was linked to prolonged disease-free survival A dissemination risk index based on the opposing influences of PAI1 and PAI2 on uPA demonstrated the shorter disease-free survival associated with the increasing ratio of PAI1:PAI2	64
2,780	ELISA	PAI2 expression associated with decreased lymph node involvement and tumour size High PAI2 expression is associated with prolonged relapse-free survival and overall survival In multivariate analysis, PAI2 is not associated with improved prognosis unless combined with uPA and PAI1	5
332	ELISA	Low PAI2 expression was associated with shorter relapse-free survival in multivariate analysis Worst prognosis was associated with concurrent high PAI1 and low PAI2 expression levels, whereas low PAI1 and high PAI2 was associated with favourable prognosis	115
460	ELISA	High levels of PAI2 expression were associated with prolonged disease-free survival Prognostic significance was increased when expression of PAI1 and PAI2 was combined	141
130	RT-PCR, ELISA	Low and high levels of *PAI2* mRNA tended to be associated with prolonged disease-free survival Levels of *PAI2* mRNA positively correlated with PAI2 protein	140
691	ELISA	PAI2 expression was associated with increased sensitivity to tamoxifen treatment, in contrast to uPA, uPAR and PAI1	122
148	ELISA	PAI2 expression correlated with prolonged overall and disease-free survival	113

ELISA, enzyme-linked immunosorbent assay; ICC, immunocytochemistry; IHC, immunohistochemistry; ISH, *in situ* hybridization; MSBR grade, modified Scarff–Bloom–Richardson grade; PAI, plasminogen activator inhibitor; RT-PCR, reverse transcription PCR; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

CSF1 expression by the tumour epithelium[131]. It is also worth noting that CSF1 is often overexpressed in endometrial tumours[145] and this might be related to the observation of consistently high PAI2 expression in more invasive endometrial tumours and the shorter survival time for these patients. This observation is also consistent with the significant link between high PAI1 and PAI2 expression observed in the largest study of patients with endometrial cancer [146].

In summary, it appears that the significance of PAI2 expression on prognosis in other cancer types is heavily context-dependent, generally relies on uPA expression, and is inversely related to PAI1 levels. Although a possible role for intracellular PAI2 in regulating apoptosis cannot be excluded, any extracellular PAI2 found in the tumour microenvironment might be able to compete for the binding of PAI1 to uPA. Extracellular PAI2 could thus limit plasmin generation while possibly neutralizing the alternative actions of PAI1.

Concluding remarks

Even though PAI1 can inhibit receptor-bound uPA *in vivo* and *in vitro*, the mechanism(s) linking PAI1 expression to tumour malignancy might be distinct from a direct role in inhibition of cell surface plasminogen activation. These mechanisms promote cell proliferation, migration and/or de-adhesion and involve interactions between PAI1 and vitronectin or integrins–uPAR–uPA–PAI1 and LDLRs (FIG. 2). Crucial structural differences in PAI2 preclude direct high-affinity binding to vitronectin or members of the LDLR family[6,7] and hence PAI2 does not have the capability to induce these additional cellular responses. Rather, high levels of PAI2 in the tumour microenvironment would facilitate cell surface uPA inhibition and clearance and might also counteract PAI1 stimulatory actions on tumour invasion and metastasis (FIG. 2). From a clinicopathological perspective, these structural and functional differences may thus explain, at least in part, the paradoxical biomarker data for PAI1 versus PAI2 in cancer prognosis. Therefore, inclusion of PAI2 expression in clinical analyses would be expected to increase the prognostic power of measuring uPA–PAI1 expression. Further animal model studies aimed at directly measuring the relative contributions of PAI1 and PAI2 to tumour progression are also needed (for example, measurement of growth and metastasis of spontaneous or xenografted tumours in PAI2 or PAI1- and PAI2-null mice). Detailed understanding of the functional differences between PAI1 and PAI2 will facilitate improved design of uPA-targeted therapies aimed at specifically inhibiting uPA activity while avoiding mitogenic and motogenic signalling through LDLRs.

Only two studies on the prognostic effect of PAI2 have attempted to distinguish between the two topological localizations of PAI2 (REFS 130,132), and none have determined the effect of glycosylation. In the tumour microenvironment, conditions such as hypoxia and inflammation can lead to phenotypical changes of the tumour-associated stroma (for example, cancer-associated fibroblasts[147]), potentially inducing PAI2 expression and secretion or release of PAI2 protein. It is also possible that the contentious role of intracellular PAI2 in the regulation of apoptosis may be of some prognostic influence, but this process is currently poorly understood. Hence, it is clear that further studies need to discriminate between the functions of the two topologically distinct forms of PAI2.

In conclusion, the emerging evidence for the existence of pericellular and extracellular PAI2 and the clear anti-tumour benefits of inhibition of uPA by PAI2, as opposed to PAI1, all suggest that PAI2 has an important role as an inhibitory serpin in the tumour microenvironment.

