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Host-derived plasminogen activator inhibitor-1 (PAI-1) concentration is critical for *in vivo* tumoral angiogenesis and growth

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Abstract

Plasminogen activator inhibitor type 1 (PAI-1) plays a key role in tumor progression and is believed to control proteolytic activity and cell migration during angiogenesis. We report here that host PAI-1, at physiological concentration, promotes *in vivo* tumor invasion and angiogenesis. In sharp contrast, inhibition of tumor vascularization was observed when PAI-1 was produced at supraphysiologic levels, either by host cells (transgenic mice overexpressing PAI-1) or by tumor cells (after transfection with murine PAI-1 cDNA). This study provides for the first time *in vivo* evidence for a dose-dependent effect of PAI-1 on tumor angiogenesis. Of great interest is the finding that PAI-1 produced by tumor cells, even at high concentration, did not overcome the absence of PAI-1 in the host, emphasizing the importance of the cellular source of PAI-1.

Keywords: PAI-1; cancer invasion; tumoral angiogenesis; serine protease; protease inhibitor

The Plasminogen/Plasmin system is one of the most efficient enzymatic systems used by tumor and endothelial cells to break down the extracellular matrix and to invade host tissue (Stephens *et al.,* 1998; Andreasen *et al.,* 2000; Rakic *et al.,* 2003). Urokinase-type (uPA) and tissue-type plasminogen (tPA) activators both convert plasminogen to plasmin, which degrades matrix components and activates latent metalloproteinases and latent growth factors. Their main physiological inhibitor, Plasminogen activator inhibitor type 1 (PAI-1), has been considered for a long time as a cancer inhibitor (Soff *et al.,* 1995; Stefansson *et al.,* 2001). However, recently increasing evidence demonstrated a proangiogenic role of PAI-1. PAI-1 deficiency in mice has been reported to reduce angiogenesis in two models of tumor transplantation (Bajou *et al.,* 1998; Gutierrez *et al.,* 2000), in the mouse aortic ring assay (Devy *et al.,* 2002; Masson *et al.,* 2002) and in the laser-induced choroidal neoangiogenesis assay (Lambert *et al.,* 2001). PAI-1 is thought to contribute to angiogenesis by regulating plasmin-mediated proteolysis (Bajou *et al.,* 2001; Devy *et al.,* 2002) and/or by modulating cell migration (Kjoller *et al.,* 1997; Deng *et al.,* 1996, 2001; Isogai *et al.,* 2001; Czekay *et al.,* 2003).

These findings emphasizing a proangiogenic and/or a promigratory effect of PAI-1 are in accordance with the clinical data indicating that high expression of PAI-1 in tumors correlates with poor prognosis for patients suffering from a variety of tumors (Andreasen *et al.,* 2000; Foekens *et al.,* 2000; Rakic *et al.,* 2003). However, there are apparent discrepancies between these data and (1) the lack of impact of PAI-1 deficiency on metastasis of melanoma cells (Eitzman *et al.,* 1996), on MMTV-Pym T-induced breast cancer progression (Almholt *et al.,* 2003), and (2) the antiangiogenic effect of PAI-1 observed in the chicken chorioallantoid membrane (CAM) (Stefansson *et al.,* 2001) and in the Matrigel implant assay (McMahon *et al.,* 2001). Taken together, these findings indicate that the role of PAI-1 as a determinant of tumoral angiogenesis might vary with experimental setting (tumor-type or injection-site dependent) and might depend on its cellular origin (tumor cells *versus* host cells). In addition, a dose-dependent effect of PAI-1 has been reported *in vitro* (Devy *et al.,* 2002) and *in vivo,* in a model of choroidal neoangiogenesis (Lambert *et al.,* 2003).

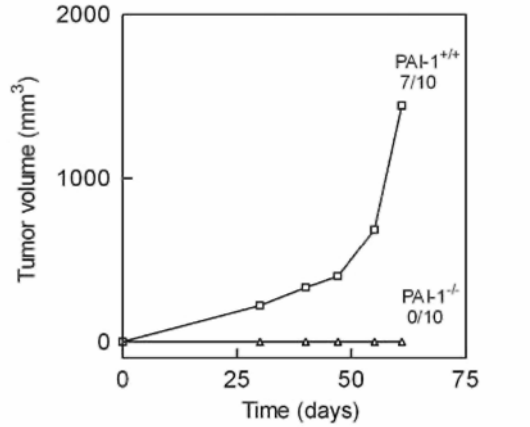
In an attempt to explain these conflicting results, malignant PDVA keratinocytes overexpressing or not PAI-1 were transplanted into mice lacking PAI-1 or expressing it at various levels. Their tumorigenicity and angiogenic phenotype were compared in the presence of different matrix components.

