Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

Research Article

Thrombin Induces Tumor Cell Cycle Activation and Spontaneous Growth by Down-regulation of $p27^{Kip1}$, in Association with the Up-regulation of Skp2 and MiR-222

Liang Hu,[1] Sherif Ibrahim,[2] Cynthia Liu,[2] Jeffrey Skaar,[2] Michele Pagano,[2] and Simon Karpatkin[1]

Departments of [1]Medicine and [2]Pathology, New York University School of Medicine, New York, New York

Abstract

The effect of thrombin on tumor cell cycle activation and spontaneous growth was examined in synchronized serum-starved tumor cell lines and a model of spontaneous prostate cancer development in TRAMP mice. BrdUrd incorporation and propidium iodide staining of prostate LNCaP cells arrested in G_0 and treated with thrombin or serum revealed a 48- and 29-fold increase in S phase cells, respectively, at 8 hours. Similar results were obtained with TRAMP cells and a glioblastoma cell line, T98G. Cell cycle kinases and inhibitors in synchronized tumor cells revealed high levels of $p27^{Kip1}$ and low levels of Skp2 and cyclins D1 and A. Addition of thrombin, TFLLRN, or serum down-regulated $p27^{Kip1}$ with concomitant induction of Skp2, Cyclin D1, and Cyclin A with similar kinetics. LNCaP $p27^{Kip1}$-transfected cells or Skp2 knockdown cells were refractory to thrombin-induced cell cycle activation. MicroRNA 222, an inhibitor of $p27^{Kip1}$, was robustly up-regulated by thrombin. The *in vitro* observations were tested *in vivo* with transgenic TRAMP mice. Repetitive thrombin injection enhanced prostate tumor volume 6- to 8-fold ($P < 0.04$). Repetitive hirudin, a specific potent antithrombin, decreased tumor volume 13- to 24-fold ($P < 0.04$). Thus, thrombin stimulates tumor cell growth *in vivo* by down-regulation of $p27^{Kip1}$. [Cancer Res 2009;69(8):3374–81]

Introduction

Experimental data from numerous reports (1–19) suggest that thrombin contributes to a more malignant phenotype *in vivo* by activating tumor-platelet adhesion, tumor adhesion to subendothelial matrix, tumor implantation, tumor growth, experimental pulmonary metastasis, and tumor-associated angiogenesis. However, there is no direct proof that thrombin enhances primary tumor growth *in vivo* because this is dependent on tumor implantation, angiogenesis, and metastasis. Neither is there any *in vitro* data on the effect of thrombin on tumor cell lines cultivated in the absence of serum, a potent growth factor.

In addition, the animal data generated from models using serum-cultured transformed tumor cell lines (with unknown chromosomal aberrations) treated with exogenous thrombin before injection do not reflect a true pathophysiologic representation. They ignore endogenous thrombin production/concentration at the tumor-host interface, and it is likely that the concentrations used are at unphysiologic levels and the exposure transient. We therefore elected to study the effect of serum-free thrombin on growth of synchronized tumor cells *in vitro* as well as the effect of thrombin on spontaneous tumor growth *in vivo*, using a transgenic TRAMP mouse programmed to develop prostate cancer, a more relevant model.

The inhibitory cell cycle regulator $p27^{Kip1}$ is an inhibitor of the G_1 phase of the cell cycle. It is a negative regulator of protein kinases cyclin-dependent kinase (cdk) 2/cyclin E and cdk2/cyclin A, which drive cells into the S phase. It is an established tumor cell marker of poor prognosis. $p27^{Kip21}$ is decreased or absent with high tumor grade and poor prognosis with several human tumors (20–28). Its regulation is often at a posttranscriptional level (21). The degradation of $p27^{Kip1}$ is carried out by the ubiquitin system (29) that delivers proteins for proteosome degradation. $p27^{Kip21}$ is degraded by a Skp1-Cyclin-F box protein ubiquitin ligase complex that contains Skp2 as the target recognizing subunit (30–32). Skp2 plays an important role in promoting entry into S phase by inducing $p27^{Kip1}$ degradation (30–32). Skp2 is commonly overexpressed in numerous human cancers and is an independent prognostic marker (33).

MicroRNAs (MiRNA) are small noncoding RNAs that control translation through binding to the 3′-untranslated region of protein coding genes. They inhibit or destabilize translation. Recent data reveal that miRNAs might affect both tumor suppressor and oncogenic potential in human cancer (34–43). It has recently been reported that miRNAs MiR-221 and MiR-222 enhance the proliferation of human prostate LNCaP cells (41) as well as glioblastoma, MDAMB-231, and U87 cell lines by down-regulation of $p27^{Kip1}$ (20). We therefore chose to investigate the effect of thrombin on $p27^{Kip1}$ as well as MiR-221 and MiR-222 in LNCaP cells.

We studied the effect of thrombin on activation of the cell cycle, using starved synchronized tumor cells in the absence of serum. We measured the up-regulation and down-regulation of several cell cycle regulators and focused on $p27^{Kip1}$. Here, we describe the down-regulation of $p27^{Kip1}$ by thrombin through up-regulation of Skp2 and miRNA MiR-222, and show this requirement for thrombin-induced cell cycle activation.

Transgenic TRAMP mice were used to determine whether thrombin enhances the spontaneous development of prostate cancer. TRAMP mice express the SV40 Large T Antigen under the control of the prostate-specific probasin promoter, leading to the spontaneous development of prostate cancer and metastasis over a period of 70 to 200 days (44). The SV40 Tag acts as an oncoprotein through interaction with p53 and Rb tumor suppressor gene products (45–47), which have been implicated in the development

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Oral Presentation at the Annual Meeting of the American Society of Hematology, San Francisco, December, 2008.

