PDGF/VEGF-related receptor affects transglutaminase activity to control cell migration during crustacean hematopoiesis

Kingkamon Junkunlo, Kenneth Söderhäll, Chadanat Noonin and Irene Söderhäll

Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden

*Corresponding author: Irene Söderhäll, Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden.

Tel.: +46-18-4712817; Fax: + 46-18-4716425. E-mail: Irene.Soderhall@ebc.uu.se

Running title: PDGF/VEGF-related receptor regulates HSC migration in crustaceans

Abstract

The platelet-derived growth factor (PDGF) receptor, a tyrosine kinase receptor whose ligand is PDGF, is crucial in the transduction of extracellular signals into cells and mediates numerous processes, such as cell proliferation, differentiation, survival and migration. We demonstrate the important roles of a receptor tyrosine kinase related to the PDGF/VEGF family protein (PVR) in controlling hematopoietic progenitor cell migration by affecting extracellular transglutaminase (TGase) activity. Pl_PVR1, GenBank accession number KY444650, is highly expressed in hemocytes and the hematopoietic tissue (HPT). Sunitinib malate was used to block the PVF/PVR downstream pathway in HPT cell culture. The addition of Sunitinib also caused the HPT cells to increase in size and begin spreading. An increase in extracellular TGase activity on the HPT cell membrane was observed in a dose-dependent manner after treatment with Sunitinib malate. The presence of crude Ast1 provided a combinatorial beneficial effect that enhanced the number of spreading cells after inhibition of the Pl_PVR downstream signaling cascade. In addition, an increased immunoreactivity for β-tubulin and elongation of β-tubulin filaments were found in Pl_PVR

signaling-inhibited cells. The potential roles of PVF/PVR signaling in controlling progenitor cell activity during hematopoiesis in crayfish were investigated and discussed.

Introduction

Platelet-derived growth factor (PDGF) is one of the numerous protein growth factors that regulate cell physiology processes such as proliferation, survival, migration, and differentiation [1]. Platelet derived-growth factor receptors (PDGFRs) are members of the type III tyrosine kinase receptor family, which is characterized by five Ig domains, a single transmembrane domain and a tyrosine kinase domain [2]. The interaction between PDGFs and its receptors on the surface of target cells is essential for the transduction of extracellular signals into cells and mediates a series of intracellular signaling cascades through its association with SH2 domain-containing adaptor proteins [3]. PDGF receptors are essential and evolutionarily conserved determinants of blood cell development and dispersal [4]. In mammals, the signals from PDGFs have been shown to drive cellular responses including proliferation, survival, migration, and the deposition of extracellular matrix (ECM) and tissue remodeling factors [5]. In *Drosophila*, PDGF-like factors and their receptors (PVR) are responsible for guiding cell migration and are involved in hemocyte production [6]. Recent studies have shown that PVR regulates hematopoiesis by serving as an equilibrium signal to control and maintain progenitor cells in *Drosophila* [7]. Moreover, the interaction between the Trol (terribly reduced optic lobes), heparan sulfate proteoglycan and the PVR signaling pathway participates in hemocyte homeostasis in *Drosophila* [8]. However, there are very few studies on the PDGF/PDGFR signaling pathway and its role in stem cell activity. Thus, the biological regulation of the PDGF/PDGFR signaling pathway in invertebrates is still poorly understood.

One of the more well-established invertebrate models in the field of blood cell (hemocyte) production, except for *Drosophila,* is the freshwater crayfish *Pacifastacus leniusculus*, in which proliferation of hematopoietic cells and their differentiation into mature hemocytes have been characterized in detail [9,10]. In this species, a technique for the culture of hematopoietic tissue and stem cells has been

successfully established [11,12]. Hematopoiesis is a complex process by which new blood cells are formed and released from hematopoietic tissue (HPT) through circulation, and the balance between the differentiation and proliferation of hematopoietic cells in hematopoiesis is tightly controlled by internal and external factors such as growth factors and the ECM [10]. In crayfish, Astakine1 (Ast1) is known as a hematopoietic growth factor that is involved in controlling the proliferation, differentiation and apoptosis of progenitor cells [13]. In regulating progenitor cell behavior, Ast1 has an effect on the ECM structure by regulation of extracellular transglutaminase (TGase) activity [14,15]. Recent studies have shown that Ast1 is an inhibitor of the TGase enzyme and also blocks the formation of ε(γ-glutamyl)-lysine crosslink bonds [16].

In this work, we have sought to characterize the function of the PVF/PVR signaling pathway in regulating hematopoiesis. Four different sequences of PVR isoforms were found in the crayfish transcriptome library, and Pl_PVR1 (GenBank accession number KY444650) was selected for further study based on its expression pattern, which mainly occurred in mature hemocytes and HPT.

Material and Methods

Animals

Freshwater crayfish, *P. leniusculus*, were obtained from Lake Erken, Sweden. The animals were maintained in an aquarium with aeration at 10 °C. Healthy intermolt male crayfish were used for the experiments.

Preparation of hematopoietic tissue (HPT) cells

HPT cells were isolated and cultured as previously described [11,17]. Briefly, after dissection of the crayfish, HPT was dissected and incubated for 20 min in 800 µl of 0.1% collagenase type I and type IV (Sigma-Aldrich, USA) in crayfish phosphate-buffered saline (CPBS; 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 mM $CaCl_2$ and 10 mM $MnCl_2$, pH 6.8) at room temperature. Next, the collagenase

solution was removed after being centrifuged at 800 x *g* for 5 min. The resulting cell pellet was washed twice with 1 ml of CPBS and then suspended in L-15 medium (Sigma-Aldrich, USA) supplemented with 1 mM phenylthiourea, 60 mg/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml gentamicin (Sigma-Aldrich, USA), and 2 mM L-glutamine. The cells were cultured in 96-well plates at a density of 5 x 10^4 cells/well at 16 °C. One third of the medium was changed every second day.

Granular cell (GC) and semi-granular cell (SGC) separation

Hemocytes were separated by Percoll gradient centrifugation as previously described [18]. In brief, 1-2 ml hemolymph was collected in a 1:1 volume of anti-coagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6 [18]. Then, GCs and SGCs were separated using a continuous gradient of 70% Percoll in 0.15 M NaCl, and centrifugation at 3000 x g for 25 min at 4 °C. The resulting cell bands were collected and suspended in 0.15 M NaCl.