PDVA cells failed to induce tumor growth when subcutaneously injected into PAI-1$^{-/-}$ mice

The collagen gel used in the transplantation chamber model (Bajou *et al.,* 1998) could be considered as abnormal conditions leading to artefactual results. Therefore, to investigate the putative effect of matrix components, PDVA cells were injected subcutaneously with Matrigel, into wild-type *(PAI-1$^{+/+}$)* and PAI-1-deficient (*PAI-1$^{-/-}$*) mice. At 1 month after cell inoculation into *PAI-1$^{+/+}$* mice, tumors were palpable in 30% of injected sites and

their volumes increased exponentially, reaching 1.4 cm^3 after 60 days. At that time, tumor incidence was 70%. In contrast, no tumors were detected in *PAI-1*$^{-/-}$ mice even after 60 days (Figure 1). The protumorigenic effect of PAI-1 is thus independent to the extracellular matrix surrounding tumor cells. These observations agreed with the reduced tumor growth reported after subcutaneous injection of murine fibrosarcoma cells into *PAI-1*$^{-/-}$ mice (Gutierrez *et al.,* 2000). However, they do not fit with the lack of effect of PAI-1 deficiency on the progression of virus oncogene (MMTV-Pym-T)-induced breast cancer (Almholt *et al.,* 2003). A possible explanation for these conflicting results are that PAI-1 does not play a key role in the MMTV-PymT model, or that compensatory mechanisms take place during endogenous tumor development, but not after challenging mice by tumor transplantation.

Figure 1 *Primary tumor growth of malignant keratinocytes. PDVA cells (2 x* 10^6 *cells/site) were mixed with an equal volume of Matrigel (10mg/ml) and injected subcutaneously into PAI-1*$^{+/+}$ *or PAI-1*$^{-/-}$ *mice. Tumor volume was calculated using the formula: width*^{2} *x length x 0.4*



Excess or absence of PAI-1 in the host-inhibited tumor invasion and vascularization

PDVA cells cultured on a collagen gel were next transplanted into *PAI-1*$^{-/-}$, transgenic mice overproducing PAI-1 (*Tg-PAI-1*) at blood concentrations ranging from 17 ng/ml to more than 50 ng/ml, and their corresponding control mice (*PAI-1*$^{+/+}$ or *Tg-WT*) producing PAI-1 at physiological concentration (from 2 to 4 ng/ml). At 2 weeks after cell transplantation into wild-type mice (*PAI-1*$^{+/+}$ and *Tg-WT*), malignant keratinocytes invaded the collagen gel, penetrated into host tissues and were surrounded by blood vessels (Figure 2a). In sharp contrast, in *PAI-1*$^{-/-}$ mice as well as in *Tg-PAI-1* mice (Figure 2b), an inhibition of tumor cell invasion and vascularization was observed. In these conditions, blood vessels remained below the collagen gel and tumor cells failed to invade the host tissue.

Quantification by image analysis showed that the average tumor invasion and angiogenesis were significantly reduced in *PAI-1*$^{-/-}$ and *Tg-PAI-1* mice as compared to their corresponding control mice (*PAI-1*$^{+/+}$ and *Tg-WT,* respectively) (Figure 2c). In *Tg-PAI-1* mice, an inhibition of both tumor invasion and vascularization was observed independently of blood PAI-1 concentration. This suggests that an excess of PAI-1 (concentration higher than 17 ng/ml *versus* 2-4 ng/ml in *WT* mice) always leads to tumor reduction. Thus, an absence as well as an excess of host PAI-1 inhibit *in vivo* the tumor development and vascularization. These observations are consistent with the bell-shape effect of PAI-1 observed *in vitro* in the aortic ring assay (Devy *et al.,* 2002) and *in vivo,* in a model of laser-induced choroidal neoangiogenesis (Lambert *et al.,* 2003). By demonstrating that *in vivo* PAI-1 effects on tumor development and vascularization are dependent on its concentration, our findings resolve apparently conflicting data indicating that PAI-1 can inhibit as well as promote angiogenesis (Soff *et al.,* 1995; Bajou *et al.,* 1998; Gutierrez *et al.,* 2000; McMahon *et al.,* 2001; Stefansson *et al.,* 2001; Curino *et al.,* 2002).