Requests for reprints: Simon Karpatkin, New York University Medical Center, 550 First Avenue, New York, NY 10016. Phone: 212-263-5609; Fax: 212-263-0695; E-mail: Simon.Karpatkin@med.nyu.edu.

doi:10.1158/0008-5472.CAN-08-4290

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

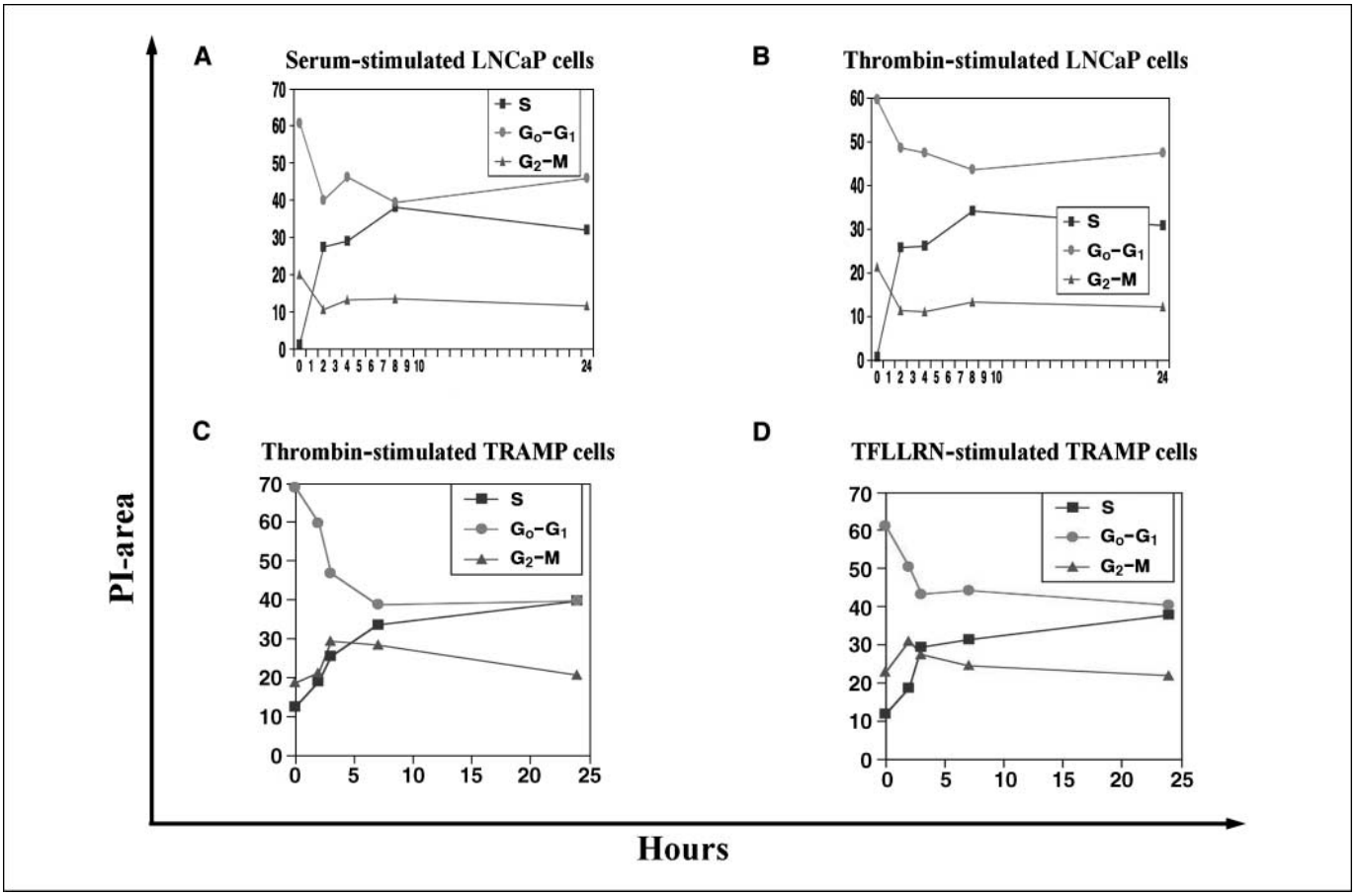

Figure 1. Effect of thrombin and serum on tumor cell cycle kinetics in LNCaP prostate and TRAMP tumor cell lines. Androgen-dependent LNCaP prostate (*A* and *B,* representative of eight experiments) and TRAMP (*C* and *D,* representative of four experiments) were assayed for BrdUrd incorporation and propidium iodide staining. Cells were serum starved for 72 h before repletion with serum or 0.5 unit/mL thrombin. *PI-Area,* propidium iodide staining of DNA during various phases of the cell cycle. BrdUrd-FITC is a measure of the kinetics of DNA synthesis of the various phases. Note increase in S and decrease in G_0-G_1 after serum or thrombin addition.

of prostate carcinoma (47, 48). These tumors are androgen dependent and regulated with sexual maturity. We monitored the effect of thrombin on *in vivo* tumor development via two methods. First, we chronically injected the mice with thrombin, i.p. Second, we investigated the loss of endogenous thrombin by chronic injection of hirudin, a highly specific, potent thrombin inhibitor. Here, we describe markedly increased spontaneous prostate cancer growth with thrombin treatment (in the absence of enhanced tumor angiogenesis) and decreased tumor growth with hirudin treatment, indicating that thrombin contributes to *in vivo* tumor growth.

Materials and Methods

Reagents. All reagents (including human thrombin and androgen-depleted serum) were purchased from Sigma. unless otherwise noted. Hirudin (Refludan) was purchased from Hoechst Marion Roussel. Culture media DMEM was obtained from Mediatech. All vascular growth factor and receptor antibodies (anti–vascular endothelial growth factor, KDR, ANG-2, Tie-2, GRO-1, and CD31) were obtained from Santa Cruz Biotechnology. Anti–cyclin D1 was obtained from Chemicon. Anti-Skp2 were purchased from Zymed Laboratories. Cyclin A is a previously described rabbit polyclonal antibody (C). BrdUrd was obtained from Amersham. Anti–BrdUrd-FITC, MoAb, was purchased from Pharmingen.

Cell lines and culture conditions. The human LNCaP prostate cancer, TRAMP C1, and T98 glioblastoma cells were purchased from American Type Culture Collection.

Mice. Transgenic C57BL/6 female TRAMP mice [heterozygous for the probasin-Tag (SV40) transgene] were a gift from Dr. D. Levy at the NYU School of Medicine, New York, NY. TRAMP mice were crossed with either C57BL/6 or FVB nontransgenic males. These mice have prostatic intraepithelial neoplasia by 12 wk, with tumor arising at 24 wk in the dorsal and lateral lobes of the prostate, appearing as well-differentiated adenocarcinomas. Metastasis to lymph nodes and lung is usually noted at 30 wk. FVB/TRAMP mice give larger tumors, with primary pathology at 12 wk, mostly high-grade. Some well-differentiated prostate cancer metastasis to lymph nodes and lung is generally seen earlier, at 18 wk. Mice were genotyped by PCR using the primers 5′AGGTCTTGAAAGGAGTGCCTGG-3′ and 5′GAGTCAGTAGCCTCATCAC-3′ to give a 654 bp fragment. Mice were injected i.p. at 6 wk with either thrombin (25 units/kg) or hirudin (10 mg/kg) for 10 d daily followed by every other day until sacrifice.