In situ hybridization of Pl_PVR1

Sense and anti-sense strand probes were generated following the manufacturer's instructions for the DIG RNA Labeling Kit (SP6/T7) (Roche, USA). A DNA template of Pl_PVR1 was amplified with Pl_PVR1 specific primer pairs. The PCR products were then purified by using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, USA) and were used as templates for DIG-labeled RNA synthesis. The labeling efficiency of the probes was verified before use according to the instructions for the DIG RNA Labeling Kit (SP6/T7) (Roche, USA). The separated GCs and SGCs were seeded on SuperFrost Plus Microscope slides (Thermo Scientific, USA), fixed with 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4) for 30 min at room temperature, washed 3 times with PBS-DEPC (PBS buffer treated with diethyl pyrocarbonate (DEPC) (Affymetrix, USA)), pre-hybridized with Hybridization Mix (-) buffer (HM (-)) (50% Deionized formamide, 5X SSC, 0.1% Tween, pH 6.0 with citric acid) at 60 °C for 1 h, and incubated with Hybridization Mix (+) (HM (+)) (50% Deionized formamide, 5X SSC, 0.1% Tween, 50 ug/ml heparin, 500 ug/ml RNase-free tRNA, pH 6.0 with citric

acid) containing 50 ng of specific RNA probes at 60 °C for 16 h. Post-hybridization washes were performed 2 times each at 60 °C for 5 min using a serial dilution of HM (-) in 2X SSC (75% HM (-), 50% HM (-), 25% HM (-) and 100% 2X SSC), and then cells were washed 2 times with 0.2X SSC at 60 °C for 30 min. The slides were washed with a serial dilution of 0.2X SSC in PBST (0.1% Tween 20 in PBS buffer) and 100% PBST at room temperature for 5 min 2 times each before being incubated with blocking buffer (1x PBST, 2% horse serum, 2 mg/ml BSA) at room temperature for 1 h and incubated with blocking buffer containing 1:1000 of sheep anti digoxigenin-AP IgG (Roche, USA) at 4 °C overnight. After being washed 6 times each at room temperature for 15 min, the slides were equilibrated with staining buffer (100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl and 0.1 Tween 20) 3 times each at room temperature for 5 min. The dark purple color was developed in the dark in staining buffer containing the NBT/BCIP substrate (Roche, USA). Slides were washed 3 times each with stop solution (1X PBS, 1 mM EDTA and 0.1% Tween 20) before they were mounted with 50% glycerol in PBS.

Total RNA extraction and RT-PCR

HPT cells were cultured as previously described and treated with DMSO alone or with 1 μM or 10 μM Sunitinib in DMSO. HPT cells were collected at 48 h and 96 h after treatment. Total RNA was extracted using Trizol LS reagent (Invitrogen) according to manufacturer's protocol and processed to eliminate contaminated DNA by RNase free DNase I (Ambion, USA) treatment. Further, cDNA was synthesized using ThermoScript (Invitrogen, USA) and the gene transcription levels were detected by RT-PCR. The transcription of a 40S ribosomal protein was used as an internal control for each sample. The primers used are shown in supplementary table 1. The transcription of a 40S ribosomal protein was used as an internal control. PCR products were analyzed on 1.5% agarose gel stained with GelRed™ (Biotium, USA).

Sunitinib malate treatment

Sunitinib malate (Sigma Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO). HPT cells were cultured as previously described and treated with DMSO alone or with 1 nM, 10 nM, 1 μM or 10 μM

Sunitinib in DMSO. The cell morphology was observed at 24 h and then continuously until 7 days after treatment. The effect of crayfish Ast1 from plasma on cell morphology after treatment with Sunitinib malate at 1 μM or 10 μM was observed at 24 h until 7 days after Sunitinib malate addition with or without 2% crude Ast1. Crude Ast1 was prepared as previously described [14]. The cell morphology was observed at 24 h and 48 h and followed to 7 days after treatment. To quantify the cells with different morphology, the number of cells with a round shape and the number of cells with a spindle-like shape and flat-shaped (spreading cells) in each treatment group were counted. The result was calculated and reported as the percentage of cells with different morphology.

Cell surface area measurement after Sunitinib malate treatment

Individual images of cells after the following treatments were obtained; control, DMSO, 1 nM, 10 nM, 1 μM or 10 μM Sunitinib in DMSO respectively. These images were imported into the NIH image analysis package for calculation of cell surface area (http://rsb.info.nih.gov/nih-image). The outer membrane border of each cell on the images was manually inscribed with a digital cursor, and the area demarcated by the tracing was calculated with a macrosuboutine (Image J) calibrated before use against a micrometer slide image captured at the same magnification as the cell image. Surface area was determined for cells from all treatments at 48 h.

Detection of extracellular TGase activity

After 48 h of culture, 1 mM of 5-(biotinamido)-pentylamine ($BPNH_2$, Pierce, USA), a substrate for TGase, was added to the cultures and then incubated for 18 h. Subsequently, HPT cells were fixed as previously described [14]. Briefly, the medium was removed, and the cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature. Next, 25 mM glycine in PBS was added to the wells, and incubated for 30 min. The cells were subsequently washed five times with PBST and blocked with 10% BSA in PBST for 1 h. For detection, HPT cells were incubated for one hour with streptavidin-FITC conjugate (GE Healthcare, USA). Then, the crosslinking of the substrate was visualized with

streptavidin-FITC addition under a fluorescence microscope. Hoechst 33258 (blue) was used as a nuclear stain.

Immunocytochemistry of β-tubulin

After treatment with the PVR inhibitor (Sunitinib malate), HPT cells were cultured as previously described and treated with DMSO alone or 10 μM of Sunitinib in DMSO for 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde in PBS for 1 h, washed three times with PBS, permeabilized for 15 min three times with 0.3% Triton X in PBS, and then blocked with horse serum (Sigma-Aldrich, USA) for 1 h. Next, the cells were incubated for 2 h with a mouse monoclonal anti β-tubulin antibody conjugated to Alexa Flour 488 (Millipore, USA, 1: 200). Subsequently, the antibodies were removed, and after washing 5 times with PBST (0.5% Tween 20 in PBS buffer), the cells were incubated with Hoechst 33258 dye at a concentration of 1 μg/ml to stain the nuclei. The localization of β-tubulin was observed under a fluorescence microscope.