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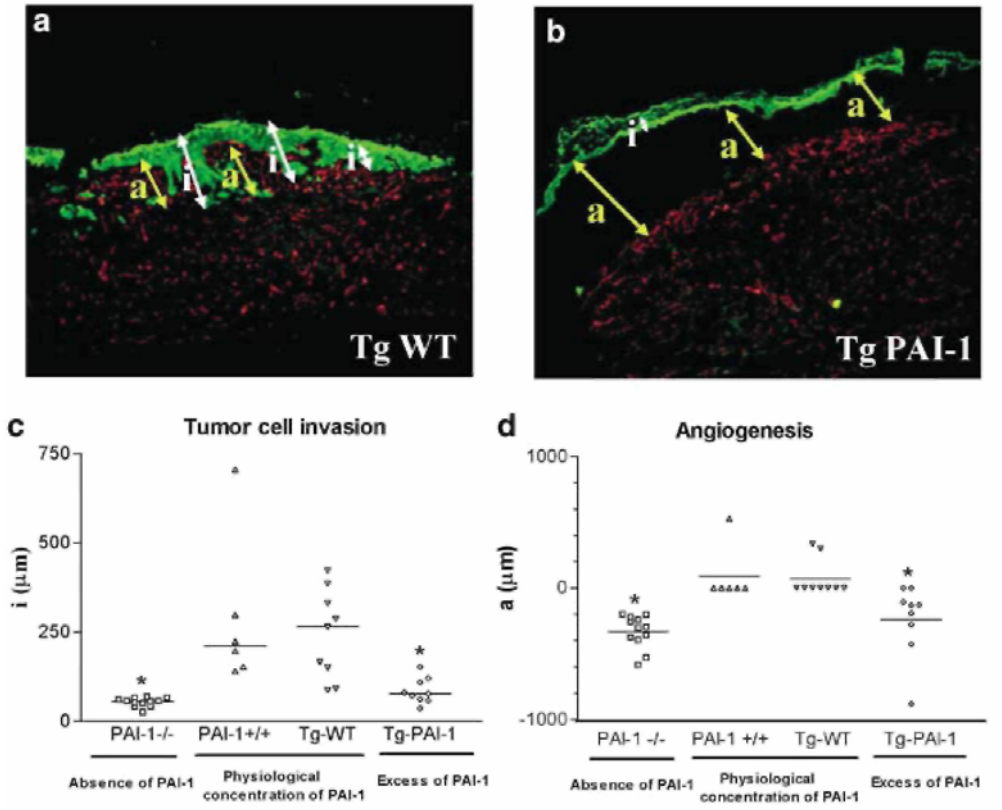
Figure 2 *Invasion and angiogenesis of malignant keratinocytes. PDVA cells (2 x 10^5) were plated on a collagen type I gel (4mg/ml) inserted in teflon rings, covered with a silicone transplantation chamber (Renner GmbH) and implanted onto the dorsal muscle fascia of mice (Bajou et al., 1998). Cells were transplanted for 2 weeks into PAI-$1^{-/-}$ mice, wild-type mice (**a**) and transgenic mice overexpressing PAI-1 under the control of adipocyte-specific αP2 promoter (Tg-PAI-1 mice) (**b**) (Lijnen et al., 2004). Malignant cells were labeled in green with an anti-keratin antibody, while vessels were stained in red with an anti-collagen type IV antibody. Quantification of tumor invasion (i) (**c**) and angiogenesis (a) (**d**) was performed by morphometric measurements using a computer-assisted image analysis system (Olympus Micro Image version 3.0 for Windows 95/NT, Olympus Optical CO., Europe GmBH). 'i' = the depth of tumor cell invasion corresponding to the distance between the top of the tumor cell layer to the deepest front of tumor spread; 'a' = distance separating tumor cells from the front of migrating blood vessels, 'a' is inversely related to the degree of endothelial cell migration. A zero value means that vessels have reached the basal tumor layer, 'a' is negative when vessels did not reach the tumor cells, and positive when vessels were intermingled with tumor cells. In all, 10-12 measurements of each parameters ('i' and 'a') were performed for each tumor (n = 8), and the mean values are reported. The horizontal bars represent median values, P-values were generated by ANOVA test (*P ≤ 0.05)*



Overexpression of PAI-1 by tumor cells reduced *in vitro* and *in vivo* tumor development, but did not compensate for PAI-1 deficiency in the host

