Protease-Activated Receptor-1 (hPar1), A Survival Factor Eliciting Tumor Progression

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Abstract
Although ample evidence point to the central involvement of protease activated receptor-1 (PAR1) in tumor progression, little is known about the fate of the tumor when hPar1 is being silenced. We observed that hPar1 antisense clones exhibit low PAR1 levels, attenuated cell proliferation and invasion in vitro, and tumor formation in vivo. These clones showed noticeably reduced paxillin phosphorylation compared with the parental A375SM cells, whereas no change in the integrin levels was noticed. Antisense clones injected into the mice resulted in very few and only occasional small tumors, whereas advanced and vascularized tumors were observed in A375SM cells.

The antisense-derived tumor sections expressed active caspase-3, increased terminal deoxynucleotidyl transferase–mediated nick-end labeling staining, and a markedly reduced proliferating cell nuclear antigen level compared with A375SM cell–derived tissue sections. Likewise, ablation of the hPar1 gene in a tetracycline-inducible hPar1 system leads to apoptosis in immature blood vessels, whereas mature vessels were unaffected.

The activation of PAR1-induced pAkt/protein kinase B abrogated serum-deprived BimEL induction and also markedly reduced Bax levels. On the other hand, small interfering RNA silencing of the hPar1 gene induced the expression of BimEL, a direct substrate of Akt/protein kinase B and also induced expression of active caspase-9 and caspase-3. These results altogether identify PAR1 as a survival factor that protects cells from undergoing apoptosis. We conclude that whereas PAR1 gene expression correlates with tumor progression, its neutralization effectively initiates an apoptotic pathway leading at least in part to significantly reduced tumor formation. (Mol Cancer Res 2007;5(3):229–40)

Introduction
Protease-activated receptors (PAR) are seven transmembrane G-coupled receptors comprising four genes (PAR1-PAR4). Members of the family are uniquely activated by proteolytic cleavage of their extracellular portion exposing tethered ligands specific for each PAR (1, 2). PAR1, the prototypic receptor of the family, is activated via a spectrum of serine proteases taking part in the thrombotic/hemostasis cascade, including thrombin (3), plasmin (4), Xa (5), and activated protein C (6). Human Par1 (hPar1) plays a distinct role in the progression of malignant epithelia as well as in the physiologic invasion process of trophoblast implantation in the uterus decidua (7-10).

The overexpression of PAR1 is a striking feature of epithelia malignancy, whereas none or very little PAR1 expression is observed in normal epithelial cells, shown in a wide array of cell lines and tissue biopsy specimens (7, 8). Accumulating evidence suggests that prolonged survival machinery is mostly responsible for enhanced tumor invasion process.

Members of the phosphatidylinositol 3’ kinase/Akt signaling axis are central mediators of the prosurvival pathway. Phosphatidylinositol 3’ kinase, a lipid kinase, catalyzes and recruits inactive cytosolic molecules to the membrane where they undergo activation. One of these is Akt, a proto-oncogene originally discovered as the cellular homologue of the v-Akt oncogene (11). Upon stimulation, the inactive Akt undergoes subsequent phosphorylation by upstream kinases that lock it in an active conformation. Akt is then released into cellular compartments where it acts as a kinase and phosphorylates numerous substrates such as Forkhead transcription factors (FOXO), glycogen synthase kinase-3, Bcl-associated dimer, and murine double minute 2 (12). Hyperactivation of Akt tips the balance in favor of prosurvival pathways, whereas reduced activity of Akt tips the balance toward apoptosis. Apoptosis can be triggered in vivo by stress events like hypoxia and growth factor deprivation or by activation of the death receptor family (e.g., tumor necrosis factor–related apoptosis-inducing ligand, refs. 13, 14). These conditions lead to activation of caspases, a family of cysteine aspartyl proteases that are involved in a cascade of cleavage events that ultimately result in apoptosis. Thus, prosurvival modulation and the apoptotic machinery pathway are both key in forming the appropriate balance for normal cell maintenance. For example, factor VIIa/tissue factor, apart from its role in coagulation, also induces tumor progression partly via eliciting a survival pathway and inhibiting apoptosis (15, 16).

We wished to elucidate the prosurvival potential of the hPar1 gene in the context of tumor progression. For this, we generated stable clones expressing hPar1 antisense and compared their properties with the parental super-metastatic melanoma cell lines. In parallel, in a tetracycline hPar1-inducible system, ablation of the hPar1 gene led to a markedly reduced number of blood vessels, mediated primarily by
selective initiation of apoptosis in immature blood vessels, whereas the mature vessels remain unaffected. Reduction of hPar1 levels was achieved also by the use of an RNA interfering gene silencing approach. Together, these strategies enabled analyses of the consequences of PAR1 modulation in Matrigel invasion in vitro and tumor formation in mice in vivo. We examined proapoptotic and prosurvival mediators under conditions of modulated PAR1 levels (e.g., overexpressed versus neutralized hPar1 levels). Our data underscore the significance of the hPar1 gene in tumor metastasis and mark it as a prime candidate for anticancer therapy. We hereby propose that activation of PAR1 leads to prolonged survival processes in both endothelial cells and epithelia and hence contributes to tumor development.