Knock-in and knockdown experiments with $p27^{Kip1}$ and Skp-2. The $p27^{Kip1}$ knock in plasmid was a gift from Dr. M. Pagano's laboratory, NYU School of Medicine, New York, NY. The $p27^{Kip1}$ cDNA encoding protein was subcloned into the EcoR1 site of expression vector pcDNA3 (Invitrogen Life Technologies). See Supplementary Figure S1 for data.

Skp2 shRNA was introduced into the shRNA-RetroQ retrovirus (BD Biosciences; Clontech) at the BamHI and EcoRI ligation sites according to the manufacturer's directions, as we have described (49). shRNA

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

Oligonucleotides were derived from the murine Skp2 sequence and synthesized after derivation from the computer program supplied by BD Biosciences.

Forward strand sequence 5′-gatccGGGAGTGACAAAGACTTTGttcaagagaCAAAGTCTTTGTCACTCCCtttttt-3′.
Reverse strand sequence 5′-aattcaaaaaaGGGAGTGACAAAGACTTTGtctcttgaaCAAAGTCTTTGTCACTCCCg-3′.

Scrambled shRNA oligo pairs.

Forward strand sequence 5′-gatccGGCGTAACATGAGGATAGTttcaagagaACTATCCTCATGTTACGCC-ttttttg-3′.
Reverse strand sequence 5′-aattcaaaaaaGGCGTAACATGAGGATAGTtctcttgaaACTATCCTCATGTTACGCCg-3′.

Immunoblots. Immunoblotting was performed as previously described (9). Briefly, cells were starved for 72 h in 0.1% bovine serum albumin before repletion with serum or treatment with thrombin. Cells were then lysed in RIPA buffer (20 mmol/L Tris-HCI, 150 mmol/L NaCI, 1% Triton X-100, 5 mmol/L EDTA, 1% deoxycholate, 0.5% SDS, 1 mmol/L Na_3VO_4, plus 1 μg/mL Aprotin, 1 μg/mL leupeptin, 50 μg/mL phenylmethylsulfonyl fluoride, and 1% NP40) and 25 μg of extract in 25 μL applied to SDS-PAGE. The immunoprecipitate was washed and boiled in SDS sample buffer. After SDS-PAGE and transfer to nitrocellulose membranes, Western blotting was performed with the appropriate antibody, secondary antibody, and enhanced ECL reagents (Supersignal/West Pico; Pierce).

Immunohistochemistry. Ten percent formalin-fixed tumor was embedded in paraffin and stained for von Willebrand factor (50) or Ki67 as previously described (1, 8). Angiogenesis was evaluated in a blinded fashion by two different observers.

Tumor pathology. Mean tumor area (mm^2) was obtained by averaging the areas from 10 of 100 serial slices of tumor (every 10th slice).

Cell cycle analysis. Cells (1×10^6) were serum starved for 72 h in 0.1% fetal bovine serum in DMEM media. Thrombin (0.5 unit/mL) was added for varying time intervals of 0 to 24 h. Cells were then centrifuged, stained with