HPT cell migration assay

To assay for the stimulation of cell migration, a BD Falcon^{TM} cell culture insert containing polyethylene terephthalate (PET) tract-etched membranes (8.0 μm pore size) (BD Biosciences, USA) was used, and inserted into 24-well culture plates filled with 800 μl L15 medium supplemented with 2% crude Ast1. Then, suspended HPT cells were gently seeded on the PET membrane at a concentration of 7.5 x 10^4 cells/350 μl. Sunitinib malate was added into the inserts at a concentration of 5 μM Sunitinib and DMSO was added as a control. After 48 h, the cells that had migrated to the bottom side of the membrane were fixed with 4% paraformaldehyde in PBS for 1 h and washed three times with PBS. Next, cells were stained with Nuclear Fast Red H 3403 (Vector Laboratories Inc., CA) for 30 min and counted under the microscope.

Statistical analysis

The percentage of round or spreading cells (spindle-like cells and flat-shaped cells) and the cell migration number are shown as the mean ± SD, and the statistical analysis was performed using the one-way ANOVA followed by Duncan's new multiple range test and Tukey's test. For comparisons between two groups, a t-test was used, and statistical significance was considered at $P<0.05$. The number of animals in each experiment was 3-7 crayfish per group.

Results

PVGF/VEGF-related receptor (Pl_PVR) in *P. leniusculus*

Four different sequences of PVR isoforms were found in our crayfish transcriptome libraries. The partial sequence of Pl_PVR1, contig CL6450Contig1, was selected for further studies based on the mRNA tissue expression. The Pl_PVR1 gene transcript was dominantly expressed in hemocytes, and a lower level of expression was detected in HPT (data not shown). The full-length of Pl_PVR1 was cloned and submitted to GenBank with accession number KY444650. The protein domain structure of Pl_PVR1 is evolutionarily conserved and consists of three immunoglobulin domains (IG), one transmembrane domain (TM) and one catalytic domain (tyrosine kinase) (Figure 1). A comparison of Pl_PVR1 to other PDGF/VEGF protein family members revealed that most proteins of this family reported so far contain a tyrosine-specific protein kinase (TyrKc) domain. However, Pl_PVR contains a nonspecific kinase domain (STYKc), which can phosphorylate the OH group of serine, threonine or tyrosine amino acid residues (Figure 1). In situ hybridization of Pl_PVR1 in separated hemocytes (granular or semi-granular) and HPT cells was consistent with the mRNA tissue expression, showing that Pl_PVR1 is mainly expressed in hemocytes. Interestingly, not all hemocytes and HPT cells expressed Pl_PVR1 mRNA (Figure 2).

Pl_PVR inhibition induced HPT cell spreading

To examine the mechanism by which hematopoietic cells responded to Pl_PVR1, Sunitinib malate (SU011248 or Sutent, Sigma-Aldrich, USA) a multi-targeted receptor tyrosine kinase (TK) inhibitor was

used to inhibit PVR downstream signaling. First, we examined whether Sunitinib malate could be used to reduce the expression of Pl_PVR1 mRNA in cultured cells (*in vitro*) by RT-PCR. At 48 h and 96 h after treatment with 1 μM and 10 μM Sunitinib malate, a minor decrease in Pl_PVR1 expression compared with that in the control (DMSO and L15 treatment) was observed (data not shown). At 48 h, HPT cells supplemented with 1 μM and 10 μM Sunitinib malate were more spread out and were attached to the bottom of the wells (Figure 3A). In addition, the spreading morphology of the Sunitinib-treated HPT cells was different from the "migrating" morphology (spindle-shaped cells), which has been reported in Lin et al. 2008 [14]. The morphological differences of HPT cells are shown in Figure 4. After HPT cells were isolated from the HPT, they usually exhibited a round shape with less cytosolic space (Figure 3A and 4A). The HPT cells after TGase knockdown or supplementation with crude Ast1 were shaped like spindles, which are narrow, elongated, and less round in the middle with two ends that are pointed (Figure 4B). After Sunitinib treatment, HPT cells appeared to be flat with several protrusions (Figure 3A and 4C). Thus, the cells were increased in their surface area as a result of Sunitinib malate addition (Figure 3A and Figure 4). At lower concentrations of Sunitinib malate (1 nM and 10 nM), the cell surface area was not significantly different from control and DMSO-treated cells (Figure 5). However, in 1 μM and 10 μM Sunitinib malate-treated cells, the surface area was significantly higher ($P<0.01$ and $P<0.001$) than control and DMSO-treated cells (Figure 5). Furthermore, the number of spreading cells (spindle-shaped and flat-shaped) increased in a dose-dependent manner with the addition of Sunitinib malate to the cultures (Figure 3B and 3C). In 10 μM Sunitinib malate-treated cells, the percentage of spreading cells was significantly higher than ($P<0.001$) in control and DMSO-treated cells (Figure 3B and 3D). At lower concentrations of Sunitinib malate (1 nM and 10 nM), there was no difference in the spreading of the HPT cells compared to that in the control or DMSO-treated cells (Figure 3). Cell viability assays using trypan blue staining and MTT assay showed that the number of surviving cells did not differ between Sunitinib malate-treated, L15-treated (control) and DMSO-treated cells (data not shown). This result suggests that PVR downstream signaling may be required to maintain cells in the round-shaped stage in the tissue, whereas the inhibition of the PVR pathway caused cell spreading and release from the HPT.