Results

Stable hPar1 Antisense Clones Exhibit Attenuated Matrigel Invasion Properties and Impaired Paxillin Phosphorylation

We have previously suggested that the molecular mechanism underlying the involvement of PAR1 in tumor invasion and metastasis is mediated in part by increased adhesion properties, cytoskeletal reorganization, and accelerated focal adhesion complex formation (9). To evaluate the effect of PAR1 gene silencing on tumor progression, we established stable clones expressing hPar1 antisense orientation plasmid. These clones were characterized on the basis of their ability to silence PAR1 expression. As shown in Fig. 1A, two stable clones showed reduced hPar1 RNA and protein expression, as shown by Northern blots and fluorescence-activated cell sorting analyses, respectively (Fig. 1A and B). These clones were termed antisense clones AS-3 and AS-4 (Fig. 1A, lanes D and E, respectively) and were compared with parental or mock-transfected cells for mRNA and protein levels (Fig. 1A, lanes A and B, respectively, and Fig. 1B). Analyses of the growth rates of the parental A375SM melanoma cells and AS-3 (or AS-4; data not shown) under serum deprivation conditions showed ~50% inhibition by day 5 and 67% inhibition by day 9. These data indicate that silencing of the hPar1 gene leads to impaired proliferation of the antisense clones.

When established melanoma cell lines with known degrees of metastasis (9, 17) were used to determine the levels of PAR1 expression, a differential expression pattern was observed. High hPar1 RNA levels are seen in the aggressively super-metastatic A375SM cell line (Fig. 1D, lane D), whereas SB-2 nonmetastatic cells have very low levels (Fig. 1D, lane A). Similar differential expression was also found in protein levels (Fig. 1E), showing a direct correlation with the metastatic potential of these cell lines (e.g., low levels in SB-2 nonmetastatic cells; clone 5 and clone 28 of low and high metastatic potentials, respectively; and elevated expression in A375SM aggressive cells).

Next, we analyzed the level of paxillin phosphorylation following PAR1 activation. A marked induction in A375SM cells was seen after PAR1 activation (60 min, 10 nmol/L). In contrast, no phosphorylation was observed in low-PAR1 expressing, nonmetastatic SB-2 cells (Fig. 2A, lane D). In the hPar1 antisense clone AS-3, a significant reduction in paxillin phosphorylation was observed after PAR1 activation, relative to mock-transfected or parental A375SM cells (Fig. 2A, lanes A-C). Cell surface integrin expression was not altered.
in any of A375SM parental, mock-transfected, or AS-3 antisense clone (Fig. 2B). This indicated that merely silencing of hPar1 expression is sufficient to impair integrin-mediated signaling (as manifested by paxillin phosphorylation). In addition, when the antisense clones were tested for their ability to invade Matrigel-coated filters, we found that whereas A375SM cells aggressively invaded the filters (see Fig. 2C), only a few AS-3 cells (or AS-4 cells; data not shown) were capable of penetrating the Matrigel layer. The invasion was quantified under phase-contrast microscopy and shown by representative histograms (Fig. 2C).

**hPar1 Antisense Clones Display Low Tumorigenic Potential in Nude Mice**

To further substantiate the inhibitory role of hPar1 gene silencing in animal models *in vivo*, cells representing antisense clones were implanted subcutaneously into nude mice (*n* = 6 per group). As seen in Fig. 3A and B, xenografts established from AS-3 cells developed to a lesser extent than the parental metastatic A375SM cells. In contrast, when highly invasive A375SM cells were injected into mice, large tumors appeared in all the injected mice (Fig. 3A and C). The mean weight value of tumors was 0.17 g (compared with 0.011 g tumor weight derived from AS-3; Fig. 3A, C, and E), and the mean volume was 145 mm³ (compared with 15 mm³ for AS-3-derived tumors; Fig. 3F). Five-micrometer sections taken from either A375SM-derived tumors or hPar1 antisense clone (AS-3) were analyzed for the presence of blood vessels. Immunohistostaining using anti–von-Willebrand factor (anti-vWF) showed the presence of distinct blood vessels in the A375SM tumors, whereas very few were found in the hPar1 antisense slow-developing tumors (Fig. 4C; vWF). H&E staining of the tumor sections showed compact dense nuclei in the antisense tumor section compared with euchromatic and proliferating nuclei within the A375SM tumor section. It is observed that apoptosis is occurring in the intratumoral region as indicted by the morphology of the nuclei, localized in the inner part of the tumor section. The peri-tumoral region in contrast is of normal appearing cells (Fig. 4A). Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining shows abundant and high levels of apoptotic cells in the antisense section, whereas very little in the A375SM parental cells (Fig. 4B, *i* and *ii*). In