In our tumor transplantation system, the lack of tumor invasion and vascularization in PAI-1-deficient mice is a consequence of the absence of PAI-1 derived from host cells. Indeed, re-establishment of PAI-1 expression in host cells by adenovirus-mediated gene transfer restored the angiogenic and invasive phenotype of PDVA cells (Bajou *et al.,* 1998, 2001). In order to determine whether an overproduction of PAI-1 by tumor cells could overcome the host deficiency, PDVA cells were transfected with murine PAI-1 cDNA. A significant increase of PAI-1 mRNA expression was assessed by RT-PCR (Figure 3a). PAI-1 overproduction was confirmed by immunostaining (data not shown) and ELISA analysis revealing a threefold increase of PAI-1 released by transfected cells. PAI-1 overexpression by PDVA cells led to a significant reduction of *in vitro* invasion ($P \leq 0.05$) (Figure 3b). An inhibition of tumor invasion and angiogenesis was observed when PAI-1-overexpressing cells were transplanted into wild-type mice or PAI-1 transgenic mice (*Tg-PAI-1*) ($P \leq 0.025$) (Figure 4a, b). Therefore, inhibition of tumor development was achieved by an overproduction of PAI-1 by tumor cells themselves (Figure 4) ($P \leq 0.025$), by host cells (in *PAI-1-Tg* mice) or by both tumor cells and host cells (Figure 2).

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Figure 3 *PAI-1 expression and in vitro invasion of PAI-1 transfected PDVA cells. Cells were transfected with pCDNA-3.1 vector alone (Invitrogen) or pCDNA-3.1 vector containing the full length cDNA of murine PAI-1. Stable populations of cells overexpressing PAI-1 ('PDVA PAI-1 ') and their corresponding control cells ('PDVA-CTL') were selected with the neomycin-analogous G418 (400µg/ml; GIBCO BRL). (**a**) RT-PCR analysis of PAI-1 expression in PAI-1-transfected cells (lane 2) and control cells (lane 1). RT-PCR products were separated on 10% acrylamide gels and analysed using a Fluor-S Multilmager (Bio-Rad, Hercules, CA, USA) after staining with Gelstar dye (FMC BioProducts, Rockland, ME, USA). The expected product size is 197bp for murine PAI-1 and 212bp for murine 28S. (**b**) In vitro invasion test. Cells (6 x 10^4) suspended in 300 µl of serum-free medium containing 0.1% (w/v) bovine serum albumin (fraction V, Sigma) were seeded in the upper compartment of Transwell cell culture inserts (8 µm pore size, Costar, Cambridge, MA, USA) coated with Matrigel (25 µg/filter). The lower compartment contained DMEM supplemented with 20% serum and 1% BSA. Results expressed as mean cell number in the lower face of a filter (x 400) (from triplicate samples) are those of one representative experiment among three separate assays*

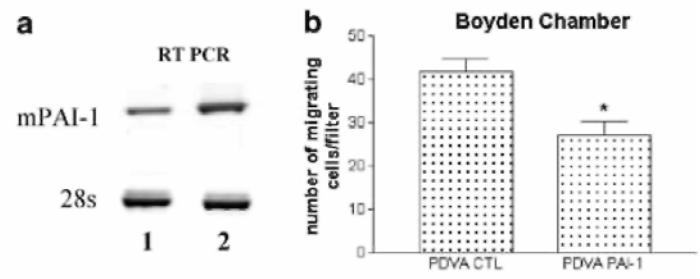
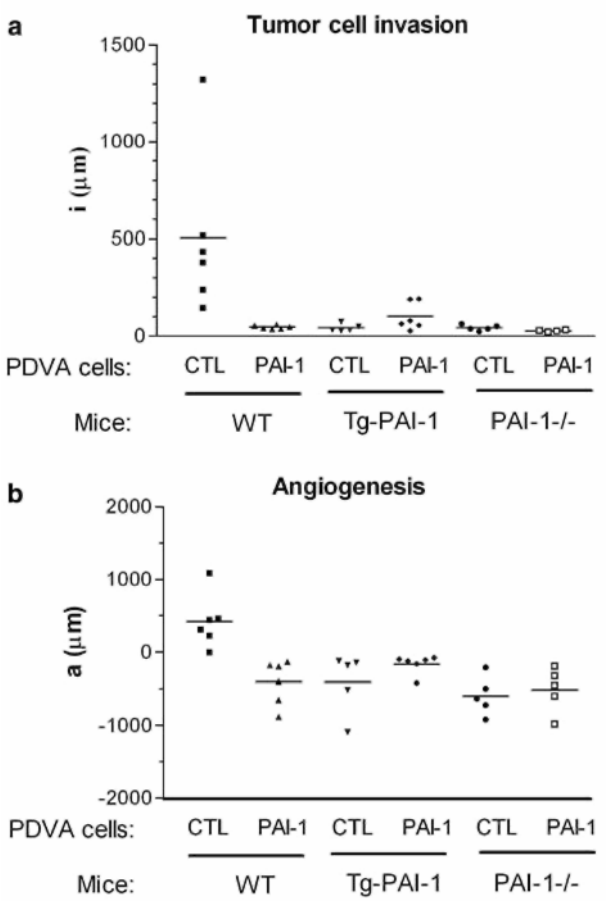