TGase activity was affected by Sunitinib treatment

TGase is one of the most abundant proteins in crayfish HPT, and high extracellular TGase activity has been shown to maintain the HPT cells in their stem-cell form in the tissue [14,15]. Higher levels of extracellular TGase activity were found in round-shaped cells than in migrating cells (spindle-shaped cells) [14]. Furthermore, we found that after knockdown of the mRNA expression of TGase in HPT cells, the expression of Pl_PVR1 (examined by real-time PCR) was slightly increased but not significantly different from dsGFP as a control (data not shown). Thus, we hypothesized that the extracellular TGase activity may be involved in controlling cell spreading. As a result of Sunitinib treatment, higher levels of extracellular TGase activity were seen on the HPT cell surface than in the control and DMSO conditions (Figure 6). Interestingly and surprisingly, extracellular TGase was increased in a dose-dependent manner according to the number of spreading cells caused by Sunitinib malate treatment (Figure 6).

Crude Ast1 enhanced cell spreading after Sunitinib malate treatment

A recent study showed that Ast1 had a direct inhibitory effect on the crosslink formation catalyzed by the TGase enzyme [16]. For this reason, the effect of crude Ast1 after inhibition of Pl_PVR downstream signaling was investigated by carrying out *in vitro* experiments. Without crude Ast1, the effect of 10 μM Sunitinib malate treatment on cell morphology was clearly observed at 48 h (Figure 3A). However, cell spreading (spindle-shaped and flat-shaped morphology) was also observed at 24 h (Figure 7A) with 10 μM Sunitinib malate treatment. Without crude Ast1, a few spreading cells was observed at 24 h and 48 h with 1 μM Sunitinib malate. The number of spreading cells increased in a dose-dependent manner and was significantly different from that in the cells treated with the lower concentration of Sunitinib malate and control cells (DMSO and L15) (Figure 3B and 7B). When the HPT cell culture was supplemented with crude Ast1, the stimulating effect of Sunitinib malate was enhanced (Figure 7A and B). Spreading cell morphology could be clearly observed at an earlier time point (at 24 h) and at lower concentration of (1 μM) Sunitinib malate supplemented with crude Ast1 compared with without crude Ast1 (Figure 7A). A

significant difference in the number of spreading cells (spindle-shaped and flat-shaped) and round-shaped cells was observed at 24 h in cells after treatment with 1 μM Sunitinib malate compared with that in cells without the addition of crude Ast1 (Figure 7B and 7C). In cells treated with 10 μM Sunitinib malate, the spreading cell number was not significantly different with or without supplementation with crude Ast1 (Figure 7B). The addition of crude Ast1 to the HPT cell culture had no effect on TGase mRNA expression [14] or Pl_PVR1 mRNA expression (data not shown). These results indicate that crude Ast1 may act as an enhancer of the PVR/PVF signaling pathway and appears to function together with the PVR/PVF signaling pathway to promote the differentiation of hematopoietic progenitor cells.

Inhibition of the Pl_PVR signaling pathway stimulated HPT cell migration

To understand more about HPT progenitor cell behavior after Pl_PVR downstream signaling inhibition, a cell migration assay was performed. Without crude Ast1, only a few HPT cells migrated in cultures treated with 5 μM Sunitinib malate or DMSO. However, after the addition of crude Ast1 to the HPT cell cultures, cell migration was induced. With the addition of crude Ast1, the number of migrating cells increased in the presence of 5 μM Sunitinib malate compared with that in the DMSO-treated control cells (Figure 8). The migration of cells is controlled by the regulation of the cytoskeleton structure. Accordingly, we observed increased immunoreactivity for β-tubulin after addition of 5 μM Sunitinib malate compared to that in DMSO-treated cells in which β-tubulin was not elongated (Figure 9). The change in shapes of HPT cells after 5 μM Sunitinib malate treatment was associated with increase in labeling of β-tubulin. According to these results, PVR signaling is suggested to be important for controlling progenitor cell behavior, and crude Ast1 may enhance the effect of PVR signaling in inducing cell migration.

Discussion

Hematopoietic progenitor cells are produced and differentiated in lobules in the HPT [10]. The homeostatic regulation of the balance between self-renewal and the commitment of progenitor cells is

controlled by the combination of intrinsic and external cell regulatory factors [19]. Multiple growth factors, cytokines and ECM proteins are involved in this regulation [20]. Our previous studies showed that Ast1 is an important factor that induces proliferation and differentiation of crayfish progenitor cells [10, 13, 21]. In mammals, PDGF/PDGFR signaling is well known as a chemotactic and mitogenic factor involved in the physiological regulation of hematopoiesis [22]. In crustaceans, the factors and mechanisms that regulate the activity of this ligand/receptor in controlling hematopoiesis are unknown. Here, we describe the regulation of Pl_PVR (a receptor tyrosine kinase related to PDGF/VEGF family protein) in controlling hematopoietic cell migration through extracellular TGase and Ast1.

From a crayfish transcriptome library, four different partial sequences showing similarity to PDGFR were found. Based on the mRNA expression pattern, Pl_PVR1 (GenBank accession number KY444650), which was highly expressed in hemocytes and HPT, was selected for further studies. *In situ* hybridization by using Pl_PVR1 mRNA probes showed that not all GCs and SGCs expressed Pl_PVR1. Lower expression of Pl_PVR1 was observed in HPT cells (immature cells) than in mature hemocytes (HCs). In *Drosophila*, PVR is mainly expressed in differentiating cells and plays an important role in activating progenitor cell proliferation and differentiation [7]. The Pl_PVR1 protein domain structure shows similarity with other PDGF/VEGF family proteins, but the crayfish Pl_PVR1 contains a nonspecific STYKc, which can phosphorylate serine/threonine/tyrosine amino acid residues. Since Pl_PVR1 mRNA expression was rarely found in HPT cells and highly expressed in hemocytes, it is reasonable to believe that this receptor protein has a role in hematopoiesis. Moreover, more mature cells exhibited higher expression than HPT cells, suggesting that Pl_PVR1 can be used as a cell marker of maturation.

We next investigated the function of PVF/PVR in hematopoiesis by inhibiting the PVF/PVR downstream signaling pathway using Sunitinib malate. Sunitinib malate is well known to be a multi-targeted receptor tyrosine kinase inhibitor. Sunitinib malate has antitumor and antiangiogenic activity and, thus, has been used in the treatment of human malignancies [23]. PVR signaling inhibition with Sunitinib malate has

also been reported in *Drosophila* and leads to a decrease in cell expansion and hemocyte number [24]. In contrast, at high concentrations of Sunitinib malate (1 μM and 10 μM), the HPT cells developed a flat spreading morphology and increased their cell surface area. The morphology change induced in HPT cells as a result of PVF/PVR signaling pathway inhibition may be a sign of induced cell migration.