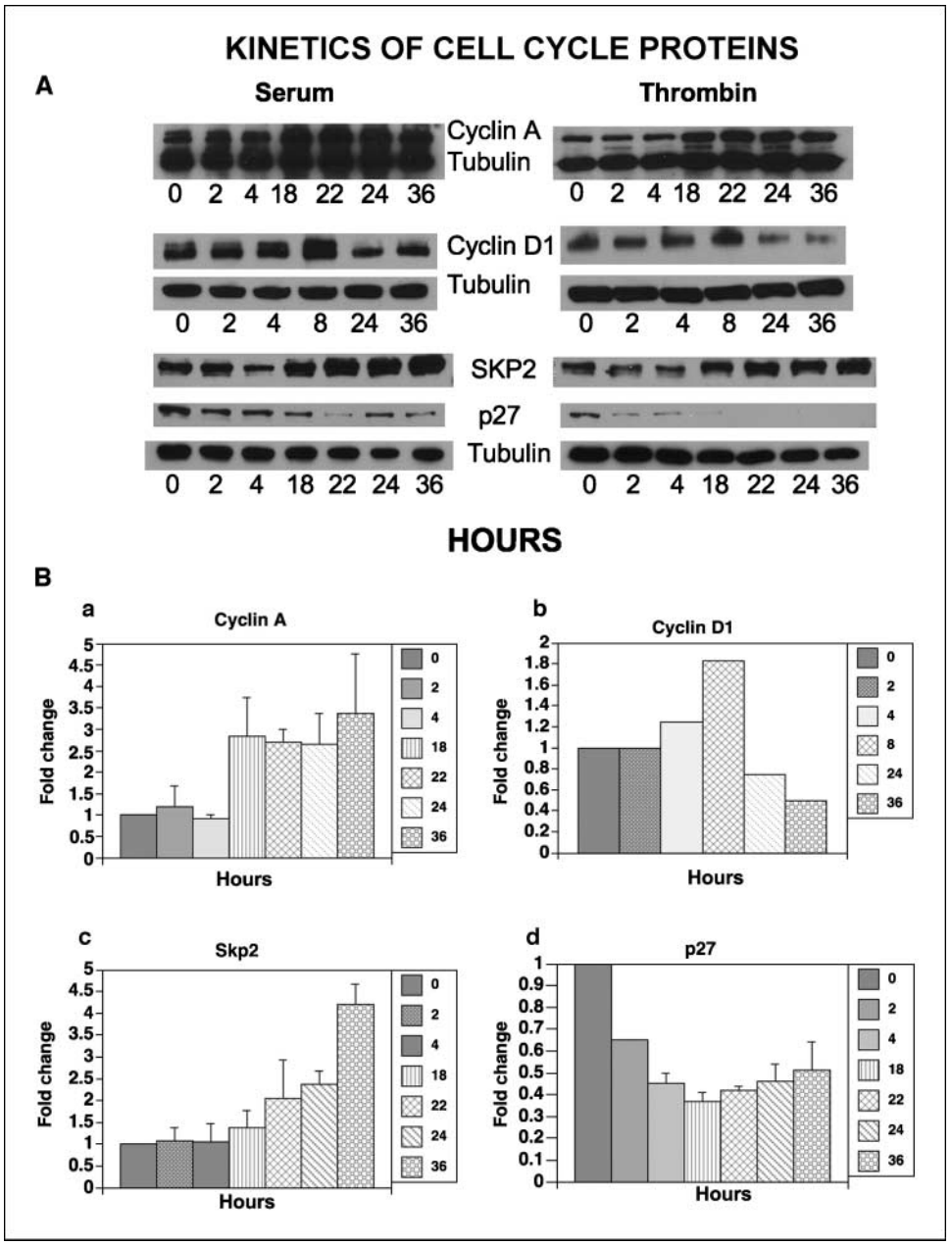

Figure 2. Protein analysis of cell cycle kinases and inhibitors (cyclin A, cyclin D1, skp2, and $p27^{Kip1}$). *A,* immunoblot of effect of serum and thrombin (0.5 μL/mL). Note transition of high to low $p27^{Kip1}$ in association with increase in cyclin A, D1, and Skp2 after serum repletion or thrombin addition in glioblastoma cells. Representative experiment. *B,* bar graphs of relative density measurements (protein/internal control) are given for 1 to 3 experiments at 0 to 36 h. SE is given.

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

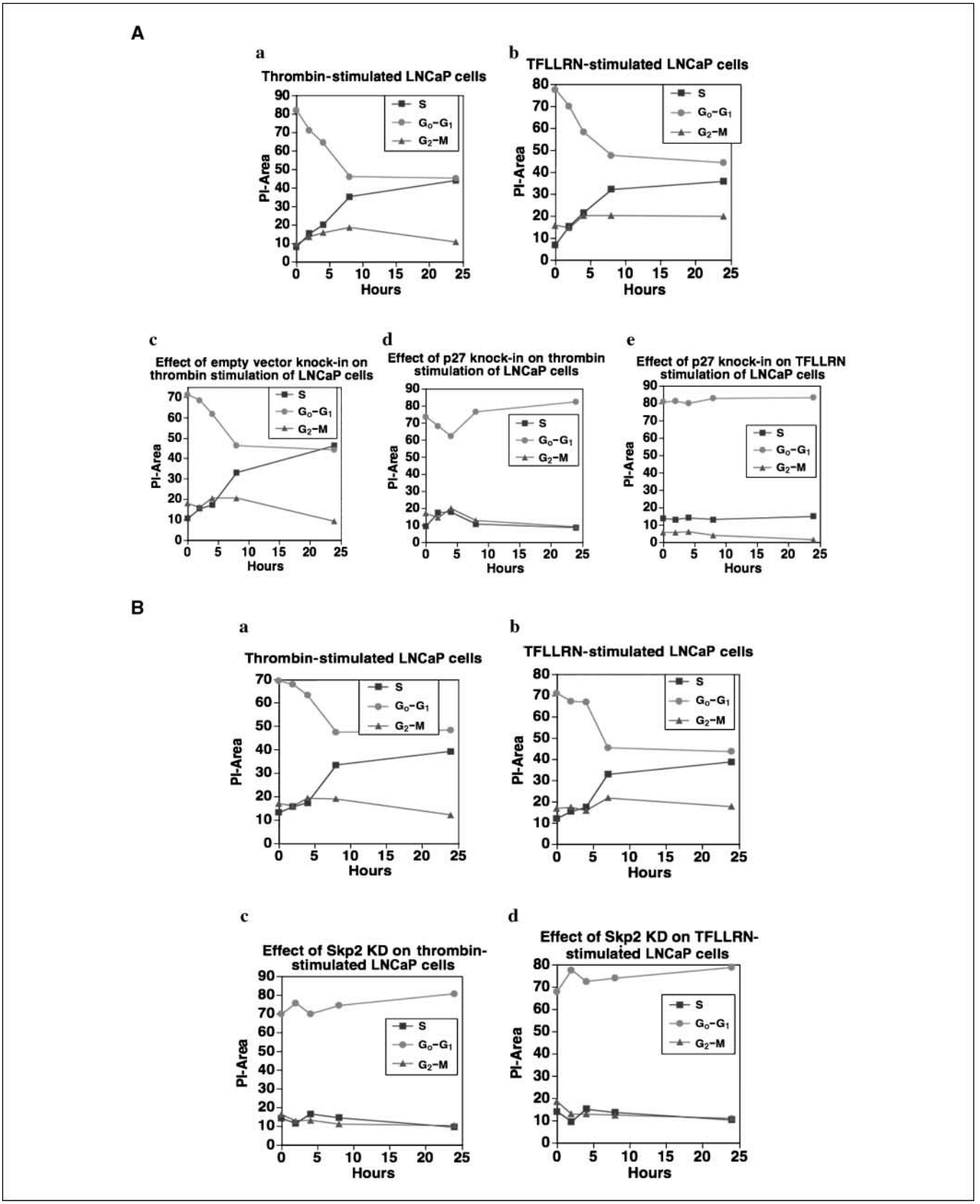

Figure 3. Effect of $p27^{Kip1}$ or Skp2 transfection on thrombin or TFLLRN-activated cell cycle kinetics. *A,* $p27^{Kip1}$ knock-in experiments show the effect of 0.5 unit/mL thrombin (*a*) or 10 μmol/L TFLLRN (*b*) in untrasnsfected LNCaP cells, and the effect of empty vector (*c*), thrombin (*d*), or TFLLRN (*e*) on $p27^{Kip1}$-transfected LNCaP cells. Representative of two experiments. Note inhibition of thrombin/TFLLRN cell cycle stimulation with $p27^{Kip1}$-transfected cells. *B,* Skp2 knockdown experiments show the effect of thrombin (*a*) or TFLLRN (*b*) on untransfected LNCaP cells and the effect of thrombin (*c*) or TFLLRN (*d*) on Skp2 knockdown LNCaP cells.

