

Thrombospondin-1 Triggers Cell Migration and Development of Advanced Prostate Tumors

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Abstract

The antitumor effects of pharmacologic inhibitors of angiogenesis are hampered in patients by the rapid development of tumor resistance, notably through increased invasiveness and accelerated metastasis. Here, we reevaluated the role of the endogenous antiangiogenic thrombospondin 1 (TSP1) in prostate carcinomas in which angiogenesis is an active process. In xenografted tumors, we observed that TSP1 altogether inhibited angiogenesis and fostered tumor development. Our results show that TSP1 is a potent stimulator of prostate tumor cell migration. This effect required CD36, which also mediates TSP1 antiangiogenic activity, and was mimicked by an antiangiogenic TSP1-derived peptide. As suspected for pharmacologic inhibitors of angiogenesis, the TSP1 capacities to increase hypoxia and to trigger cell migration are thus inherently linked. Importantly, although antiangiogenic TSP1 increases hypoxia *in vivo*, our data show that, in turn, hypoxia induced TSP1, thus generating a vicious circle in prostate tumors. In radical prostatectomy specimens, we found TSP1 expression significantly associated with invasive tumors and with tumors which eventually recurred. TSP1 may thus help select patients at risk of prostate-specific antigen relapse. Together, the data suggest that intratumor disruption of the hypoxic cycle through TSP1 silencing will limit tumor invasion. *Cancer Res*; 71(24); 7649–58. ©2011 AACR.

Introduction

Studies over the past 30 years have shown that angiogenesis is a key process in progression of cancer from an *in situ* lesion to invasive and metastatic disease, providing the rationale for the development of antiangiogenic therapies. Several antiangiogenic molecules are already in use or under clinical trial to treat various cancers, including prostate tumors (1). These treatments, which mostly target the VEGF pathway, can lead to disease stabilization and longer periods of progression free survival, but substantial benefits remain unrealized for the vast majority of patients in terms of overall survival because tumors elicit evasive resistance (2). Recent evidences in preclinical

models suggest that anti-VEGF therapies, although inhibiting the growth of primary tumors, promote tumor invasiveness and metastasis (3, 4). Treatment of mice with the antiangiogenic sunitinib was recently shown to foster the implantation into lungs of intravenously injected tumor cells (4). Interestingly, a comparable effect was reported more than 20 years ago with the endogenous antiangiogenic molecule Thrombospondin 1 (TSP1; ref. 5), and the TSP1-derived antiangiogenic peptide ABT-510 failed to show antitumor effects in 2 phase II clinical trials (6, 7). We hypothesized that the capacities to increase hypoxia and to trigger cell migration were inherently linked and that this link was shared by endogenous and pharmacologic inhibitors of angiogenesis. We thus reevaluated in experimental and clinical prostate tumors the role of TSP1, the first antiangiogenic molecule characterized (8).

TSP1 is a large—450 kDa—trimeric calcium-binding molecule composed of several domains (9), which binds to numerous ligands and receptors, including several integrins and the scavenger receptor CD36. TSP1 inhibits *in vitro* the migration of endothelial cells and induces their apoptosis (10). TSP1 expression is inhibited in a large number of tumors, including breast and androgen-dependent prostate carcinomas (ADCaP) in which there is an inverse correlation between TSP1 expression and microvessel density (MVD; refs. 11–13). The growth of prostate tumors is initially strictly dependent on androgens, and androgen ablation efficiently inhibits tumor growth. However, tumors eventually become resistant to castration, and recur with a poor prognosis. We previously reported that TSP1 expression is high, and no longer associated with a reduced MVD, in castration-resistant prostate carcinomas (CRCaP;

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ref. 11), and in breast metastases (12), suggesting that TSP1 might stimulate tumor development. We therefore analyzed in prostate tumors the functions of TSP1 and the mechanisms controlling its expression, notably by regulators of calcium homeostasis and hypoxia.

It is now well established that calcium, which binds to the type III repeats of TSP1 and modifies its folding and properties (9, 14), regulates proliferation, differentiation, and apoptosis of cancer cells (15). The role of calcium signaling in migration and metastasis becomes now more and more evident. Indeed, the cyclic morphologic and adherence changes observed during cell migration are accompanied by repetitive changes in intracellular cytosolic calcium concentration ($[Ca^{2+}]_i$) depending on Ca^{2+} influx through channels located on the plasma membrane. The molecular nature of these channels in migrating cells, and even more so for metastatic cancer cells, is still largely unknown. Accumulating evidences suggest that the development of some cancers involves ion channel aberrations, notably in the prostate in which several nonvoltage-dependent cationic channels of the transient receptor potential (TRP) family were shown to be key players in calcium homeostasis (15). For instance, TRPM8 (16), TRPV6 (15), and TRPV2 (17) are differentially expressed between normal prostate and prostate carcinomas, and androgens regulate the expression of TRPM8 and TRPV3 (18). We therefore studied the role of calcium and calcium-permeable channels in the regulation of TSP1 and in the migration of prostate cancer cells.

Patients, Materials, and Methods

Reagents and siRNA

Camphor, thapsigargin, and $COCl_2$ were purchased from Sigma-Aldrich (Saint-Quentin Fallavier). TSP1 antibodies (Ab-1, Ab-4, and Ab-11) were from Neomarkers (Interchim), tubulin antibody from Sigma-Aldrich, collagen IV antibody from Novotec, hamster anti-CD31 clone 2H8 from Pierce, and Alexa-Fluor goat anti-rabbit 488 and Alexa-Fluor goat anti-mouse 568 were purchased from Molecular probes. siRNAs were purchased from Sigma-Aldrich. Sequences used are indicated in Supplementary Table S1. ABT-510 was a generous gift from Abbott laboratories.