Initial experiments showed a potential role for the PVR signaling pathway in controlling HPT cell behavior, especially the spreading of cells. In crayfish HPT cell culture, crude Ast1 was used as a supplement to maintain stem cell activity during *in vitro* cell culture. Pl_PVR signaling inhibition with Sunitinib malate in the presence of crude Ast1 induced a combinatorial effect on cell spreading. Sunitinib malate is a small molecule inhibitor that can penetrate through cell membranes and target the ATP-binding site of the intracellular TK domain. As a competitive inhibitor of ATP, PVR signaling inhibition results in increased concentrations of ATP [25]. In dendritic cells (DCs), extracellular ATP is required for cell survival and differentiation of dendritic cell precursors into mature DCs [26]. The importance of extracellular ATP in cell migration was shown in lung cancer cells, A549 cells. Autocrine signaling through exocytosis of ATP was required for activated P2 receptor and amplified TGF-β 1-induced migration of lung cancer cells [27]. Ast1 was reported to interact with the β-subunit of ATP synthase on HPT cell membranes and resulted in a block in ATP formation [28]. Therefore, the increase in the number of spread cells when cells were supplemented with crude Ast1 together with PVR signaling inhibition might be a result of changes in ATP level.

TGase is a Ca^{2+}-dependent crosslinking enzyme that has both non-enzymatic and enzymatic functions [29]. We have shown that extracellular TGase activity is required for maintaining progenitor cells inside HPT [14,15]. Higher extracellular TGase activity was found in round-shaped HPT cells than in migrating cells. In NHDF fibroblast cells, the co-localization of TGase with PDGFR and integrin has been reported. The integrin-associated extracellular TGase acts as a physical link between integrin and PDGFR on the cell membrane to promote both adhesion-mediated and growth factor-induced PDGFR signaling [30].

Extracellular TGase stabilizes the interaction of PDGFR with integrin by bridging the two receptors on the cell surface and stabilizing their association [30]. We have shown that PVR pathway inhibition caused HPT cell spreading. The spreading morphology of HPT cells after PVR signaling inhibition was different from the "migrating morphology", which has previously been reported. Surprisingly, PVR downstream signaling pathway inhibition resulted in an increase in the extracellular TGase activity on the HPT cell surface. These results suggest a role for extracellular TGase in the control of HPT cell spreading through the PVR signaling pathway. We hypothesize that the accumulation of extracellular TGase on the HPT cell surface might occur through the association of TGase with integrin and PVR on the HPT cell surface. Here, we show evidence that extracellular TGase is not only required to maintain cells in their undifferentiated stage but also may be involved in cell spreading. Recently, we have detected one additional TGase protein with 41 % identity to *P. leniusculus* TGase1 (Accession number AAK69205.1) in the HPT, and this dual role of TGase can possibly be explained by the presence in HPT of two different TGase activities. However, the role of these different enzymes needs further investigation.

In *Drosophila*, the signaling pathway downstream of PVR is required for guiding cell migration [31]. Furthermore, PVR expression serves as an equilibrium signal produced by differentiated cells to control the differentiation of *Drosophila* progenitor cells [7]. In crayfish, PVR signaling inhibition could induce HPT cell spreading and increase the extracellular TGase activity on the cell surface. The cytoskeleton of a cell is made up of microtubules, actin filaments, and intermediate filaments that help to organize the cell compartment and provide a basis for movement [32]. Interestingly, an increase in immunoreactivity and elongation of β-tubulin was observed after PVR signaling was inhibited. Furthermore, inhibition of PVR signaling stimulated HPT progenitor cell migration. Taken together, an increase in extracellular TGase activity on the HPT cell surface may be involved in cell migration as a result of inhibition of PVR signaling. The results of the present studies provide new information and show that PVF/PVR signaling is important for regulating extracellular TGase activity in hematopoiesis.

Conclusion

In conclusion, we have shown that the PVF/PVR signaling pathway plays an important role in controlling progenitor cell behavior, especially cell migration, during crayfish hematopoiesis.

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Figure Legends

Fig. 1. The evolutionarily conserved *Pacifastacus* PVGF/VEGF-related receptor 1 (Pl_PVR1) protein domain compared with the *Drosophila* PDGF/VEGF-like receptor (Dm_Pvr). The Pl_PVR1 sequence, GenBank accession number KY444650, consists of 3 immunoglobulin (IG) domains, as shown in the gray circle; a transmembrane (TM) domain, indicated by the blue square; and a Ser/Thr/Tyr kinase catalytic (STYKc) domain, shown in the orange square. The Dm_Pvr sequence, GenBank accession number NM_001201792.2, consists of 6 immunoglobulin (IG) domains, as shown in the gray circle; a transmembrane (TM) domain, indicated by the blue square; and a Tyr kinase catalytic (TyrKc) domain, shown in the red square.

Fig. 2. Higher expression of Pl_PVR1 mRNA in mature hemocytes, granular cells (GCs) and semi-granular cells (SGCs), than in immature HPT cells shown by in situ hybridization. Pl_PVR1 expression was detected with anti-sense strand DIG-labeling RNA probes and with a sense strand probe as a negative control. Pl_PVR1 expression is shown as the dark color, which developed in staining buffer containing the NBT/BCIP substrate. A) Granular cells (GCs), B) Semi-granular cells (SGCs), and C) HPT cells.

Fig. 3. The effect of PVR inhibitor (Sunitinib malate) treatment on HPT cell morphology. Inhibition of PVR downstream signaling caused HPT cells to spread. A) Change in the morphology of HPT cells at 48 h after treatment with 1 nM, 10 nM, 1 μM and 10 μM of Sunitinib malate. DMSO in L15 medium served as a control. B) Percentage of spreading cells (spindle-shaped cells and flat-shaped cells) relative to the total number of HPT cells. C) Percentage of round cells relative to the total number of HPT cells. The percentages of cells were calculated by dividing the spreading cells (spindle-shaped cells and flat-shaped cells) or round cells with the total number of cells. The column represents the mean from 5 crayfish from 5 separate experiments, and the error bar represents the SD value. * $P<0.05$, ** $P<0.01$ and

*** P<0.001 indicate a significant difference compared to the control. Five crayfish were used in each experimental group.