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

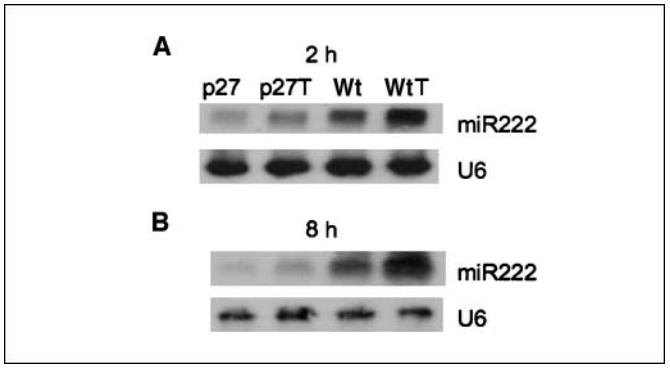

Figure 4. Effect of thrombin on up-regulation of miRNA222. Northern analysis of thrombin-induced up-regulation of MiR-222 in LNCaP cells. *A,* effect at 2 h. *Lane 1,* $p27^{Kip1}$ transfection. *Lane 2,* $p27^{Kip1}$ transfection plus thrombin. *Lane 3,* wild-type (*wt*) control. *Lane 4,* wild-type + thrombin (*wtT*; 0.5 unit/mL). *B,* effect at 8 h, similar lanes. RNA loading control is given for U6. Representative of four experiments.

anti–BrdUrd-FITC for 30 min at room temperature, recentrifuged, washed, and stained with propidium iodide before fluorescence-activated cell sorting analysis (with a kit purchased from BD Biosciences).

Northern analysis. LNCaP cells were extracted for RNA with TRizol (Invitrogen). Fifteen micrograms of total RNA were loaded onto a 10% denaturing polyacrylamide gel. Immobilon-Ny + Transfer Membranes (Millipore Corp.) were used for electrotransfer of RNA. Probes were labeled with T4 polynucleotide kinase- [32P]ATP. Hybridization buffer (Clontech) was used for Northern blot hybridization according to the manufacturer's instructions. The probe sequences were as follows: miR-222, 5′-gagacccagtagccagat-3′; U6.5′-cacgaatttgcgtgtcatccttgcgca-3′.

Results

Effect of thrombin on tumor mitogenesis and activation of the cell cycle in human LNCaP prostate and T98 glioblastoma tumor cell lines as well as a murine TRAMP prostate tumor cell line. The potential mitogenic effects of thrombin were examined in three tissue culture systems in serum-starved synchronized tumor cells. First, the effect of thrombin administration on LNCaP prostate cancer cells was analyzed. After 72 hours of androgen starvation, LNCaP cells arrest in the G_0 phase of the cell cycle, so we assessed the ability of thrombin to cause arrested LNCaP cells to re-enter the cell cycle using BrdUrd incorporation and propidium iodide staining. Addition of thrombin (0.5 u/mL) had essentially the same effect as addition of androgen-containing serum (Fig. 1*A* and *B*), inducing cells to leave G_0, enter G_1, and progress to S phase. By 8 hours posttreatment, the number of S phase cells increased dramatically from baseline for both the serum (29-fold) and thrombin-treated cells (48-fold). We further analyzed the S phase changes between 2 and 24 hours for thrombin or PAR-1 agonist TFLLRN. This increased 2.2-fold ($P < 0.04$) and 3.5-fold ($P < 0.004$), respectively ($n = 6$). To confirm these results, we performed similar studies with TRAMP (Fig. 1*C* and *D*) and glioblastoma cell lines, T98G (Supplementary Fig. S2), which arrest in G_0 in response to serum starvation. Similar to the results in LNCaP cells, serum-starved TRAMP cells responded to thrombin as well as its PAR-1 activation peptide, TFLLRN by increasing 2.8-fold for both ($P < 0.001$; $n = 4$). T98G cells also entered S phase in response to both serum and thrombin, suggesting that thrombin is a general mitogen. Thus, thrombin per se is a potent stimulator of the cell cycle compared with the resting state.

Examination of cell cycle kinases and inhibitors. We further analyzed the effect of thrombin on the cell cycle by performing immunoblots on cell cycle components known to be modulated during cell growth and proliferation (Fig. 2*A*). In cells synchronized by serum/androgen starvation, levels of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, are high, whereas levels of cyclins D1 and A, the activating subunits for cyclin-dependent kinases are low.