Cell culture and transfection

LNCaP and C4-2 cells were grown in RPMI containing 10% fetal calf serum, PC-3 cells in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum. Hiperfect reagent (Qiagen) was used to transfect cells in 24-well plates with the indicated siRNAs (10 nmol/L) as recommended by the manufacturer. For hypoxic conditions, cells were cultured at 37°C with 5% CO_2 , 94% N_2 , and 1% O_2 in a hypoxic incubator (Binder GmbH). In some experiments, to mimic hypoxia, cells were grown in the presence of 300 μ mol/L cobalt chloride.

Migration assay

Migration capacity was measured by Transwell assays (BD Biosciences). Cells (40,000) were seeded in RPMI 1% FBS in the upper part of a cell culture-chamber-insert system

separated from the lower chamber by a 8 μ m PET membrane. RPMI 10% FBS, or cell-conditioned medium, was added in the lower compartment as indicated. Eighteen hours later, nonmigrating cells in the upper compartment were scrapped off with a cotton swab. Cells on the lower side of the membrane were fixed with methanol at $-20^\circ C$ and stained with Hoechst 33258 (Sigma-Aldrich). Membranes were then excised, mounted on a glass slide with glycerol (Dako) and cells counted.

Real-time reverse transcriptase PCR, siRNA, and mRNA analysis

Total RNA was isolated with TRIzol reagent (Invitrogen). RNA was retrotranscribed using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was quantified by real-time PCR using the *Power* SYBR Green PCR Master Mix (Applied Biosystems). Human cyclophilin A was used as an internal control. Sequences of the PCR primers are indicated in Supplementary Table S2.

ELISA

TSP1 and VEGF protein contents in cell homogenates and supernatant were measured by ELISA (Quantikine, R&D systems).

Calcium imaging

Cytosolic Ca^{++} concentration was measured using ratio-metric dye Fura-2 (Invitrogen Ltd.) and quantified as described (19). Cells were loaded with 1 μ mol/L Fura-2/AM for 45 minutes, washed and bathed for at least 10 minutes in a solution containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose, before measuring Ca^{2+} .

Animals, siRNA injection, and tumorigenicity assays

Studies involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with the local animal ethical committee in the Institut André Lwoff in Villejuif, France. Tumor cells (2×10^6 cells/mouse) were injected subcutaneously in 50% (v:v) Matrigel (BD Biosciences) to 6- to 8-week-old male nude mice and tumors were measured every day. When tumors grew exponentially, siRNA diluted in PBS was injected i.p. on a daily basis (120 μ g/kg). Tumor volume was estimated by the formula: length \times width² \times 0.5.

Subjects

Prostate tissue samples were obtained from 35 patients who underwent radical prostatectomy. Written consent was obtained from each patient. Immediately after prostate removal, small pieces of tissues were gross dissected by the pathologist, snap frozen, and stored in liquid nitrogen until analysis in tumor banks of the Centre hospitalier Lyon Sud (Lyon, France), groupe hospitalier Cochin-Saint Vincent de Paul (Paris, France), or CHU Henri Mondor (Créteil, France). Histologic analysis of a frozen section was conducted for each sample by the same pathologist before RNA extraction. The fragments fully constituted of cancerous glands were

selected and named "tumor" samples, whereas those that did not contain cancerous tissue were selected and named "peritumoral tissue."

Statistical analysis

Results are expressed as mean \pm SEM. Differences between means were studied using a 2-tailed *t* test or ANOVA when 2 groups or more than 2 groups were compared respectively. *In vivo* experiments were analyzed by 2-way ANOVA with repeated measures followed by a Bonferroni post test. Statistically significant results are labeled as follows in all figures: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Statistical analysis was done with GraphPad Prism5 (GraphPad Software Inc.).

Results

TSP1 is repressed in ADCaP but reexpressed in CRCaP and AICaP cell lines

The TSP1 promoter is methylated in the ADCaP cell line LNCaP (20) and accordingly, TSP1 mRNA and protein levels are very low (Fig. 1A-B). In contrast, we found a sustained TSP1 expression in C4-2 cells, which were established from LNCaP tumors recurring in mice after castration (21). TSP1 was also found expressed, at the mRNA (Fig. 1A) and protein (Fig. 1B) levels, in the CRCaP cell line 22RV1, and in the androgen-independent (AICaP) PC3 cell line which does not express the androgen receptor.