Fig. 4. Round-shaped cell, spindle-shaped cell and flat-shaped cell morphology in HPT cell culture. Three different types of HPT cell morphology were observed during different culture conditions. A) The round-shaped cell morphology of HPT cells cultured in normal condition or without crude Ast1. B) The spindle-shaped cell morphology of HPT cells supplemented with crude Ast1 or knockdown of mRNA for TGase. C) The flat-shaped cell morphology of HPT cells treated with 10 μM Sunitinib malate.

Fig. 5. The effect of Sunitinib malate on cell surface area. Individual images of HPT cells at 48 h after treatment with 1 nM, 10 nM, 1 μM or 10 μM of Sunitinib malate were captured at identical magnification. The cell membrane borders were manually traced, and the total surface area was calculated using a calibrated algorithm (http://rsb.info.nih.gov/nih-image) by Image J program. The column represents the mean of cells from 6 crayfish from 6 separate experiments, and the error bars represent the SD value. * P<0.05, ** P<0.01 and *** P<0.001 indicate a significant difference. Six crayfish were used in each experimental group.

Fig. 6. PVR downstream signaling inhibition resulted in increased extracellular TGase activity on the membrane of HPT cells. The effect of the PVR inhibitor (Sunitinib malate) at 72 h after 1 nM, 10 nM, 1 μM, and 10 μM Sunitinib malate treatment on extracellular TGase activity was determined after incubating the HPT cells with TGase substrate 5-(biotinamido)-pentylamine ($BPNH_2$) for 16 h. DMSO in L15 medium served as a control. The crosslinking of the substrate was visualized with the addition of streptavidin-FITC (green) under a fluorescence microscope. Hoechst 33258 (blue) was used as a nuclear stain. The experiments were repeated 3-5 times.

Fig. 7. Crude Ast1 resulted in an enhanced effect on cell spreading after PVR inhibitor (Sunitinib malate) treatment. Supplementation of HPT cell culture with crude Ast1 rapidly enriched the effect of Sunitinib malate at a lower concentration (1 μM) on HPT cell spreading. A) HPT cell morphology at 24 h after 1 μM and 10 μM Sunitinib malate treatment in the presence or absence of crude Ast1. DMSO and Control (L15 medium) served as a control. B) Percentage of spreading cells (spindle-shaped and flat-shaped cells) relative to the total number of HPT cells. C) Percentage of round cells relative to the total number of HPT cells. The percentage of cells was calculated by dividing the spreading cells (spindle-shaped cells and flat-shaped cells) or round cells with the total number of cells. The column represents the mean of 5 crayfish in each experimental group and the experiments were repeated 3-5 times. The SD value. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate a significant difference.

Fig. 8. PVR pathway inhibition induced the migration of HPT cells in the presence of crude Ast1. HPT cells were cultured in a culture chamber containing polyethylene terephthalate (PET) tract-etched membranes. Cells were incubated with 5 μM Sunitinib malate for 48 h in the presence of crude Ast1. Cells that migrated to the bottom side of the membrane were counted. The column represents the mean of 7 separate experiments with 7 crayfish in each experimental group. The error bars represent the SD value. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate a significant difference.

Fig.9. PVR inhibitor (Sunitinib malate) treatment increased the immunoreactivity for β-tubulin and elongation of β-tubulin filaments. The β-tubulin organization of round and flat-shaped cells (spreading cells) after Sunitinib malate treatment or DMSO (as control), was detected by immunocytochemistry using a β-tubulin antibody (green) in HPT cells. Hoechst 33258 (blue) was used as a nuclear stain. Four crayfish were used in each experimental group, and the experiment was repeated 3 times.

Author Disclosure Statement

The authors hereby certify that they have no financial or commercial associations that might create a conflict of interest in connection with this article.