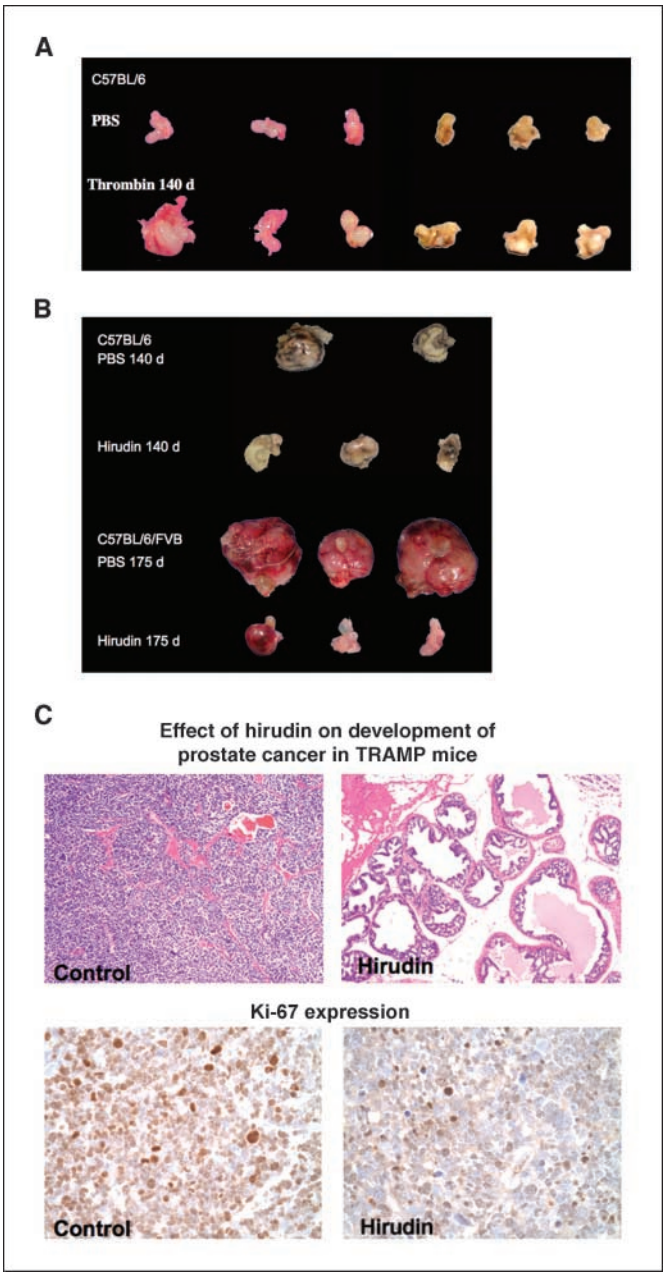

Figure 5. Effect of chronic treatment with thrombin or hirudin on tumor size and mitotic index in C57BL/6 TRAMP or C57BL/6/FVB TRAMP mice. *A,* effect of thrombin on C57BL/6 mice. Thrombin (25 units/kg) was given at 6 wk, daily for 10 d, and then every other day until sacrifice on day 140 ($n = 6$ mice in each group). *B,* effect of hirudin, 10 mg/kg in both strains of mice on day 140 and 175 ($n = 5$ to 6 mice in each group). *C, top*, histopathology of prostate from a C57BL/6/FVB mouse treated with PBS (*control*) or hirudin, sacrificed at 175 d; magnification, ×400. *Bottom*, Ki-67 staining (mitogenesis) of TRAMP tumor in PBS and hirudin-treated TRAMP mice. Note inhibition of Ki-67 staining (surrogate for mitogenesis) with hirudin treatment of C57BL/6 mice for 140 d; magnification, ×200.

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

Table 1. Effect of thrombin or hirudin on spontaneous tumor growth in transgenic mice

A. Effect of thrombin on spontaneous tumor growth of prostate adenocarcinoma in TRAMP mice*

	n	Pos/total	Tumor area DXD, mm^2	% Tumor	*n*	Prostate tumor weight, grams
PBS	9	2/9	0.014 ± 0.01	11 ± 9	6	0.19 ± 0.04
Thrombin	9	7/9	0.42 ± 0.22	62 ± 15	6	1.46 ± 0.74
Fold increase			3×	5.6×		7.7×
P		0.028+	0.020	0.035		0.028

B. Effect of hirudin on spontaneous tumor growth of prostate adenocarcinoma in TRAMP mice †

	n	Pos/total	Tumor area DXD, mm^2	% Tumor	Prostate tumor weight, grams
PBS	12	7/10	1.06 ± 0.38	50.9 ± 16.1	6.42 ± 1.03
Hirudin	10	4/12	0.14 ± 0.01	2.12 ± 1.12	0.51 ± 0.11
Fold decrease			7.6×	24×	12.6×
P		0.09+	0.03	0.03	0.04

Abbreviations: *DXD,* diameter × diameter of tumor sections examined. *% tumor,* % tumor in total prostate.
*C57BL/6 TRAMP mice were injected i.p. every other day starting at 6 wk with PBS or 25 units/kg thrombin for 140 d. SE is given. *P,* Wilcoxon signed-rank test unless noted with +, which indicates Fisher's exact test (140 d).
†C57BL/6 TRAMP mice were injected i.p. every other day starting at 6 wk with PBS or 10 mg/kg hirudin. Treatment time varied at 8 d (C57BL/6), 120, and 175 d (C57BL/6FVB). *P,* Wilcoxon signed-rank test unless noted with +, which indicates Fisher's exact test (140 and 175 d).

Confirming the effects of thrombin on these cells, both thrombin and serum addition result in down-regulation of $p27^{Kip1}$ with the concomitant induction of Skp2, the E3 ubiguitin ligase for $p27^{Kip1}$. Cyclins D1 and A are induced with similar kinetics by both thrombin and serum, with cyclin D1 up-regulation by 4 to 8 hours and cyclin A by 4 to 18 hours. Quantification of thrombin effect by density measurements is given in Fig. 2*B*. These results confirm that thrombin has a mitogenic effect on cells, ruling out an *in vivo* angiogenesis requirement for thrombin-induced tumor growth.

Thrombin activates the cell cycle through the ligation of PAR-1. We next examined the thrombin-induced mechanism of cell cycle activation by using the thrombin PAR-1 activation peptide TFLLRN. Figure 3*Aa* and *Bb* show similar TFLLRN activation kinetics as found with pure thrombin, including the down-regulation of $p27^{Kip1}$ for LNCap cells. We wondered whether $p27^{Kip1}$ is rate limiting for thrombin activation of the cell cycle. We therefore studied the inhibitory role of $p27^{Kip1}$ on thrombin-induced cell cycle activation. Transfected $p27^{Kip1}$ LNCaP cells inhibited the stimulation effect of thrombin, Fig. 3*Ac* and *Ad* or TFLLRN (Fig. 3*Ae*) on cell cycle activation. The effect of thrombin on $p27^{Kip1}$ and Skp2 was next examined because Skp2 is involved in the degradation of $p27^{Kip1}$. Thrombin (Fig. 3*Ba*) or TFLLRN-stimulated (Fig. 3*Bb*) cell cycle was inhibited in Skp2 KD LNCaP cells (Fig. 3*Bc* and *Bd*).

Thrombin down-regulates $p27^{Kip1}$ through the up-regulation of MiR-222. MiRNA-222 is up-regulated in numerous cancers, many of which are accompanied by $p27^{Kip1}$ loss or mutation (34, 42, 43). A recent report on prostate cancer cell lines reveals an inverse relationship between $p27^{Kip1}$ with MiR-222 (41). We therefore hypothesized that thrombin up-regulates MiR-222. This hypothesis was tested by Northern analysis of thrombin-activated LNCaP cells with and without $p27^{Kip1}$ transfection. Figure 4 shows up-regulation of MiRNA-222 in wild-type cells ~2-fold but not in $p27^{Kip1}$-transfected cells at 2 and 8 hours ($P > 0.1$). Thus, we can confirm an inverse relationship between $p27^{Kip1}$ and MiR-222 and now show that thrombin down-regulation of $p27^{Kip1}$ requires up-regulation of MiR-222.