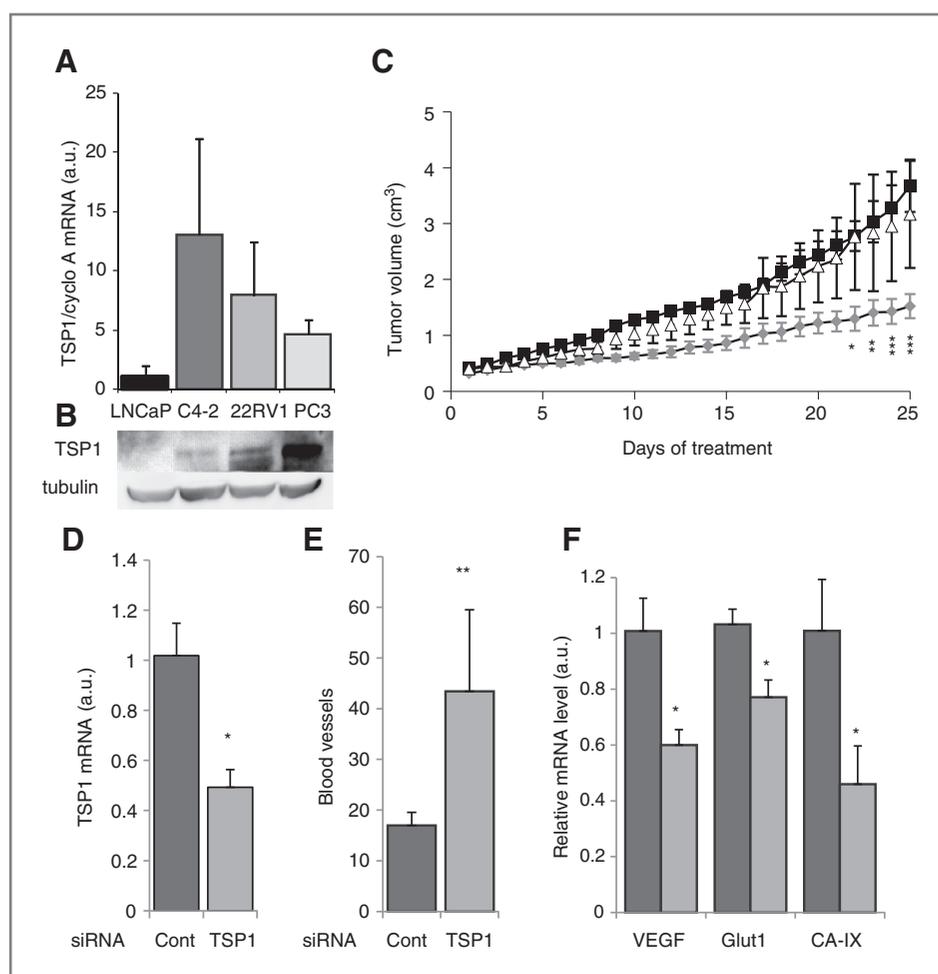


Figure 1. TSP1 is antiangiogenic but fosters tumor development in castration-resistant prostate tumors. **A**, TSP1 mRNA level in androgen-dependent (LNCaP), castration-resistant (C4-2 and 22RV1), or androgen-independent (PC3) prostate tumor cells. Results (mean \pm SEM, $n > 3$) are normalized to the cyclophilin A mRNA level and expressed in arbitrary units (LNCaP set to 1). **B**, corresponding TSP1 protein level in the same prostate cell lines. Tubulin was used as a loading control. **C**, mice bearing exponentially growing C4-2 tumors were treated daily with i.p. injections of PBS (white triangles), control (black squares), or TSP1-siRNA (grey diamonds). All siRNAs were diluted (120 μ g/kg) in PBS. Tumor volume is expressed in cm³ (mean \pm SEM, 6 mice per group). The experiment was repeated 3 times with comparable results. Tumor volumes in TSP1-siRNA-treated mice are significantly different from cont-siRNA-treated group (2-way ANOVA followed by a Bonferroni test: $P < 0.05$ on day 22, < 0.01 on day 23, < 0.001 on days 24 and 25). **D**, TSP1 mRNA level, normalized to cyclophilin A, in C4-2 tumors collected at the end of the experiment depicted in **C**. **E**, CD-31-positive blood vessels in C4-2 tumors collected at the end of the experiment shown in **C** were quantified in hot spots of vascularization. **F**, VEGF, GLUT1, and CA-IX mRNA levels, normalized to cyclophilin A, in control (black bars) or TSP1-siRNA-treated C4-2 tumors (gray bars) collected at the end of the experiment depicted in **C**. Cont, control.

***In vivo* silencing of TSP1 increases MVD but inhibits the growth of castration-resistant prostate tumors**

We used RNA interference *in vivo* to address the role of TSP1 in C4-2 tumors. Tumor cells were xenografted into nude mice. Once tumors were exponentially growing, mice were randomized for treatment and received daily a PBS i.p. injection, or 120 $\mu\text{g}/\text{kg}$ of either control-, or a TSP1-siRNA diluted into PBS and injected i.p. as previously described (22). As compared with tumors treated with PBS, or with a control siRNA with no known mRNA target, TSP1-siRNA reduced TSP1 mRNA levels in tumors (Fig. 1D) and significantly inhibited tumor growth (Fig. 1C). The TSP1-siRNA did not activate the innate immune system (Supplementary Fig. S1A). Similarly, TSP1-siRNA injected into mice bearing PC3 tumors inhibited tumor development (Supplementary Fig. S1B and S1C). Although the growth of C4-2 tumors was blunted by TSP1-siRNA, MVD was significantly higher than in controls in C4-2 (Fig. 1E, Supplementary Fig. S1D) and in PC3 tumors (Supplementary Fig. S1E). Several genes, including *VEGF*, *GLUT1*, and *CA-IX* are induced under hypoxic conditions (23). The increased MVD paralleled a reduced expression of these 3 genes in TSP1-siRNA-treated tumors (Fig. 1F and Supplementary Fig. S1F), strongly suggesting that blood vessels were functional. These results establish that the antiangiogenic properties of TSP1 are maintained in castration-resistant and androgen-independent prostate tumors. They also show that TSP1 stimulates the development of these tumors, and that this property is dominant over its antiangiogenic effects.

TSP1 stimulates the migration of prostate tumor cells

We used the above TSP1-siRNA (TSP1a-siRNA) and a second one (TSP1b-siRNA) to study the mechanism of the protumoral effect of TSP1 in prostate tumor cells *in vitro*. The 2 TSP1-siRNAs silenced TSP1 expression over 70% at the mRNA and protein levels (Supplementary Fig. S2A–S2B), with no effect on cell proliferation or apoptosis (Supplementary Fig. S2C–S2D). In contrast, TSP1 silencing strongly inhibited the migration of C4-2 cells in a Transwell assay (Fig. 2A). This effect was not dependent upon the expression of the androgen receptor because the migration of PC3 cells was also strongly affected by TSP1 silencing (Fig. 2A). Conversely, the migration of LNCaP cells, which do not express TSP1 and are poorly motile, was stimulated when cells migrated towards C4-2 cells-conditioned medium (Fig. 2B). To further establish the role of TSP1 on the migration of prostate tumor cells, we took advantage of a cell line, JT8, in which the production of TSP1 is under the control of a tetracycline-repressible promoter (24). We prepared conditioned media from TSP1-induced or TSP1-repressed JT8 cells (Supplementary Fig. S2E) and compared their effect on the migration of C4-2 cells. The presence of TSP1 strongly increased the capacity of cells to migrate (Fig. 2C).