Figure 1

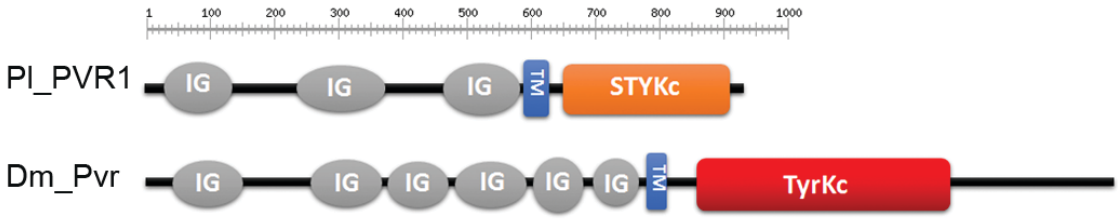


Figure 2

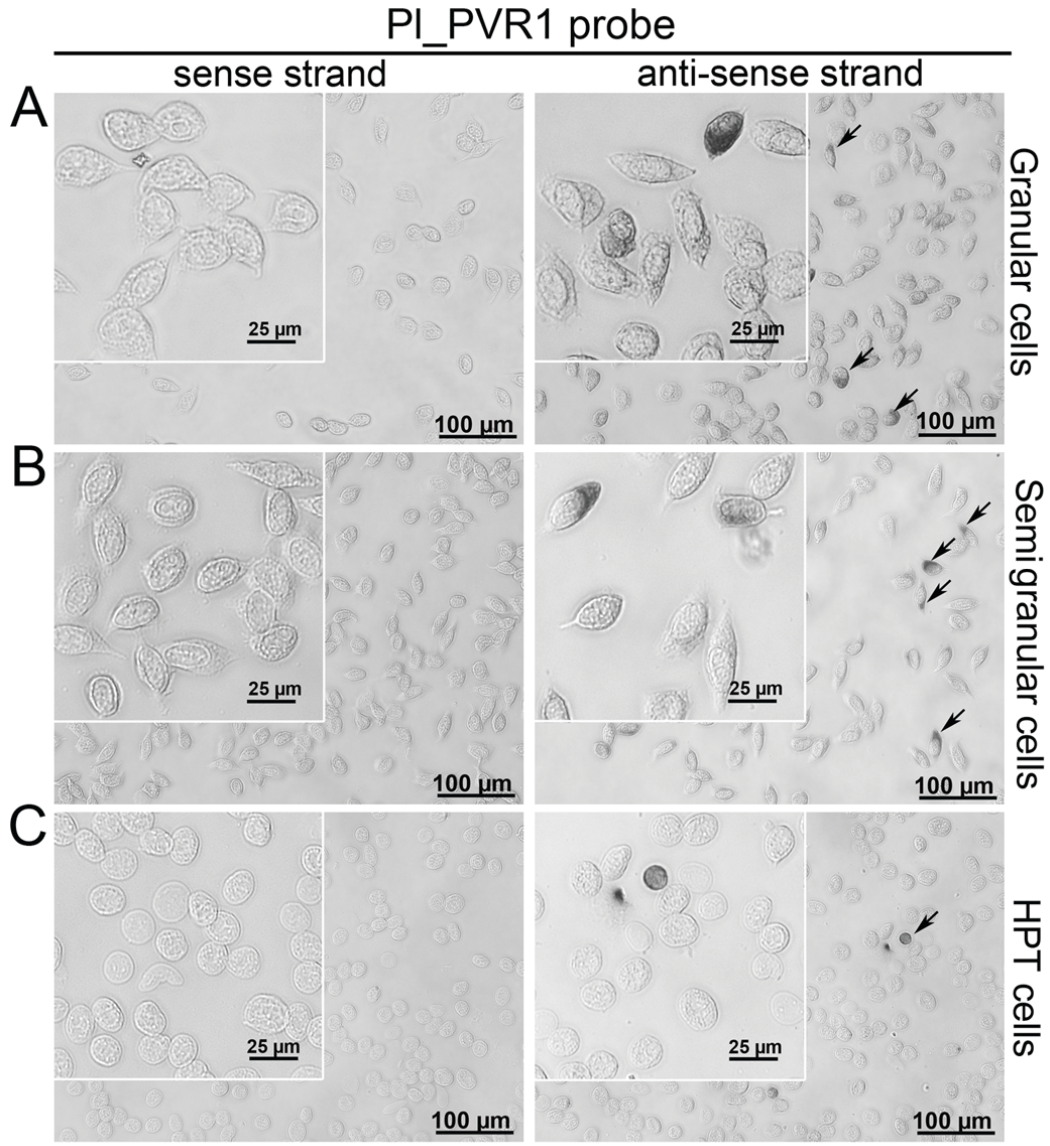


Figure 3

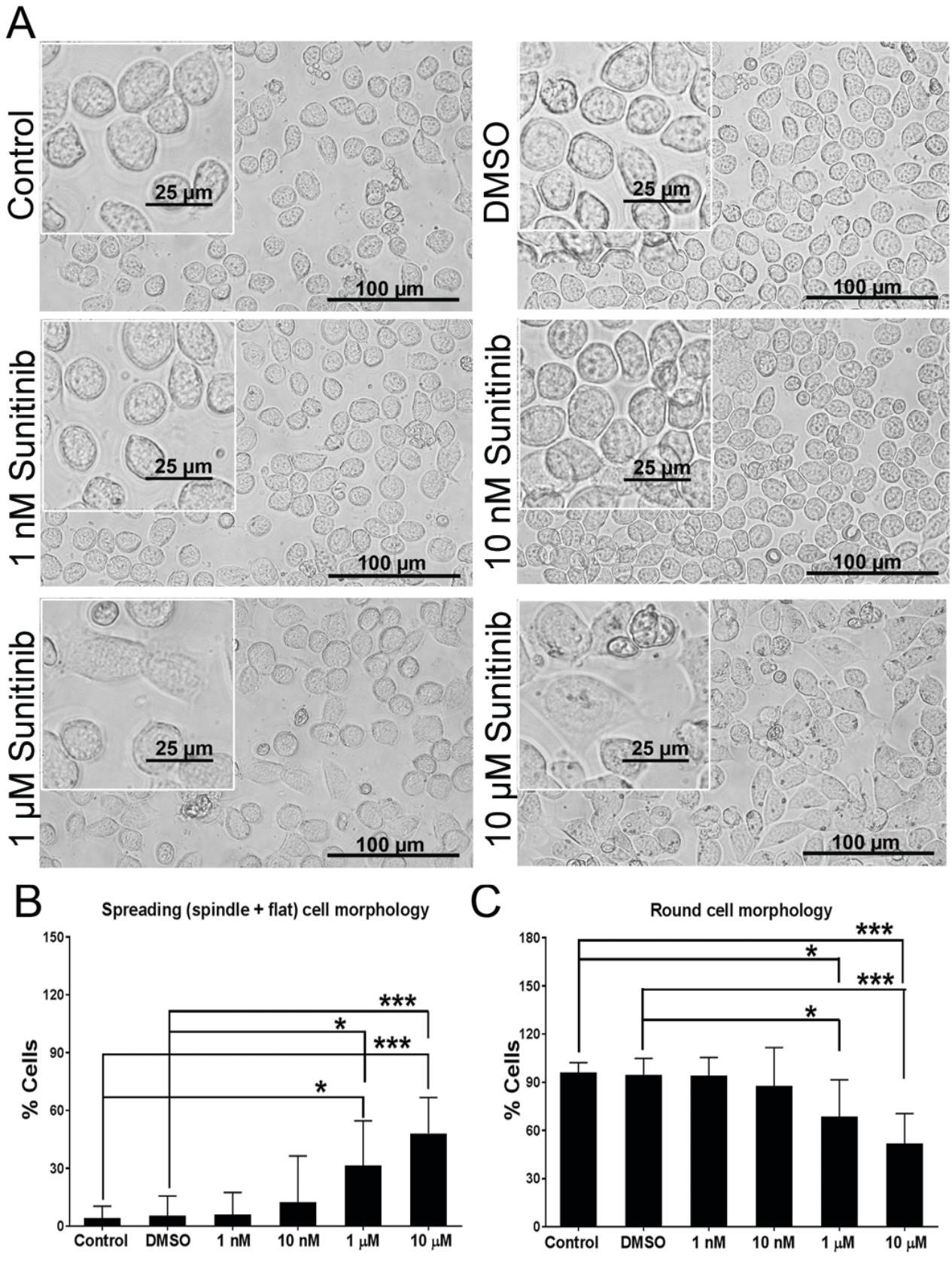

A
Control
DMSO
1 nM Sunitinib
10 nM Sunitinib
1 µM Sunitinib
10 µM Sunitinib
25 µm
100 µm
B
Spreading (spindle + flat) cell morphology
% Cells
150
120
90
60
30
0
Control
DMSO
1 nM
10 nM
1 µM
10 µM
***
*
***
*