Effect of thrombin on spontaneous tumor growth in TRAMP mice. We next designed an *in vivo* experimental program to test the effect of chronic thrombin treatment on the development of spontaneous tumor growth in TRAMP mice, obviating the effect of *in vitro* thrombin-tumor culture followed by *in vivo* injection of a large unphysiologic bolus of tumor. Animals were treated at 6 weeks with 25 units/kg thrombin or 10 mg/kg hirudin/mouse every day for 10 days followed by every other day until age 140 to 175 days, as indicated. Six experiments were performed with five to six animals in each group, using two different genetic backgrounds, C57BL/6 and C57BL/6/FVB. Measurements of tumor incidence, tumor area, tumor size versus whole prostate (% tumor), and mean prostate weight was recorded for each mouse.

Effect of thrombin on prostate cancer and angiogenesis in TRAMP mice. The result of repetitive thrombin treatment is shown in Fig. 5*A* and Table 1*A*. Tumor area, tumor percentage and prostate/tumor weight increased 3-fold ($P = 0.02$), 5.6-fold ($P = 0.035$), and 7.7-fold ($P = 0.028$), respectively. The incidence of carcinoma was also greater in thrombin-treated compared with PBS-treated mice (7 of 9 versus 2 of 9, respectively; $P = 0.028$). Although the angiogenesis index was enhanced 1.64-fold (1.1 ± 0.1 versus 1.8 ± 0.15; $P < 0.02$) in 9 livers of thrombin-treated animals, no enhancement was noted in 9 tumor specimens ($P > 0.1$; Supplementary Fig. S3). Because no thrombin enhancement was noted for tumor associated angiogenesis (von Willebrand factor staining) the data support the concept that *in vivo* thrombin-induced growth is independent of angiogenesis.

Effect of hirudin on the mitotic index and prostate cancer in TRAMP mice. To test the effect of endogenous thrombin, animals were treated with repetitive hirudin (potent antithrombin). To examine the effect of endogenous thrombin on the mitotic index of tumors, we first compared hirudin-treated TRAMP mice with PBS-treated controls using Ki-67 staining to monitor cell proliferation.

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

Figure 5*B* and Table 1*B* show the hirudin-induced inhibition of prostate tumor growth in TRAMP of both mouse strains (C57BL/6 and C57BL/6 FVB). Tumor area, tumor percentage, and prostate tumor weight decreased 7.6-fold ($P = 0.03$), 24-fold ($P = 0.03$), and 12.6-fold ($P = 0.04$), respectively. The incidence of carcinoma was also lower in hirudin-treated animals compared with PBS-treated control (4 of 12 versus 7 of 10; $P = 0.09$). The lack of significance is likely due to a longer time period chosen for observation as well as a more susceptible mouse strain (C57BL/6/FVB). Figure 5*C* (*top two*) confirms the beneficial effect of hirudin on tumor infiltration. Note the PBS-treated poorly differentiated prostate tumor growing in a diffuse pattern compared with the hirudin-treated tumor demonstrating early atypical preneoplastic changes with focal glandular hyperplasia, cribiform features, and mild atypia. Figure 5*C* (*bottom*) clearly shows inhibition of tumor mitogenesis by hirudin. Positive staining with Ki-67 represents nuclear staining in a proliferating cell. It is conceivable that the hirudin effect was nonspecific. However, we think this unlikely because hirudin had no effect on tumor growth *in vitro* and hirudin is a highly specific inhibitor of thrombin.

Discussion

These studies clearly show that thrombin is a growth factor that stimulates spontaneous prostate mitogenesis by inducing activation of the cell cycle from G_0-G_1 to S by down-regulation of $p27^{Kip1}$, after activation of PAR-1. Of particular interest is the potent growth stimulating effect of pure thrombin, which was equipotent with serum (a mixture of growth factors). Thrombin down-regulates $p27^{Kip1}$, a negative regulator of the cell cycle, in association with the up-regulation of Skp2 and MiR-222, which decrease $p27^{Kip1}$ by different mechanisms. Thus, thrombin operates posttranscriptionally by inactivating $p27^{Kip1}$.

The *in vitro* cell cycle data were confirmed *in vivo* with a spontaneous prostate cancer phenotype found in TRAMP mice. These studies, along with the cell cycle *in vitro* studies, show that thrombin can contribute to tumor growth. Indeed, tumor-associated angiogenesis was not significantly different between thrombin-treated and PBS-treated animals, suggesting that the tumor itself induces sufficient potentially maximal angiogenesis in the TRAMP model. Thrombin-treated animals increased their tumor volume 6- to 8-fold, whereas hirudin (potent-specific inhibitor of endogenous thrombin) decreased tumor volume 13- to 24-fold. The very potent effect of hirudin reflects the importance of endogenous generation of thrombin during spontaneous tumor development. These data are consistent with our previous reports that show the rate-limiting effect of the thrombin PAR-1 receptor on experimental pulmonary metastasis (a highly artificial pathophysiologic situation; ref. 5), as well as the effect of hirudin on external tumor implantation, seeding, and spontaneous metastasis (an artificial implantation situation; ref. 8). Our present data reflect a more relevant spontaneous tumor development situation.

These new observations accentuate the importance of thrombin in spontaneous tumor growth and development—and could be responsible for activation of a dormant tumor state, as suggested in a recent review (1).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 11/11/08; revised 2/5/09; accepted 2/9/09; published OnlineFirst 4/7/09.