![FIGURE 2. Levels of paxillin phosphorylation, cell surface integrins, and Matrigel invasion properties. A. Paxillin phosphorylation. Top, the tyrosine phosphorylation (pY) levels of paxillin were evaluated in A375SM cells before (−) and after (+) PAR1 activation. Bottom, paxillin phosphorylation levels analyzed in SB-2 cells (lane D) and in highly metastatic A375SM cells (lane A), both after PAR1 activation. Detection was evaluated following immunoprecipitation with paxillin antibody and Western blot analysis of anti-phosphotyrosine mAb (4G10; Upstate Biotechnology). Reduced paxillin phosphorylation in hPar1 antisense clone was observed under similar conditions (lane C) compared with PAR1-activated A375SM cells (lane A) or PAR1-activated mock-transfected A375SM cells (lane B). B. Fluorescence-activated cell sorting analysis of cell surface integrins in parental A375SM versus antisense PAR1, AS-3 clone. Integrin expression levels were measured by flow cytometry in parental A375SM cells, mock-transfected cells (vector), and its antisense-derived clone AS-3, each activated with thrombin at a concentration of 1 unit/mL. The levels of α<sub>3</sub>, α<sub>5</sub>/α<sub>6</sub>, and α<sub>3</sub>/α<sub>6</sub> were detected by incubating the cells with the secondary isotype-specific mouse IgG. White peaks, expression levels of control secondary isotype-specific mouse IgG antibodies. C. Matrigel invasion of A375SM and AS-3. AS-3 stable hPar1 antisense clone exhibits markedly reduced Matrigel invasion properties. Although potent Matrigel invasion is observed in A375SM highly metastatic melanoma cells, only a few cells of AS-3 clone invaded the Matrigel-coated filters. Representative histogram summarizes the number of invading cells.](mcr.aacrjournals.org/doi-fig)
In a tetracycline-inducible hPar1 expression system of rat prostate cancer, we found an essentially similar outcome showing blood vessel–induced apoptosis after hPar1 gene withdrawal. We reported previously (18) that subcutaneous injection of AT2.1/Tet-On/hPar1 C14 clone into rats provided with drinking water containing doxycycline (to induce hPar1 gene levels) resulted in large and vascular tumors, whereas control rats injected with the same cells, but maintained without doxycycline (hPar1 is not expressed), developed very small tumors similar to the mock-transfected cells. In the present study, we show that in the large tumors that developed, blood vessel number is also high. For example, 58 blood vessels per field in the presence of hPar1 is observed compared with 18 blood vessels developing over the same time period under conditions where hPar1 gene was ablated (Fig. 5B). Next, experiments were carried out in which tumors were allowed to develop (doxycycline for 2 weeks), and then the hPar1 gene was withdrawn (elimination of doxycycline from the drinking water) for two additional weeks compared with tumor growth for 4 weeks (continuous addition of doxycycline). The following results were observed when the blood vessel network was analyzed in xenografts of each group. In the ablated hPar1 gene group, consistently fewer blood vessels were seen, specifically in the immature young sprouting vessels, whereas mature vessels were essentially unaffected. This finding was based on differential and selective staining of mature blood vessels with smooth muscle α-actin, but not of the immature vessels that contain only endothelial cells (Fig. 5A). We hypothesized that the reduced number of immature blood vessels is, in fact, the direct outcome of apoptosis induced by hPar1 withdrawal. Indeed, by focusing on one mature blood vessel per field, no effect following hPar1 shut down is observed. This, however, is not the case when immature blood vessels are the focus of the treatment. After withdrawal of the hPar1 gene, the topographical location of an immature blood vessel shows the appearance of “active caspase-3,” whereas there is none in the mature blood vessel (see Fig. 5A).
These data indicate that silencing of hPar1 initiates an apoptotic pathway in that may be also the direct outcome of blood vessel ablation primarily following hPar1 withdrawal. Ablation of hPar1 leads to a markedly reduced number of immature blood vessels and thus reduced delivery of nutrients to the center of a tumor and the subsequent formation of an apoptotic center.