Figure 4 *Analysis of tumor cell invasion (**a**) and angiogenesis (**b**) of PDVA cell overexpressing PAI-1 or control cells. PDVA cells ('CTL') or PAI-1 cDNA-transfected cells ('PAI-1') were transplanted into wild-type mice (WT), transgenic mice (Tg-PAI-1) and PAI-1-deficient mice (PAI-$1^{-/-}$). Tumor invasion (i) and angiogenesis (a) were determined as described in Figure 2 legend*



To determine whether PAI-1 produced by tumor cells could compensate for the absence of host PAI-1, PAI-1-overexpressing PDVA cells were also transplanted into PAI-1-deficient mice. Interestingly, whatever the amounts of PAI-1 produced by tumor cells, blood vessels remained below the collagen gel and tumor cells failed to invade the PAI-1-deficient host tissue. These findings clearly demonstrate that the specific site of PAI-1 production (stromal cells rather than tumor cells) is a critical factor in tumor angiogenesis rather than the total amount of PAI-1 in tumor.

The proangiogenic effect of PAI-1 produced by host cells could be related to a protection of the extracellular matrix against an excessive degradation induced by tumor cells. Our previous work demonstrated that the function of PAI-1 in angiogenesis was related to controlled plasmin-mediated proteolysis during tumoral angiogenesis, rather than to a modulation of cell interaction with vironectin (Bajou *et al.,* 2001). PAI-1 might stabilize the matrix scaffold required for endothelial cell migration and the assembly of endothelial cells into capillaries (Montesano *et al.,* 1990). Indeed, a critical balance between proteases and their inhibitors is thought to be essential for optimal angiogenesis (Pepper and Montesano, 1990) and tumor cell invasion (Liu *et al.,* 1995). The sequestration of PAI-1 in the matrix is consistent with such a role (Bajou *et al.,* 1998). This model implies that angiogenesis requires PAI-1 levels within an optimal range and fits perfectly with the present demonstration of a dose-dependent effect of PAI-1 on angiogenesis.

The antiangiogenic effect of PAI-1 used at therapeutical concentrations agreed with the data reported in the CAM, Matrigel implant and aortic ring assays (McMahon *et al.,* 2001; Stefansson *et al.,* 2001; Devy *et al.,* 2002). Accordingly, when PDVA cells were transfected with murine PAI-1 cDNA to overproduce PAI-1, a significant reduction of tumor cell invasion was observed *in vivo* after transplantation into *WT* mice. This effect could be ascribed to a reduced capacity of invasion as assessed *in vitro* in Transwell assay. These findings are in accordance with the results obtained after PAI-1 transfection of human prostate carcinoma cells and showing a reduction in tumor-associated angiogenesis (Soff *et al.,* 1995).

The present study highlights for the first time the importance of local variations of PAI-1 concentration and of the cellular source of PAI-1 (tumor cells *versus* host cells) for its opposite effects *in vivo* on tumor invasion and vascularization. Our demonstration of a dose-dependent effect of PAI-1 on tumoral angiogenesis confirms our previous observations in another pathological angiogenic process, the choroidal neoangiogenesis (Lambert *et al.,* 2003). Altogether, these findings have important implications in antiangiogenic strategies and sound a note of warning for the pharmacological use of PAI-1 agonists/antagonists.

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References

Almholt K, Nielsen BS, Frandsen TL, Brunner N, Dano K and Johnsen M. (2003). *Oncogene,* 22, 4389-4397.

Andreasen PA, Egelund R and Petersen HH. (2000). *Cell Mol. Life Sci.,* 57, 25-40.