Antiangiogenic TSP1 peptides foster prostate cell migration

TSP1-derived peptides inhibiting endothelial cell migration and neovascularization *in vivo* have been characterized (25). A stabilized derivative of one of these peptides, which retains the antiangiogenic activity of TSP1 (26) was further

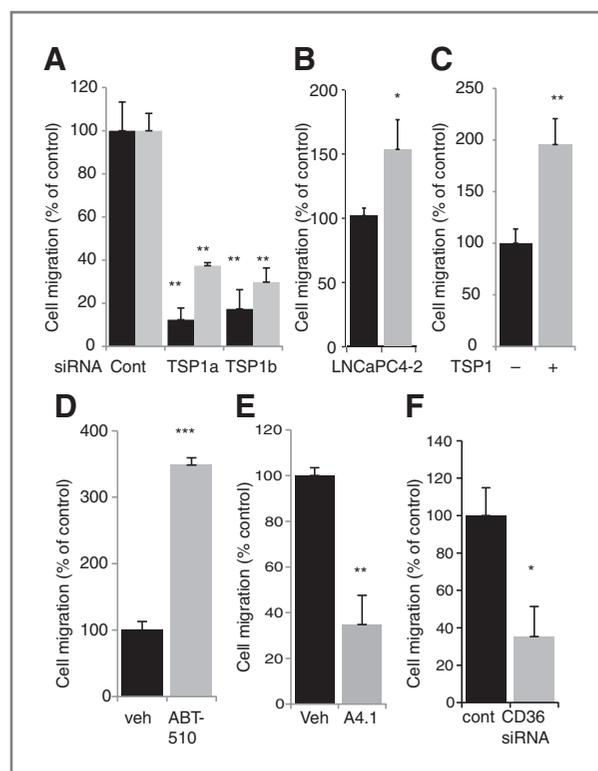


Figure 2. TSP1-stimulated migration of tumor cells requires CD36. A, C4-2 (black bars) or PC3 cells (gray bars) were transfected with the indicated siRNAs and their migration capacity toward culture medium containing 10% FBS measured 3 days later in a Transwell assay. The experiment was repeated 3 times with comparable results. B, LNCaP (black bars) or C4-2 cells (gray bars) were plated in the lower part of Boyden chambers. Two days later, LNCaP cells were seeded in the upper chamber and the number of migrating cells counted 18 hours later. C, migration of C4-2 cells toward medium conditioned by TSP1-tet-repressible JT8 cells grown in the presence of doxycycline (TSP1-) or in absence of doxycycline (TSP1+). D, migration of LNCaP cells toward 10% FBS-containing medium (veh) or the same medium complemented with 100 nmol/L of ABT-510. E, migration of C4-2 cells toward culture medium in the presence of vehicle (veh) or TSP1-Ab1 antibody A4.1 (1 $\mu\text{g}/\text{mL}$) in the upper compartment. F, C4-2 cells were transfected with cont-siRNA or CD36-siRNA as indicated, and their migration capacity measured 3 days later. Results in each panel are expressed as the number of migrating cells reported to cells migrating in control condition set to 100 (mean \pm SEM, $n = 3$). A representative result of 3 independent experiments is shown.

developed by Abbott as ABT-510 for clinical applications. In a Transwell assay, ABT-510 induced a 3.5-fold increase in the number of LNCaP migrating cells (Fig. 2D).

TSP1-induced migration requires CD36

The antiangiogenic properties of TSP1 are mediated by its binding to the CD36 receptor (26, 27) which we previously found induced by androgen ablation and expressed in CRCaP tumors in patients (11). TSP1 antibody A4.1 inhibits the interaction between TSP1 and CD36 and blocks TSP1 antiangiogenic activity (25). This antibody inhibited by 65% the migration of C4-2 cells (Fig. 2E). Similarly, transfection of C4-2 cells with an siRNA silencing CD36 (Supplementary Fig. S2F) cells also inhibited cell migration by 65% (Fig. 2F). Altogether

these results show that the promigratory and antiangiogenic properties of TSP1 are closely linked at the molecular level.

TSP1 expression and secretion are regulated by cytosolic calcium concentration

We next sought the mechanisms inducing TSP1 in prostate tumors. Each TSP1 molecule binds approximately 35 calcium ions (28), which modifies its folding. TSP1 silencing significantly increased the basal cytosolic calcium concentration ($[Ca^{2+}]_i$) in C4-2 (Fig. 3A) and PC3 cells (Supplementary Fig. S3A), showing that, directly or indirectly, TSP1 contributes to regulate calcium concentration in prostate tumor cells. Because a 2-hour treatment with thapsigargin, an inhibitor of SERCA pump which increases $[Ca^{2+}]_i$, induced TSP1 secretion (Supplementary Fig. S3B–S3C), we investigated whether reciprocally the cytosolic calcium concentration in prostate tumor cells could regulate the expression and secretion of TSP1 and cell migration.

Several channels of the TRP family, which are deregulated during prostate tumor progression (15), actively regulate $[Ca^{2+}]_i$ in prostate cells, in particular TRPV2, TRPV6, and TRPM8 (16, 17, 29, 30). *TRPV3* was recently described as an androgen-receptor target gene (18) and we found this channel expressed in prostate tumor cell lines (Fig. 3B), with a reduced

expression in LNCaP cells, as observed for TSP1 (Fig. 1A). In C4-2 cells, the basal $[Ca^{2+}]_i$ was not significantly affected by TRPV3 silencing but was increased 1.6-fold by thapsigargin-induced store depletion (Fig. 3C), indicating that this channel was regulating calcium homeostasis, possibly through an ER membrane localization. TRPV3 silencing in PC3 cells increased the basal $[Ca^{2+}]_i$ level but only marginally affected thapsigargin-induced store depletion (Supplementary Fig. S3D). Treatment of C4-2 cells with camphor, a well-established agonist of TRPV3 and TRPV1 (31, 32), induced after 2 hours a dose-dependent secretion of TSP1 in the culture medium (Fig. 3D), followed after 6 hours by a dose-dependent increase in TSP1 mRNA level (Fig. 3E). These results suggested that TRP channels might regulate TSP1 expression and cell migration. Indeed, silencing TRPV2, TRPV3, TRPV6 or TRPM8, resulted into a reduced TSP1 level in C4-2 cells (Fig. 3F and Supplementary Fig. S3E and S3F). In contrast, TRPC4 and TRPC6 silencing increased TSP1 mRNA level in C4-2 cells, whereas TRPC1, TRPC3 or ORAI had no significant effect (Fig. 3F). In addition, silencing TRPV3 or TRPM8 inhibited C4-2 cells migration as efficiently as TSP1-siRNA, whereas silencing TRPC4 or TRPC6 stimulated cell migration (Fig. 3G). We chose to further analyze the regulation of TSP1 expression by TRPV3