Activation of PAR1 Induces Prosurvival Signaling

Next, we examined the possibility that PAR1 activation induces cell survival pathway. For this, we used a stable clone of nonmetastatic SB-2 melanoma transfected with the full-length hPar1 gene, C113 (9). Activation of PAR1 in C113, induced phosphorylation of Akt/protein kinase B (PKB; Fig. 6A), whereas no effect is observed in a control epithelia cell line of no PAR1 expression. Akt, also known as PKB, has emerged as a central regulator of widely divergent cellular processes. Disruption of the normal Akt/PKB signaling occurs frequently in several human cancers, and the enzyme seems to play an important role in cancer progression and cell survival. In parallel, when serum-starved 293-HEK cells were analyzed for the expression of the proapoptotic “BH3-only” protein (Bim-Bcl-2, interacting mediator of cell death) Bim<sub>HEL</sub>, higher levels were induced. Nevertheless, activation of PAR1 (either by thrombin or SFLLRN) protected cells from undergoing apoptosis as manifested by the abrupt down-regulation of Bim<sub>HEL</sub> expression back to basal levels (Fig. 6B). Next, when 293-HEK monolayers were infected with hPar1 small interfering RNA (siRNA) constructs to reduce hPar1 expression, a marked re-elevation in Bim<sub>HEL</sub> expression was noted. These results provide strong evidence that silencing of PAR1 effectively initiates apoptotic pathways, similar to the

**FIGURE 4.** Properties of paraffin-embedded tumor sections derived from A375SM and AS-3. A. H&E staining of tumor derived tissue sections. The stainingshowapoptoticcellsintheAS-3–derivedtumorsbutnotintheA375SM-derivedtumorsections. The apoptosis in mainly intratumoral regions and not peri-tumoral regions. B. Histologic analysis of apoptosis shown by TUNEL i, histologic sections of tumors dissected were stained with the Dead End Fluorometric TUNEL System reagent (green, left) and with propidium iodide (blue, right) for nuclear morphology. Magnification, ×400. ii, histogram representing apoptotic nuclei. C. Levels of blood vessels and apoptosis. Five-μm sections were used for immunostaining of tumors derived from A375SM and hPar1 antisense/AS-3 occasionally derived tumors. Either anti-vWF (vWF) or anti--active caspase-3 antibodies (active caspase-3) were used as primary antibodies and compared with staining with a secondary antibody only (control, bottom). Staining of blood vessels is observed in A375SM but not in AS-3 (middle). Active caspase-3 staining is seen in AS-3 but not in A375SM (top). The majority of the slides analyzed (90%) showed negative staining (<1) of active caspase-3 in A375SM tumor sections and positive for vWF (+3). In contrast, 90% of the AS-3–derived tumor sections scored positive staining (+3) for active caspase-3 and negative (<1) for vWF. cii, representative histogram showing levels of proliferating cell nuclear antigen (PCNA) in the nuclei of A375SM- and AS-3–derived tumor sections. D. Proliferating cell nuclear antigen staining of paraffin-embedded tissue sections. i, tumor sections derived from A375SM- and AS-3–derived tumors were examined for proliferating cell nuclear antigen level. ii, representative histogram demonstrating the proliferation index, defined as the number of proliferating cell nuclear antigen–positive nuclei per total number of nuclei. Columns, average proliferation index for three different representative tumor sections; bars, SD.
initiation observed following serum starvation. The presence of PAR1, as well as initiation of PAR1 signaling machinery, protected cells from undergoing apoptosis under conditions of serum starvation.

Caspases play a central role in apoptosis. These proteins are synthesized as latent zymogens organized in a cascade system that, upon activation, stimulates apoptosis. Indeed, inhibition of apoptosis is accomplished either by inhibiting the activity of caspase(s) or by preventing the conversion of the zymogen to the active form. To obtain clues to the mechanism by which hPar1 inhibits apoptosis, we investigated the activation of a major apoptotic effector, caspase-3. This was carried out by Western blot analysis using an antibody recognizing both the zymogen and the active enzyme. We showed that serum removal induced time-dependent appearance of an 18- to 20-kDa caspase-3 protein band (Fig. 6C), representing the activated caspase-3 levels. Increased levels of active caspase-3 was evident 12 h after serum removal and reached maximum levels at 24 h. In a similar manner, active caspase-9 appears after 12 h of serum deprivation and markedly increased by 24 h (Fig. 6C). In the presence of 10 nmol/L thrombin or SFLLRN, however, clear attenuation of either active caspase-9 or "active caspase-3" levels was observed. This indicates that activation of PAR1 is sufficient to induce a prosurvival signaling pathway that inhibits apoptosis via down-regulation of "active caspase-9" as also "active caspase-3" levels downstream. We have analyzed levels of another apoptotic protein, Bax. While under serum deprivation (6-24 h), Bax is highly expressed; a decrease in Bax levels is observed after PAR1 activation (12 h of SFLLRN). The Bax levels remain significantly reduced when analyzed after 24 h. Representative histograms show quantification of the marked decrease in Bax after PAR1 activation compared with the steady unchanged levels of Bcl-2 and β-actin (Fig. 6D).