Bajou K, Masson V, Gerard RD, Schmitt PM, Albert V, Praus M, Lund LR, Frandsen TL, Brunner N, Dano K, Fusenig NE, Weidle U, Carmeliet G, Loskutoff D, Collen D, Carmeliet P, Foidart JM and Noel A. (2001). *J. Cell Biol.,* 152, 777-784.

Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D and Foidart JM. (1998). *Nat. Med.,* 4, 923-928.

Curino A, Mitola DJ, Aaronson H, McMahon GA, Raja K, Keegan AD, Lawrence DA and Bugge TH. (2002). *Oncogene,* 21, 8830-8842.

Czekay RP, Aertgeerts K, Curriden SA and Loskutoff DJ. (2003). *J. Cell Biol.,* 160, 781-791.

Deng G, Curriden SA, Hu G, Czekay RP and Loskutoff DJ. (2001). *J. Cell Physiol.,* 189, 23-33.

Deng G, Curriden SA, Wang S, Rosenberg S and Loskutoff DJ. (1996). *J. Cell Biol.,* 134, 1563-1571.

Devy L, Blacher S, Grignet-Debrus C, Bajou K, Masson V, Gerard RD, Gils A, Carmeliet G, Carmeliet P, Declerck PJ, Noel A and Foidart JM. (2002). *FASEB J.,* 16, 147-154.

Eitzman DT, Krauss JC, Shen T, Cui J and Ginsburg D. (1996). *Blood,* 87, 4718-4722.

Foekens JA, Peters HA, Look MP, Portengen H, Schmitt M, Kramer MD, Brunner N, Janicke F, Meijer-van Gelder ME, Henzen-Logmans SC, van Putten WL and Klijn JG. (2000). *Cancer Res.,* 60, 636-643.

Gutierrez LS, Schulman A, Brito-Robinson T, Noria F, Ploplis VA and Castellino FJ. (2000). *Cancer Res.,* 60, 5839-5847.

Isogai C, Laug WE, Shimada H, Declerck PJ, Stins MF, Durden DL, Erdreich-Epstein A and DeClerck YA. (2001). *Cancer Res.,* 61, 5587-5594.

Kjoller L, Kanse SM, Kirkegaard T, Rodenburg KW, Ronne E, Goodman SL, Preissner KT, Ossowski L and Andreasen PA. (1997). *Exp. Cell Res*., 232, 420-429.

Lambert V, Munaut C, Carmeliet P, Gerard RD, Declerck PJ, Gils A, Claes C, Foidart JM, Noel A and Rakic JM. (2003). *Invest Ophthalmol. Vis. Sci.,* 44, 2791-2797.

Lambert V, Munaut C, Noel A, Frankenne F, Bajou K, Gerard R, Carmeliet P, Defresne MP, Foidart JM and Rakic JM. (2001). *FASEB J.,* 15, 1021-1027.

Lijnen HR, Van Hoef B, Umans K and Collen D. (2004). *J. THromb. Haemost.,* 2, 16-22.

Liu G, Shuman MA and Cohen RL. (1995). *Int. J. Cancer,* 60, 501-506.

Masson V, Devy L, Grignet-Debrus C, Bernt S, Bajou K, Blacher S, Roland G, Chang Y, Fong T, Carmeliet P, Foidart JM and Noel A. (2002). *Biol. Proc. Online,* 4, 24-31.

McMahon GA, Petitclerc E, Stefansson S, Smith E, Wong MK, Westrick RJ, Ginsburg D, Brooks PC and Lawrence DA. (2001). *J. Biol. Chem.,* 276, 33964-33968.

Montesano R, Pepper MS, Mohle-Steinlein U, Risau W, Wagner EF and Orci L. (1990). *Cell,* 62, 435-445.

Pepper MS and Montesano R. (1990). *Cell Differ. Dev.,* 32, 319-327.

Rakic JM, Maillard C, Jost M, Bajou K, Masson V, Devy L, Lambert V, Foidart JM and Noel A. (2003). *Cell Mol. Life Sci.,* 60, 463-473.

Soff GA, Sanderowitz J, Gately S, Verrusio E, Weiss I, Brem S and Kwaan HC. (1995). *J. Clin. Invest.,* 96, 2593-2600.

Stefansson S, Petitclerc E, Wong MK, McMahon GA, Brooks PC and Lawrence DA. (2001). *J. Biol Chem.,* 276, 8135-8141.

Stephens RW, Brunner N, Janicke F and Schmitt M. (1998). *Breast Cancer Res. Treat.,* 52, 99-111.