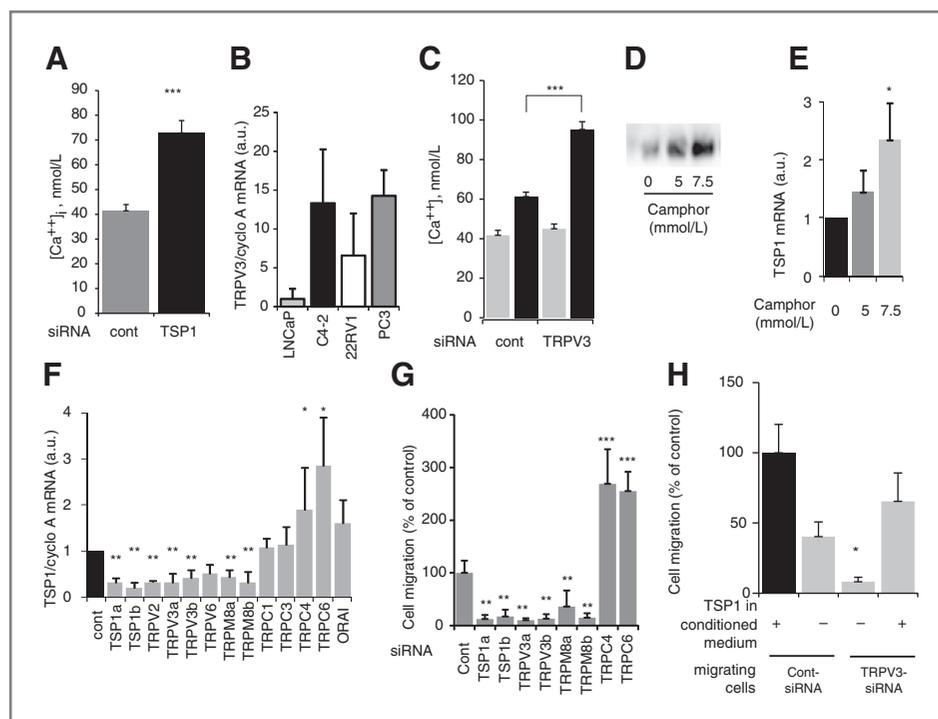


Figure 3. TSP1 regulation by calcium regulators. A, cytosolic calcium concentration in C4-2 cells placed for 5 minutes in calcium-free medium 48 hours after transfection with control- or TSP1-siRNA. B, TRPV3 mRNA level, normalized to cyclophilin A mRNA, in prostate tumor cell lines. Results are expressed in arbitrary units, TRPV3 level in LNCaP cells set to 1 (mean \pm SEM, $n = 3$). C, cytosolic calcium concentrations in basal condition (gray bars) or after thapsigargin-induced store depletion (peak concentration, black bars) recorded in C4-2 cells transfected 2 days earlier with control-siRNA or TRV3-siRNA. D, Western blot detection of TSP1 secreted in the culture medium of C4-2 cells 2 hours after treatment with camphor at the indicated concentration. E, TSP1 mRNA level, normalized to cyclophilin A mRNA in C4-2 cells 6 hours after treatment with camphor at the indicated concentrations. F, C4-2 cells were transfected by the indicated siRNAs and the TSP1 mRNA level, normalized to cyclophilin A, was quantified 2 days later (mean \pm SEM, $n > 3$). To target TRPV3, TRPM8, and TSP1, 2 different siRNAs, noted a and b, were used for each gene. The TSP1 mRNA level in control cells was set to 1 (mean \pm SEM, $n = 3$). G, migration in a Transwell assay toward medium containing 10% FBS of C4-2 cells transfected 3 days before with the indicated siRNAs. Results are expressed as in Fig. 2. H, C4-2 cells were transfected with control- or TRPV3-siRNA. Three days later, their migration toward conditioned medium prepared from control- or TSP1-siRNA-transfected C4-2 cells was quantified in a Transwell assay. Results expressed as the percentage of cells migrating in control condition (mean \pm SEM, $n = 3$).

because this channel is expressed at all stages of prostate tumor progression (Fig. 3B), and because, in contrast with TRPV6 (30) and TRPM8, TRPV3 silencing did not affect cell proliferation (Supplementary Fig. S3G), a phenotype which could bias the interpretation of migration assays.

TSP1 mediates the regulation of prostate tumor cell migration by TRPV3

To further analyze the relative contributions of TSP1 and TRPV3 in cell migration, we prepared conditioned medium from control- or TSP1-siRNA-transfected C4-2 cells (Supplementary Fig. S3H). We then measured in Transwell assays the migration of control, or TRPV3-silenced C4-2 cells, toward control, or TSP1-depleted media. Consistent with the results shown in Fig. 3G, migration of TRPV3-silenced cells toward TSP1-depleted medium was inhibited by 86% (Fig. 3H). Importantly, addition of TSP1 in the lower compartment fully restored migration of TRPV3-silenced cells (Fig. 3H), showing that the stimulation of cell migration by TRPV3 is mediated by the secreted TSP1.

Using the same protocol as for TSP1-siRNA, we treated mice bearing exponentially growing C4-2 or PC3 tumors with TRPV3-siRNA daily. TRPV3-siRNA treatment was as efficient as TSP1 to inhibit tumor development in the 2 tumor models (Supplementary Fig. S4A and S4B).