**FIGURE 5.** Ablation of hPar1 gene expression in a Tet-on hPar1-inducible rat prostate carcinoma AT2.1 leads to apoptosis of immature blood vessels. A. AT2.1/Tet-On/hPar1 CI4 cells injected subcutaneously allowing tumors to develop either for a period of 4 wks [doxycycline (Dox) in the drinking water] or for 2 wks doxycycline and withdrawal of doxycycline for an additional 2 wks (i; ref. 18). Tumor sections (5 μm) were processed and analyzed for levels of angiogenesis. Although total blood vessel network is detected following vWF (DAKO), the staining with smooth muscle (SM) α-actin detects mature blood vessels (middle). Positive staining of active caspase-3, however, is seen only in immature blood vessels, but not in a mature smooth muscle α-actin–positive vessel. Representative histograms showing the statistics of blood vessels in the different sections (ii). B. Increased blood vessels are seen in sections grown in the presence of the hPar1 gene (presence of doxycycline; CI4 + Dox) compared with the section exhibiting no hPar1 expression (CI4). Right, histogram represents 58 vessels per slide in the presence of hPar1 (CI4 + Dox) compared with 18 vessels in the slide without hPar1 expression (CI4).
**Discussion**

Tumor growth and invasion are largely regulated by the imbalance between prosurvival and proapoptotic pathways. In fact, initiation of the oncogenic process and the early stages of metastasis are largely the direct outcome of aberrant regulation of the apoptotic pathway. Here, we present data that connect hPar1 gene expression and signaling, enhanced survival properties, and inhibition of apoptosis in the context of tumor progression.

The mechanisms by which growth factor receptor overexpression induces tumor cell invasion are not fully understood. One important early event implicated in a molecular switch for growth factor-induced cell migration is the activation of integrins via central activation of the non-receptor kinase focal adhesion kinase (FAK). FAK is a major protein of the focal adhesion complex, playing a key role in cell migration and matrix survival signals (19, 20). FAK is activated by numerous growth factors, including ErbB ligands. Epidermal growth factor (21, 22) and herregulin (23), followed by integrin clustering in response to components present in the extracellular cell matrix.

The interaction between FAK and the adaptor protein paxillin is critical for the activation of signaling cascades involved in the control of cell survival and motility (24). FAK binds paxillin through its COOH-terminal focal adhesion targeting region (25). This focal adhesion targeting region is also contained within a naturally occurring fragment of FAK (26), termed FAK-related non-kinase. Indeed, transfection with FAK-related non-kinase blocks the interaction of endogenous FAK with paxillin at focal adhesions, decreasing survival and motility (26, 27). The expression of paxillin is elevated, like that of FAK, in a collection of tumors and in a wide spectrum of highly metastatic cell lines. There is a direct correlation between paxillin phosphorylation and degree of metastasis. Knocking down of paxillin by siRNA resulted in effective attenuated motility of highly metastatic cell lines. Similarly, paxillin was identified as a molecule of outstanding differential phosphorylation state between low- and high-metastatic cell lines. It is thus a good candidate for the design of cancer therapy drugs, among several phosphotyrosine-containing proteins that are differentially phosphorylated in the highly metastatic cell lines, but nearly not in low-metastatic lines. Although paxillin has no intrinsic enzymatic activity, it has multiple domains that interact with cytoskeletal and signaling molecules, and it functions as a scaffold protein at focal adhesions (24, 28). We characterized two stable hPar1 antisense clones and showed that whereas PAR1 levels are considerably neutralized, it caused also a markedly decreased levels of paxillin phosphorylation. This is in contrast to the high and elevated levels of paxillin phosphorylation found in overexpressing hPar1 clones (9).

We present data showing that tumor apoptosis initiated by hPar1 silencing takes place on two levels. The first is via the increased expression of several proapoptotic proteins, such as Bim and Bax, as also active caspase-3 and caspase-9. The second is the down-regulation of PAR1-dependent vascular endothelial growth factor expression, leading to regression of immature blood vessels (see Fig. 5). This is shown by the use of a tetracycline-inducible system of hPar1 gene expression, whereby withdrawal of the hPar1-inducing agent (e.g., doxycycline) from the drinking water of mice led to apoptosis. The apoptotic machinery is observed primarily in immature vessels as indicated by the appearance of active caspase-3, located at a site where prior young sprouting blood vessel was present as judged by the combined staining of vWF and smooth muscle actin (for mature vessel).