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DATABASES
Entrez Gene:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
cathepsin D | CSF1 | EGFR | ERBB2 | FAS | FGFR1 | FPR2 | IRF3 | LRP | LRP2 | MAPK1 | MAPK3 | p53 | PLAT | PLAU | PLAUR | PRPF8 | protein kinase Cε | PSMB1 | PTK2 | RB | SERPINB2 | SERPINE1 | SERPINF2 | TNFα | vitronectin | VLDLR | ZMYM2
National Cancer Institute: http://www.cancer.gov/
bladder cancer | breast cancer | colorectal cancer | endometrial cancer | gastric cancer | head and neck cancer | lung cancer | oral cancer | ovarian cancer | pancreatic cancer | squamous cell carcinoma
National Cancer Institute Drug Dictionary:
http://www.cancer.gov/drugdictionary/tamoxifen
Pfam: http://pfam.sanger.ac.uk/PF00079
Protein Databank: http://www.rcsb.org/pdb/home/home.do
PAI1 | PAI2
Simple Modular Architecture Research Tool:
http://smart.embl.de/SM00093

FURTHER INFORMATION
M. Ranson's homepage:
http://www.uow.edu/science/biol/academics/
Movie of serpin inhibitory mechanism:
http://huntingtonlab.cimr.cam.ac.uk/Movies/serpin_mech05.mov
Serpin structures in Protein Data Bank: http://www.pdb.org/pdb/static.do?p=education_discussion/molecule_of_the_month/pdb53_report.html
Structural Medicine database of serpin mutations:
http://www-structmed.cimr.cam.ac.uk/Serpins/serp_regions/table2.html
Whisstock laboratory serpin page:
http://en.wikipedia.org/wiki/Serpin

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OPINION

Exploring the role of cancer stem cells in radioresistance

Michael Baumann, Mechthild Krause and Richard Hill

Abstract | Radiobiological research over the past decades has provided evidence that cancer stem cell content and the intrinsic radiosensitivity of cancer stem cells varies between tumours, thereby affecting their radiocurability. Translation of this knowledge into predictive tests for the clinic has so far been hampered by the lack of methods to discriminate between stem cells and non-stem cells. New technologies allow isolation of cells expressing specific surface markers that are differentially expressed in tumour cell subpopulations that are enriched for cancer stem cells. Combining these techniques with functional radiobiological assays holds the potential to elucidate the role of cancer stem cells in radioresistance in individual tumours, and to use this knowledge for the development of predictive markers for optimization of radiotherapy.

A cancer stem cell is defined as a cell within a tumour that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumour[1]. This definition directly implies that an anticancer therapy can cure a tumour only if all cancer stem cells are killed[1,2]. In the context of this article about radiotherapy the definition of a cancer stem cell translates into a cell which, when left after irradiation in its natural environment, has the capacity to cause a tumour recurrence. Different researchers studying cancer stem cells have used terms such as (functional) tumour stem cells, tumour-rescuing units, tumour- or cancer-initiating cells, cancer stem-like cells or cancer stem cells[3–8]. In some cases these terms have been preferred because of the nature of the techniques used to test tumour-derived cells for the stem-like properties, in others because of the concern that use of the term implies that the origins of such cells are normal tissue stem cells, which may not be the case. The fundamental premise of the present article is that a tumour would not recur if no cancer stem cells had survived treatment. Therefore, we have preferred to use the term 'cancer stem cells' that was defined by the American Association for Cancer Research Workshop on Cancer Stem Cells[1], because the primary focus of our discussion concerns therapeutics, and one of the major rationales cited by the workshop and most authors for the study of such cells is their potential influence on the effects of cancer treatment. We acknowledge that the term 'cancer stem cells' in this article may be controversial and that others might prefer to use different terms for describing the results obtained by different assays. We also recognize that it is currently unknown whether a 'cancer stem cell' that can initiate a tumour after transplantation is the same that can cause a recurrence after anticancer treatment or whether a 'cancer stem cell' that can cause a recurrence after irradiation may be different from a 'cancer stem cell' that has the capacity to regrow a tumour after chemotherapy. We believe the latter is unlikely, but it is theoretically possible if it is supposed that treatment may cause recruitment of early progenitor (non-stem) cells back into the cancer stem cell pool. To our knowledge, this issue has not been addressed in the literature.

A recent milestone in cancer research was the introduction of flow sorting techniques to isolate cell populations on the basis of cell surface markers that are differentially expressed in tumour cell subpopulations that have enriched cancer stem cell-like properties, namely the ability to regrow a tumour on transplantation. Application of this technology has been postulated to allow discrimination of stem cells and non-stem cells on an individual basis[1]. The concept of a cancer stem cell is evolving and, for example, it is not yet certain that some progenitor cells may not be able to re-establish stem cell properties.

Radiotherapy is typically applied in a course of multiple fractions over several weeks, although recent technical advances are allowing the investigation of treatment with a few large fractions for