Hypoxia modifies calcium homeostasis in C4-2 cells and induces TSP1 expression

The antitumoral properties of antiangiogenic molecules rely on their capacity to increase hypoxia. One of the constant early responses to hypoxia in almost all cell types is an increase in $[Ca^{2+}]_i$ (33). We thus analyzed how hypoxia affected calcium homeostasis and TSP1 expression in prostate tumor cells. As compared with normoxia, hypoxia induced in C4-2 cells a 2.2-fold increase in $[Ca^{2+}]_i$ level after thapsigargin-induced store depletion (Fig 4A). TRPV3 silencing further increased both resting and thapsigargin-induced $[Ca^{2+}]_i$ levels in hypoxia. Similar results were obtained in PC3 cells (Supplementary Fig. S5A). These data suggested that TSP1 could also be induced by hypoxia. We thus measured in C4-2 cells grown in 1% or 20% oxygen, the expression and secretion of TSP1. We observed a time-dependent increase of both TSP1 and VEGF mRNA levels in hypoxia as compared with normoxia (Fig. 4B). TSP1 protein was synthesized in hypoxia (Fig. 4C) and secreted in the cell culture medium (Fig. 4D). TSP1 was also induced by hypoxia in PC3 cells (Supplementary Fig. S5B) and its secretion in cell culture medium was induced by thapsigargin and Colbalt Chloride treatments (Supplementary Fig. S5C).

TSP1 expression is associated with invasion and cancer recurrence after radical prostatectomy

In prostate tumors, TSP1 inhibits angiogenesis and thus increases hypoxia. Our results show that in turn, hypoxia induces TSP1 which fosters cell migration. A vicious circle is thus likely to take place as tumors progress toward more advanced and invasive stages. To test this hypothesis, we quantified TSP1 mRNA level in 35 frozen radical prostatectomy specimens from patients with clinically localized prostate

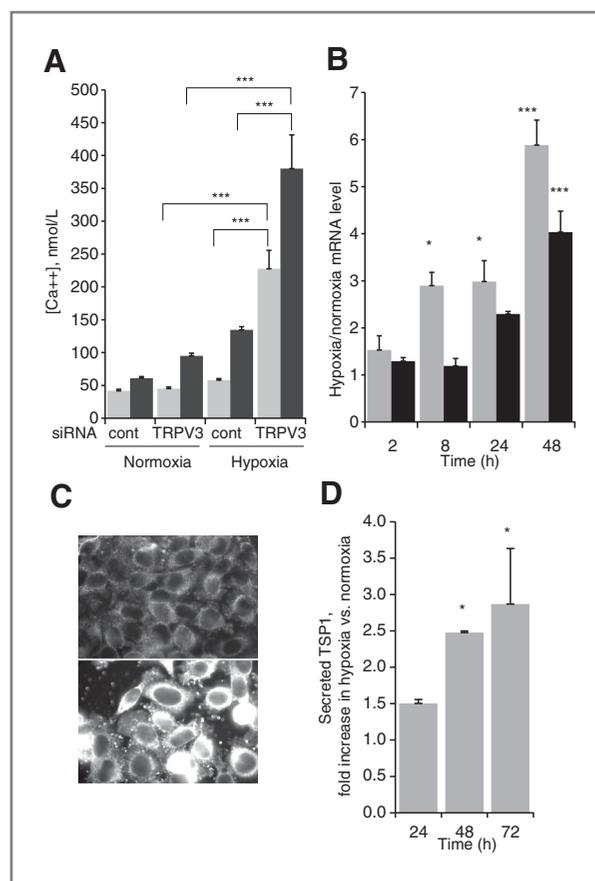


Figure 4. Regulation of calcium homeostasis and TSP1 by hypoxia. A, C4-2 cells were transfected with control- or TRPV3-siRNA. Two days later, half of the cells were maintained in normoxia while COCl₂ 300 μmol/L was added to sister plates to mimic hypoxia. Cytosolic calcium concentrations in basal condition (gray bars) or after thapsigargin-induced store depletion (peak concentration, black bars) were recorded 24 hours later (48 hours after transfection). B, VEGF (gray bars) and TSP1 (black bars) mRNA levels in C4-2 cells incubated in 20% (normoxia) or 1% oxygen (hypoxia) for up to 48 hours. Results are normalized to the cyclophilin A mRNA level and expressed in arbitrary units (mean ± SEM, n = 3 independent experiments). C, TSP1 immunodetection in cells grown for 48 hours in control medium (top) or in the presence of 300 μmol/L COCl₂ (bottom). D, TSP1 protein content measured by ELISA in conditioned medium of C4-2 cells grown in 1% or 20% oxygen for up to 72 hours. Results are expressed as (secreted TSP1/protein in cell homogenate in hypoxia)/(secreted TSP1/protein in cell homogenate in normoxia) (mean ± SEM, n = 3 independent experiments).

cancer who did not receive any radiotherapy and/or hormonal ablation treatment before surgery. Pairs of tumor and peritumoral tissue were analyzed in 32 specimens. There was no significant association between either tumoral or peritumoral TSP1 mRNA level and patients' age, Gleason score, or serum prostate-specific antigen (PSA) level before surgery (Table 1). As compared with prostate-localized pT2 tumors, and to pT3a tumors, in which the tumor has spread through the prostatic capsule, there was a significant increase in pT3b tumors, which invade seminal vesicles, indicating an association between TSP1 expression and invasion in patients (Fig. 5A; Table 1). Of the 35 patients included in this study, 9 experienced PSA

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Table 1. TSP1 mRNA level in peritumoral and tumoral tissue from radical prostatectomy specimens