Antiapoptotic or survival pathways are portrayed via the activation/phosphorylation of Akt/PKB, inhibition of the proapoptotic protein BH3-only protein BimEL and expression of “active caspase-3.” The Bim gene is induced at the transcriptional level, in part, in response to withdrawal of survival factors and inactivation of Akt/PKB (29). Although multiple Bim mRNA transcripts have been described (thought to be generated by alternative splicing; refs. 30-32), most tissues express one predominant isoform of Bim (i.e., BimEL). Under conditions that promote cell growth, Bim is bound to the dynein light chain of the microtubular motor complex and is sequestered away from other Bcl-2 family members (33). Following proapoptotic stimulus, however, Bim localizes to the mitochondria, where it initiates a cell death pathway either by directly activating Bax-like proteins (34, 35) or by binding to prosurvival Bcl-2 family members and releasing of Bax-like proteins (36, 37).

We observed that in hPar1-overexpressing cells, activation by either thrombin or the specific activating peptide SFLLRNp leads to enhanced cell survival, induced paxillin phosphorylation, elevated invasion in vitro through Matrigel, and formation of advanced tumor formation in mice in vivo. We showed that in a stable clone overexpressing the hPar1 gene, the addition of either thrombin or SFLLRNp markedly inhibits both “active caspase-9” and “active caspase-3” levels as also BimEL expression, induced by serum deprivation. Moreover, siRNA silencing of hPar1 is sufficient to restore high levels of BimEL, indicative of initiation of an apoptotic process. The high levels of BimEL induced by serum starvation were down-regulated after PAR1 activation (Fig. 5B). In addition, we show that SFLLRNp treatment markedly inhibited the levels of Bax compared with equal levels of Bcl-2 and actin. Therefore, when PAR1 is present and activated, it may provide important survival signals at least during the first stages of tumor progression. The in vitro findings are complemented by data from tumors in vivo, which show enhanced expression of “active caspase-3,” a well-known molecular marker of apoptosis, in tumor tissue sections derived from hPar1 antisense clone (AS-3 or AS-4) injected into mice. In contrast, no expression is observed in the large and advanced tumors that appear after inoculation of the parental metastatic A375SM melanoma cells. In parallel, in a tetracycline-inducible hPar1 system, ablation of hPar1 leads to selective apoptosis in immature blood vessels, indicated by “active caspase-3” expression, whereas no staining is observed in mature, pericyte-coated vessels. Overall, the withdrawal of hPar1 initiates apoptosis in immature blood vessels and subsequently in tumor epithelia. Similarly, it has been recently shown that blockade of PAR1 reduced the incorporation of 3H-thymidine in endothelial cells and increased PARP cleavage, indicative of the apoptotic pathway (38).
More recent studies have shown that the apoptotic function of Bim is regulated by phosphorylation followed by ubiquitination and degradation (39). It was shown by the use of recombinant Akt and in vitro kinase assays that BimEL is phosphorylated at Ser^87. Thus, Akt attenuates the proapoptotic pathway by phosphorylating Bim Ser^87 (40). Extracellular signal-regulated kinase also phosphorylates Bim on multiple sites via a docking domain that contains the amino acid

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Serum withdrawal leads to elevated levels of active -caspases 3 and 9 in C113

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<td>+</td>
</tr>
<tr>
<td>sRNA:</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BimEL</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>GAPDH</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

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**Figure Legends**

A. Time course of thrombin-induced apoptosis in C113 and Parental cells.

B. Western blot analysis of BimEL and GAPDH levels in C113 cells following serum deprivation and SFLRN treatment.

C. Serum withdrawal leads to elevated levels of active -caspases 3 and 9 in C113.

D. Western blot analysis of Bax and Bcl2 levels in C113 cells following SFLRN treatment.
sequence FSF, identified as DEF (docking site for extracellular signal-regulated kinase, FXFP; ref. 41). When acting as an enzyme, Akt also phosphorylates forkhead transcription factors, causing them to be retained in the cytoplasm (42). Inhibition of Akt activity (by LY294002) allows forkhead proteins to translocate into the nucleus, where they are known to be direct activators of Bim gene expression (43–45). Therefore, Akt activation can down-regulate Bim transcription via phosphorylation of forkhead proteins. The direct phosphorylation and sequestration of BimEL by Akt (40) would seem to complement the long-term effect of Akt on Bim transcription by immediately neutralizing preexisting Bim within a cell.