Grant support: NIH grants HL 13336 and DA 04315 and the Hildegarde D. Becher Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Nierodzik ML, Karpatkin S. Thrombin induces tumor growth, metastasis, and angiogenesis: evidence for a thrombin-regulated dormant tumor phenotype. Cancer Cell 2006;10:355–62.
2. Nierodzik M, Plotkin A, Kajumo F, Karpatkin S. Thrombin stimulates tumor-platelet adhesion *in vitro* and metastasis *in vivo*. J Clin Invest 1991;87:229–36.
3. Nierodzik M, Kajumo F, Karpatkin S. Effect of thrombin treatment of tumor cells on adhesion of tumor cells to platelets *in vitro* and metastasis *in vivo*. Cancer Res 1992;52:3267–72.
4. Nierodzik ML, Bain RM, Liu L-X, et al. Presence of the seven transmembrane thrombin receptor on human tumor cells: effect of activation on tumor adhesion to platelets and tumor tyrosine phosphorylation. Br J Haematol 1996;92:452–57.
5. Nierodzik M, Chen K, Takeshita K, et al. Protease-activated receptor 1 (PAR-1) is required and rate-limiting for thrombin-enhanced experimental pulmonary metastasis. Blood 1998;92:3694–700.
6. Caunt M, Huang Y, Brooks P, Karpatkin S. Thrombin induces neoangiogenesis in the chick chorioallantoic membrane. J Thromb Haemost 2003;1:2097–102.
7. Camerer E, Qazi A, Duong D, et al. Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. Blood 2004;104:397–401.
8. Hu L, Lee M, Campbell W, Perez-Soler R, Karpatkin S. Role of endogenous thrombin in tumor implantation, seeding and spontaneous metastasis. Blood 2004;104:2748–51.
9. Caunt M, Hu L, Tang T, et al. Growth-regulated oncogene is pivotal in thrombin-induced angiogenesis. Cancer Res 2006;66:4125–32.
10. Dardik R, Savion N, Kaufmann Y, Varon D. Thrombin promotes platelet-mediated melanoma cell adhesion to endothelial cells under flow conditions: role of platelet glycoproteins P-selectin and GPIIb-IIIa. Br J Cancer 1998;77:2069–75.
11. Even-Ram S, Uziely B, Cohen P, et al. Thrombin receptor overexpression in malignant and physiological invasion processes. Nat Med 1998;4:909–14.
12. Fisher E, Ruf W, Mueller B. Tissue factor-initiated thrombin generation activates the signaling thrombin receptor on malignant melanoma cells. Cancer Res 1995;55:1629–32.
13. Klepfish A, Greco MA, Karpatkin S. Thrombin stimulates melanoma tumor-cell binding to endothelial cells and subendothelial matrix. Int J Cancer 1993;53:978–82.
14. Martin CB, Mahon GM, Klinger MB, et al. The thrombin receptor, PAR-1, causes transformation by activation of Rho-mediated signaling pathways. Oncogene 2001;20:1953–63.
15. Miller G, Bauer K, Howarth D, et al. Increased incidence of neoplasia of the digestive tract in men with persistent activation of the coagulant pathway. J Thromb Haemost 2004;2:2107–14.
16. Mueller B, Reisfeld R, Edgington T, Ruf W. Expression of tissue factor by melanoma cells promotes efficient hematogenous metastasis. Proc Natl Acad Sci U S A 1992;89:832–6.
17. Shi X, Gangadharan B, Brass LF, Ruf W, Mueller BM. Protease-activated receptors (PAR1 and PAR2) contribute to tumor cell motility and metastasis. Mol Cancer Res 2004;2:395–402.
18. Tsopanoglou NE, Maragoudakis ME. On the mechanism of thrombin-induced angiogenesis. Potentiation of vascular endothelial growth factor activity on endothelial cells by up-regulation of its receptors. J Biol Chem 1999;274:23969–76.
19. Zacharski L, Memoli V, Morain W, Schlaeppi J-M, Rousseau S. Cellular localization of enzymatically-active thrombin in intact tissue by hirudin binding. Thromb Haemost 1995;73:793–7.
20. le Sage C, Nagel R, Egan DA, et al. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. EMBO J 2007;26:3699–708.
21. Belletti B, Nicoloso MS, Schiappacassi M, et al. p27(kip1) functional regulation in human cancer: a potential target for therapeutic designs. Curr Med Chem 2005;12:1589–605.
22. Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. J Cell Physiol 2000;183:10–7.
23. Macri E, Loda M. Role of p27 in prostate carcinogenesis. Cancer Metastasis Rev 1998;17:337–44.
24. Tsihlias J, Kapusta L, Slingerland J. The prognostic significance of altered cyclindependent kinase inhibitors in human cancer. Annu Rev Med 1999;50:401–23.
25. Lloyd RV, Erickson LA, Jin L, et al. p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. Am J Pathol 1999;154:313–23.
26. Cheville JC, Lloyd RV, Sebo TJ, et al. Expression of p27kip1 in prostatic adenocarcinoma. Mod Pathol 1998;11:324–8.
27. Tsihlias J, Kapusta LR, DeBoer G, et al. Loss of

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-4290

cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma. Cancer Res 1998;58:542–8.
28. Yang RM, Naitoh J, Murphy M, et al. Low p27 expression predicts poor disease-free survival in patients with prostate cancer. J Urol 1998;159:941–5.
29. Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995;269:682–5.
30. Carrano AC, Eytan E, Hershko A, Pagano M. SKP2 is required for ubiquitinmediated degradation of the CDK inhibitor p27. Nat Cell Biol 1999;1:193–9.
31. Sutterluty H, Chatelain E, Marti A, et al. p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat Cell Biol 1999;1:207–14.
32. Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr Biol 1999;9:661–4.
33. Hershko DD. Oncogenic properties and prognostic implications of the ubiquitin ligase Skp2 in cancer. Cancer 2008;112:1415–24.
34. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A 2005;102:19075–80.
35. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–8.
36. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. Nature 2005;435:828–33.
37. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–66.
38. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–69.
39. Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene 2006;25:6188–96.
40. Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 2007;315:1576–9.
41. Galardi S, Mercatelli N, Giorda E, et al. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem 2007;282:23716–24.
42. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006;103:2257–61.
43. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer 2007;120:1046–54.
44. Greenberg NM, DeMayo FJ, Sheppard PC, et al. The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. Mol Endocrinol 1994;8:230–9.
45. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell 1979;17:43–52.
46. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. Nature 1979;278:261–3.
47. Bookstein R, Rio P, Madreperla SA, et al. Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma. Proc Natl Acad Sci U S A 1990;87:7762–6.
48. Rubin SJ, Hallahan DE, Ashman CR, et al. Two prostate carcinoma cell lines demonstrate abnormalities in tumor suppressor genes. J Surg Oncol 1991;46:31–6.
49. Hu L, Roth JM, Brooks P, Luty J, Karpatkin S. Thrombin up-regulates cathepsin-D which enchances angiogenesis, growth, and metastasis. Cancer Res 2008;68:1–8.
50. Hu L, Roth JM, Brooks P, Ibrahim S, Karpatkin S. Twist is required for thrombin-induced tumor angiogenesis and growth. Cancer Res 2008;68:4296–302.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Thrombin Induces Tumor Cell Cycle Activation and Spontaneous Growth by Down-regulation of $p27^{Kip1}$, in Association with the Up-regulation of Skp2 and MiR-222

Liang Hu, Sherif Ibrahim, Cynthia Liu, et al.

Cancer Res 2009;69:3374-3381. Published OnlineFirst April 7, 2009.