Criteria	n	TSP1 mRNA Mean (range)		n	TSP1 mRNA Mean (range)		pT versus PT
		PT	P		Tumor (T)	P	
All patients	32	0.38 (0.01–1.19)		35	0.71 (0.02–3.68)		0.041
Age							
≤60	16	0.37 (0.01–0.93)		18	0.95 (0.04–3.68)		0.048
>60	16	0.39 (0.01–1.19)	0.453	17	0.46 (0.02–1.37)	0.058	0.308
Gleason score							
6	6	0.26 (0.04–0.56)	0.266	6	0.86 (0.05–2.11)	0.189	0.127
7	20	0.41 (0.01–1.19)	0.404	22	0.52 (0.02–3.68)	0.280	0.307
>7	6	0.31 (0.13–0.59)	0.308	7	1.2 (0.07–3.48)	0.051	0.082
PSA before surgery							
<7	10	0.25 (0.01–0.91)	0.071	11	0.6 (0.02–3.68)	0.382	0.184
7–15	14	0.47 (0.05–1.19)	0.266	16	0.72 (0.04–3.48)	0.300	0.196
>15	8	0.36 (0.01–0.93)	0.269	8	0.84 (0.04–2.06)	0.381	0.088
Stage							
pT2	9	0.25 (0.01–0.89)	0.113	10	0.4 (0.04–1.33)	0.470	0.242
pT3a	10	0.51 (0.01–1.19)	0.206	10	0.41 (0.04–1.47)	0.041	0.329
pT3b	13	0.37 (0.02–0.93)	0.195	15	1.12 (0.02–3.68)	0.045	0.022
Relapse							
No	19	0.38 (0.01–1.19)		19	0.31 (0.02–1.33)		0.283
Yes	8	0.45 (0.13–0.93)	0.335	9	1.74 (0.27–3.68)	0.00003	0.013

relapse, whereas 19, followed for at least 30 months after surgery, did not show evidence of tumor recurrence. Importantly, at the time of radical prostatectomy, TSP1 mRNA level

in tumors, but not in peritumoral tissue, was significantly associated with PSA relapse (Table 1; Fig. 5B).

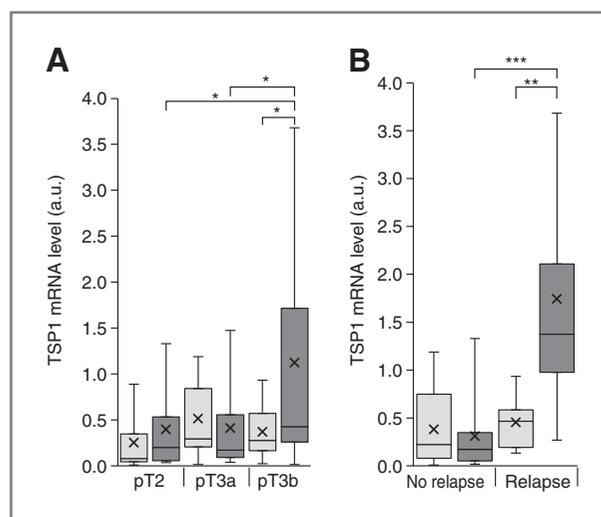


Figure 5. TSP1 mRNA in radical prostatectomy samples. TSP1 mRNA level, normalized to cyclophilin A mRNA level, in peritumoral (PT, gray bars) and tumoral (T, dark gray bars) tissues from frozen radical prostatectomy samples taken before any other treatment. Each box plot is composed of 3 horizontal lines that display the 25th, 50th (median), and 75th percentiles. The highest and lowest values are shown by error bars; mean value is indicated with an x. A, analysis with respect to the pathologic tumor stage. B, comparison in samples from patients who did not show evidence of tumor recurrence for at least 30 months (mean follow-up: 48 months, range: 30–64 months, $n = 19$) or who experienced PSA relapse ($n = 9$).

Discussion

The antiangiogenic functions of TSP1 in tumors, extensively showed in most tumor models, are exemplified by the inverse correlation between TSP1 expression and MVD in androgen-dependent prostate carcinomas (11, 13, 34). TSP1 expression is inhibited by promoter methylation in the androgen-dependent cell line LNCaP (20), but surprisingly, reexpressed in C4-2 cells, a castration-resistant derivative of LNCaP cells, and in androgen-independent PC3 cells. This TSP1 reexpression is also observed in CRCaP in patients in which angiogenesis is an active process (11). TSP1 reexpression in CRCaP and AICaP is not fortuitous as TSP1 silencing blunted tumor development *in vivo* (Fig. 1C and Supplementary Fig. S1B), showing that TSP1 actively stimulates tumor growth, as observed in other experimental (5, 35) and clinical situations (12, 36). Indeed, our experiments show that TSP1, which does not regulate CRCaP cell proliferation or apoptosis, is a potent trigger of tumor cell migration. This property was also observed in other cell types (35), and notably in a glioblastoma model, in which the overexpression of an N-terminal fragment of TSP1 gave rise to less angiogenic but more invasive tumors (37). Recently, it was also reported that the growth of primary tumors is increased in TSP1 knockout mice, whereas the number of metastases is decreased (38).

We investigated if, in addition to its stimulatory effect on tumor cell migration, TSP1 could have lost its antiangiogenic effects in CRCaP and AICaP. This might occur through an

increased secretion of proangiogenic factors (24), or through a lack of TSP1 secretion. The latter was described in renal cell carcinomas in which a decreased expression of TRPC4 impaired calcium uptake and TSP1 secretion (39). In CRCaP, however, TSP1 is secreted, and this secretion is fostered by hypoxia (Fig. 4D and Supplementary Fig. S5C) or by an increase in $[Ca^{2+}]_i$ (Fig. 3D, Supplementary Figs. S3B and S5C). In addition, silencing TSP1 increased tumor MVD (Fig. 1E, Supplementary Fig. S1D and S1E), showing that TSP1 is still antiangiogenic in CRCaP. It might seem paradoxical that silencing an antiangiogenic molecule inhibits tumor development. A similar situation was described for DLL4: the inhibition of this antiangiogenic factor increased MVD but reduced tumor development (40). However, DLL4 regulates the specification of tip cells and thus, despite an increased MVD, hypoxia was increased in DLL4-inhibited tumors because blood vessels were not functional. The antitumoral effects associated with TSP1 silencing are likely mediated by a different mechanism: TSP1, through its binding to CD36, induces apoptosis of endothelial cells (10), and in TSP1-siRNA-treated tumors the increased MVD was associated with a reduced VEGF, GLUT1, and CA-IX mRNA levels (Fig. 1F, Supplementary Fig. S1F), indicative of a reduced hypoxia, suggesting that blood vessels in TSP1-silenced tumors are functional. It thus seems that the proinvasive effects of TSP1 in CRCaP are dominant over its antiangiogenic effects.