Although PAR1 is known to activate the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway, the inactivation of Bim may take place either via extracellular signal-regulated kinase or via the Akt/PKB pathway. The Akt pathway has emerged as a major mechanism by which a long list of growth factors and oncogenes elicit cell survival (46). We showed that PAR1 activation induces Akt phosphorylation and initiation of cellular Akt/PKB signaling. Although we did not determine which of the Akt family members is induced by PAR1, it is most likely that Akt-1 is the major Akt member involved in tumor progression and angiogenesis (47). Evidence of the importance of Akt signaling in cancer comes from studies in which the overexpression and hyperactivation of Akt in a wide range of human tumors have been detected, often linked also to poor prognosis (48). However, two recent studies published independently by separate groups yielded unexpected findings showing that activated Akt1 in fact inhibits migration and Matrigel invasion (49, 50). Indeed, these data fit with studies by Bissel et al. showing that hyperactivation of Akt 1 can prevent the invasive behavior underlying breast metastasis. It is suggested that Akt phosphorylation in breast cancer cells induces phosphorylation of the tumor suppressor tuberous sclerosis complex 2, leading to reduced focal adhesions, migration motility, and invasion (51). This is in agreement with the analyses of the double-transgenic ErbB2/PKB mouse model for tumor metastasis in which metastatic lesions in lung are found only in the ErbB2 transgenes. Only primary tumors, with the distinct absence of metastatic lesions, are formed in the double-transgenic mice that also overexpress the activated Akt/PKB allele (52). These accumulating data point to the central involvement of PAR1-mediated Akt/PKB pathway activation at least in the initial phases of prosurvival tumor formation but do not provide sufficient explanation for the role of hPar1 in tumor metastasis (53). It is most likely that whereas Akt/PKB plays a significant role in the initial phases of PAR1-induced tumor formation, other pro-invasive mediators, not yet fully determined, are activated by PAR1.

### Materials and Methods

#### Cells
SB-2 human noninvasive melanoma line and A375-SM “super-metastatic” human melanoma cells (kindly provided by M. Bar-Eli, Department of Cell Biology, University of Texas, M.D. Anderson Cancer Center, Houston, TX) were grown in 10% FCS/DMEM supplemented with penicillin and streptomycin (50 units/mL; Life Technologies, Gaithersburg, MD) and maintained in a 37°C humidified incubator with 8% CO2. C113, the hPar1- stable transfectant, was maintained as previously described (9). AT2.1/Tet-On (generously provided by Dr. Hua-Quan Miao, Imclone Systems, Inc., New York, NY) were transfected with 2 μg DNA of either pAHygTet1-hPar1 or pAHygTet1 using Fugene 6 transfection reagent (Roche, Mannheim, Germany), as previously described (10). HEK-293T cells were maintained in 10% FCS/DMEM.

### Growth Curve
Analyses of the parental A375SM and antisense clones was carried out in the following manner. Cells (5 × 103) were plated in a 35-mm dish. One day after plating, the medium (10% FCS/DMEM) was replaced to 0.5% FCS in DMEM for the entire period analyzed.

#### Transfection of Cells
Cells grown to 60% to 70% confluency were transfected with 1 μg of plasmid DNA, using Fugene 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. Stable transfected clones were established after 10 days of selection with 400 μg/mL G418-containing medium. Antibiotic-resistant cell colonies were transferred to separate culture dishes and were grown in 200 μg/mL G418 medium.

### Western Blotting Analysis
Cells were solubilized in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, and protease inhibitor cocktail (Sigma, St. Louis,
MO) including 5 µg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L Na orthovanadate, for 30 min at 4°C. After centrifugation at 12,000 × g for 20 min at 4°C, the supernatants were transferred, and the protein content was measured. Lysates (50 µg) were loaded on a 10% SDS-PAGE followed by transfer to Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked and probed with the appropriate antibodies at a concentration of 1 µg/mL. Anti-PAR1 thrombin receptor monoclonal antibody Clone II aR-A (Biodesign International, Saco, ME), anti-paxillin monoclonal antibody Clone 4G10 (Upstate Biotechnology, Lake Placid, NY) and anti-phosphotyrosine monoclonal antibody Clone 4G10 (Upstate Biotechnology, Lake Placid, NY) were suspended in 3% bovine serum albumin in 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 0.5% Tween 20. After washes, blots were incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence reagent (Pierce, Rockford, IL).

Matrigel Invasion Assay

Blind well chemotaxis chambers with 13-mm-diameter filters were used for this assay. Polyvinylpyrrolidone-free polycarbonate filters, 8-mm pore size (Costar Scientific Co., Cambridge, MA), were coated with basement membrane Matrigel (25 µg per filter) as previously described (11). Briefly, the Matrigel was diluted to the desired final concentration with cold distilled water, applied to the filters, and dried under a hood. Matrigel was diluted to the desired final concentration with polyvinylpyrrolidone-free polycarbonate filters, 8-mm pore size (Costar Scientific Co., Cambridge, MA). The filters were used for this assay. Polyvinylpyrrolidone-free polycarbonate filters, 8-mm pore size (Costar Scientific Co., Cambridge, MA), were coated with basement membrane Matrigel (25 µg per filter) as previously described (11).

PAR1 Activation

Either thrombin (Jones Pharma, Inc., St. Louis, MO; 1 unit/µL) was used at concentration of 10 mmol/L or SFLLRN (BioSight Ltd., Karmiel, Israel) at 100 µmol/L.

Tumorigenicity Studies

Cells from exponential cultures of control and antisense-transfected hPar1 clone 3 were detached with trypsin, washed with PBS, and brought to a concentration of 2 × 10^7 per mL. Cell suspension (2 × 10^6 per 0.2 mL) was inoculated subcutaneously into the right flank of 5-week-old athymic nude mice CD1-W (Harlan, Jerusalem, Israel). Xenograft size was determined weekly by externally measuring tumors in two dimensions using a caliper. Tumor volume was determined by the equation $V = L \times W^2 \times 0.5$, where $L$ is the length, and $W$ is the width of xenografts. At the end of the experiments, mice were sacrificed by cervical dislocation, and xenografts were resected, weighed, and fixed in formalin and embedded in paraffin. The sections were stained with H&E or immunostained with either anti-vWF or anti–active caspase-3 antibodies, using the staining kit according to the manufacturer’s instructions (Zymed, South San Francisco, CA).

Immunohistochemistry

Sections (5 µm) from tumors were deparaffinized and rehydrated. Tissues were then denatured for 4 min in a microwave oven [in a citrate buffer, 0.01 mol/L (pH 6)]. The sections were incubated overnight at 4°C with either an antibody to vWF (DAKO, Glostrup, Denmark), anti–active caspase-3 (Cell Signaling, Beverly, MA, USA), anti–active caspase-9 (polyclonal rabbit; Cell Signaling Technology, Inc., Danvers, MA), anti–smooth muscle α-actin (Sigma-Aldrich, St. Louis, MO) at 1 µg/mL or with an irrelevant antibody or only a secondary antibody as a control (anti-Bax and anti-Bcl-2; BD Biosciences PharMingen, San Diego, CA). Color was developed using a Zymed kit according to the manufacturer’s instructions (Zymed) and counterstained with Mayer’s hematoxylin. The data are representative of at least five independent experiments carried out per group of mice. The experiments were done on serial sections.

Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling Assay

For apoptosis analysis, 5-µm-thick sections of tumor tissue were processed and stained with the Dead End Fluorometric TUNEL System (Promega Corp., Madison, WI) according to the manufacturer’s protocol and counterstained with propidium iodide. Apoptotic cells were counted using confocal microscope (magnification, ×400). A total of 10 fields per tumor was analyzed, and mean ± SD was determined. These results were subjected to statistical Student’s t test analysis.

siRNA Constructs and Lentiviral Vector Production

We used U6 promoter–driven and lentivirus (pLentilox 3.7)–mediated delivery cassette of siRNA, specific for hPar1. For this, a sequence of 19 nucleotides of the hPar1 coding region was selected for stem-and-loop oligonucleotide siRNA. The selected sequences were submitted to a BLAST search against the human genome to ensure that it was not targeted. Appropriate DNA oligonucleotides were synthesized to construct the hairpin, stem-and-loop siRNA expression cassette. The oligos comprised the following: 19 bases of hPar1 coding sequence, the loop sequence linker (9 bases), reverse complement of the 19 bases of hPar1 coding region, and a terminator sequence poly T. The sticky end of the XhoI site was added to the antisense strand oligos. Both sense and antisense sequences were phosphorylated at the 5’ ends. The sense sequence oligos were annealed to their respective antisense oligos. siRNA cassette sequences were then ligated into pLentilox 3.7 vector (Van Parij’s Laboratory). We created five such siRNA cassettes that target the hPar1 gene. The sequences of hPar1 targeted by siRNA were as follows: 5′-GGCTGAGCAGCACTTTTGGCC-3′ (542), 5′-GAAGCGCGCTGGTGTTGAGCAGCACTTTTGGCC-3′ (645), 5′-GCAGGCGTGTTTCTGGC-3′ (833),
S′-GAACCCCGTCGCGAAGGCTACTA-3′ (1003), S′-GCTCG-AAGGCTACTATGGC-3′ (1029). The lentivirus particles were generated by a three-plasmid expression system, in which 293T cells were cotransfected with the following three vectors: packaging (CMVΔ R8.91), envelope (CMV-VSV-G), and the transfer vector P lentilox 3.7. 293T cells were plated to 60% confluence 1 day before transfection. The medium was changed the next day with fresh medium, and cells were transfected with the three plasmids using Fugene 6 transfection reagent. Medium was changed to fresh medium 24 h after transfection. On days 2 and 3 after transfection, medium was collected, and the viral particles were concentrated 100-fold by centrifuging the medium for 1 h at 40,000 rpm.

References
Protease-Activated Receptor-1 (*hPar1*), A Survival Factor Eliciting Tumor Progression

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