TSP1 folding and functions depend on the binding of calcium ions (9, 14) whose intracellular concentration is tightly regulated (41). In C4-2 cells, at least 6 membrane receptors of the TRP family regulated the TSP1 expression positively—TRPM8, TRPV2, TRPV3, and TRPV6—or negatively—TRPC4 and TRPC6. Cell migration was induced by TRPC4 or TRPC6 silencing, or, conversely, inhibited by TRPM8 or TRPV3 silencing. In addition, in TRPV3-silenced cells, migration was restored by the addition of TSP1 in the extracellular medium, placing TSP1 as a central mediator of the TRPV3 effect on cell migration. These results show that intracellular calcium concentration is a major regulator of TSP1 synthesis, secretion, and capacity to induce cell migration. In patients, while prostate tumor progresses, the intracellular calcium homeostasis is disrupted, at least in part because the expression of several TRP channels is deregulated, notably TRPV2 (17), TRPV6, and TRPM8 (42). This mechanism thus most likely participates in the upregulation of TSP1 expression and secretion during prostate tumor progression.

Hypoxia, which spontaneously increases during prostate tumor progression (43), is associated with a shorter time to biochemical failure or progression free survival (44, 45). One of the early events induced by hypoxia is an increase in $[Ca^{2+}]_i$ (33), notably through TRP channels (46, 47). TRPV3 appears as an important regulator of calcium homeostasis in hypoxia in prostate tumor cells, as its silencing markedly increased $[Ca^{2+}]_i$ levels in C4-2 and PC3 cells. Because calcium homeostasis is an important regulator of TSP1 secretion and synthesis, we studied the effect of hypoxia on TSP1 expression. Importantly, hypoxia increased TSP1

protein synthesis and secretion (Fig. 4B, 4C, Supplementary Fig. S5B and S5C). TSP1 translation in hypoxia likely occurs through an internal ribosome entry site recently described in the 5' TSP1 mRNA (48). Hypoxia was also reported to induce TSP1 in heart infarction, in wound healing (49), and in several situations of ischemia in the retina, brain, leg (50), and kidney. In atherosclerotic lesions, hypoxia-induced TSP1 expression promotes migration of vascular smooth muscle cells (51), showing that the interplay between hypoxia, TSP1, and cell migration is not restricted to tumor cells. Likely, TSP1-induced migration helps cells survive escaping the hypoxic environment.

The antiangiogenic effects of TSP1 are mediated by its binding to CD36 (10, 27). The TSP1-CD36 interaction also stimulates resorption by osteoclasts (52) and induces TIMP-1 production and secretion in prostate and breast tumor cell lines (53). In addition, our results now establish that TSP1 promotes prostate tumor cell migration. This function also requires CD36 as silencing of this receptor, or inhibition of the TSP1-CD36 interaction using specific antibodies, impaired migration. The TSP1-mimetic antiangiogenic peptide ABT-510 also stimulated migration in LNCaP cells although the role of CD36 in that activity was not investigated. These results support the hypothesis that the capacity of TSP1 to stimulate cell migration, invasion, and tumor dissemination is intrinsically linked to its capacity to increase hypoxia. Similarly, hypoxia triggered by pharmacologic antiangiogenic drugs induces invasion (3, 4). These observations strongly suggest that the increased invasion and formation of distant metastasis in hypoxia occurs whatever the trigger of hypoxia. This is of particular importance in the treatment of prostate cancers because castration, the first-line treatment for invasive prostate tumors, represses VEGF (54) and induces TSP1 (11, 13, 55), creating a hypoxic stress which contributes to the antitumoral effect. Because hypoxia induces VEGF, the VEGF repression is only transient, and in the prostate of castrated rats, VEGF returns to control levels after 3 days (54). No such feedback mechanism occurs for TSP1, as castration induces TSP1 which increases hypoxia, which in turn induces TSP1 expression in a vicious circle. Castration-resistant cells express TSP1, which stimulates their migration. These cells, which proliferate in low testosterone levels, have enhanced migrating capabilities, and resist to hypoxia, are prone to give rise to aggressive metastases. Disrupting this vicious circle silencing TSP1 in patients undergoing chemical castration should help controlling cell dissemination in response to hypoxia-induced castration. In addition, our experimental data show that TSP1 silencing increases MVD. Therefore, association of TSP1 silencing with chemotherapeutic treatments should also be beneficial, improving penetration of therapeutic agents, and inhibiting invasion and metastasis dissemination.

Importantly, in radical prostatectomy specimens taken before any other treatment, we observed an increased TSP1 mRNA level in pT3b as compared with less invasive pT3a- and pT2-staged cancers and a statistically higher tumoral TSP1 expression in patients who experienced tumor recurrence. This finding may be of importance: with the

development of PSA screening, the number of so called "clinically insignificant cancers" has increased dramatically. TSP1 expression on prostate biopsies may have an incremental staging value in clinical practice, and improve identification of indolent tumors, thus avoiding unnecessary radical and possibly morbid treatment such as radical prostatectomy. On radical prostatectomy specimens, TSP1 mRNA may also be of clinical interest to select patients at risk of PSA relapse and propose additional treatments such as adjuvant radiotherapy.

Disclosure of Potential Conflicts of Interest

F. Cabon is a share holder of SeleXel. The other authors disclosed no potential conflicts of interest.

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