C
Round cell morphology
% Cells
180
150
120
90
60
30
0
Control
DMSO
1 nM
10 nM
1 µM
10 µM
***
*
***
*

Figure 4

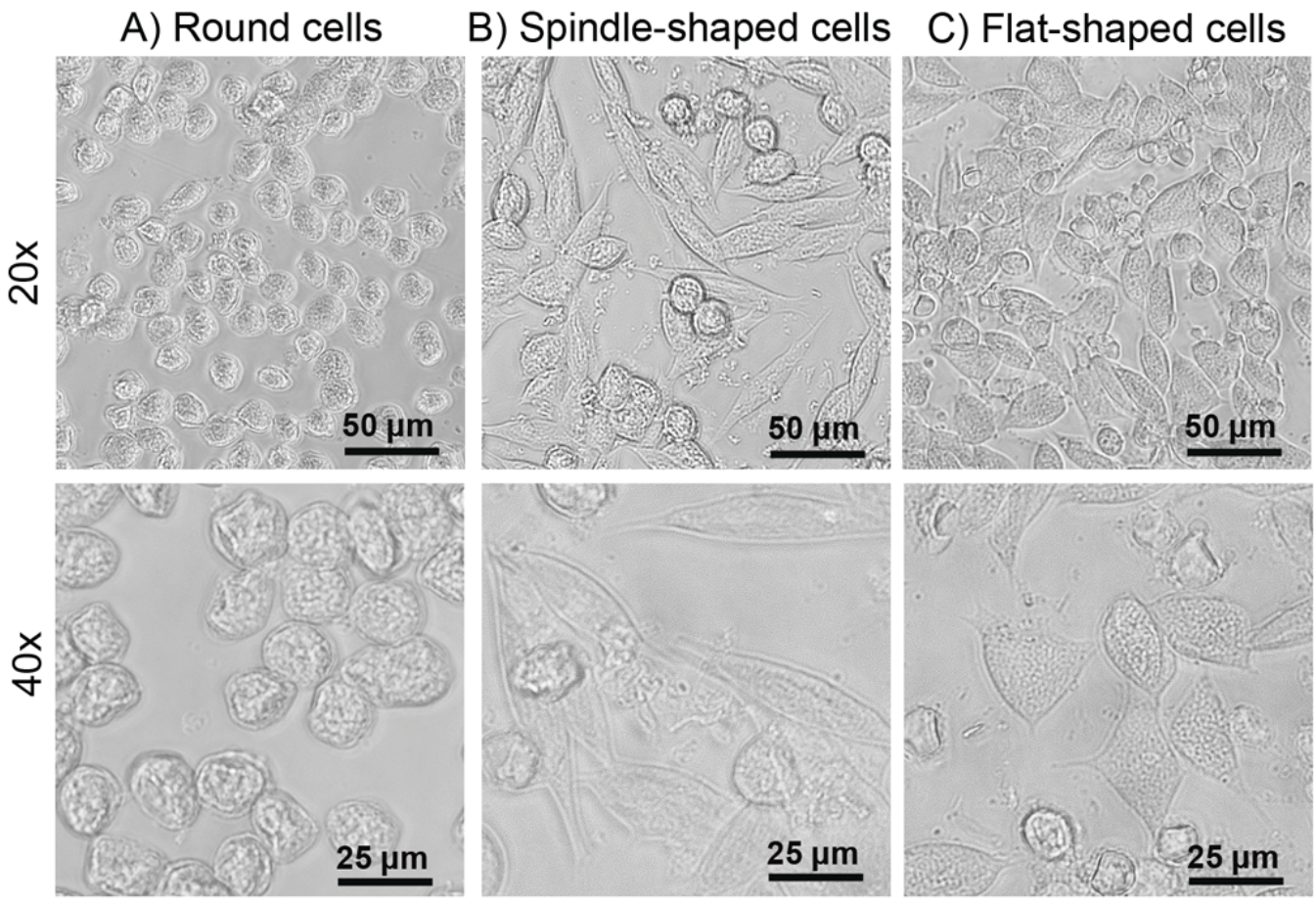
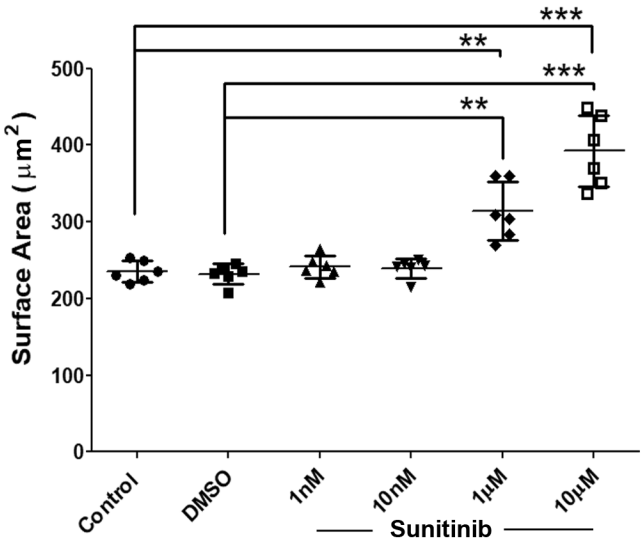

A) Round cells
B) Spindle-shaped cells
C) Flat-shaped cells
20x
40x
50 µm
50 µm
50 µm
25 µm
25 µm
25 µm

Figure 5



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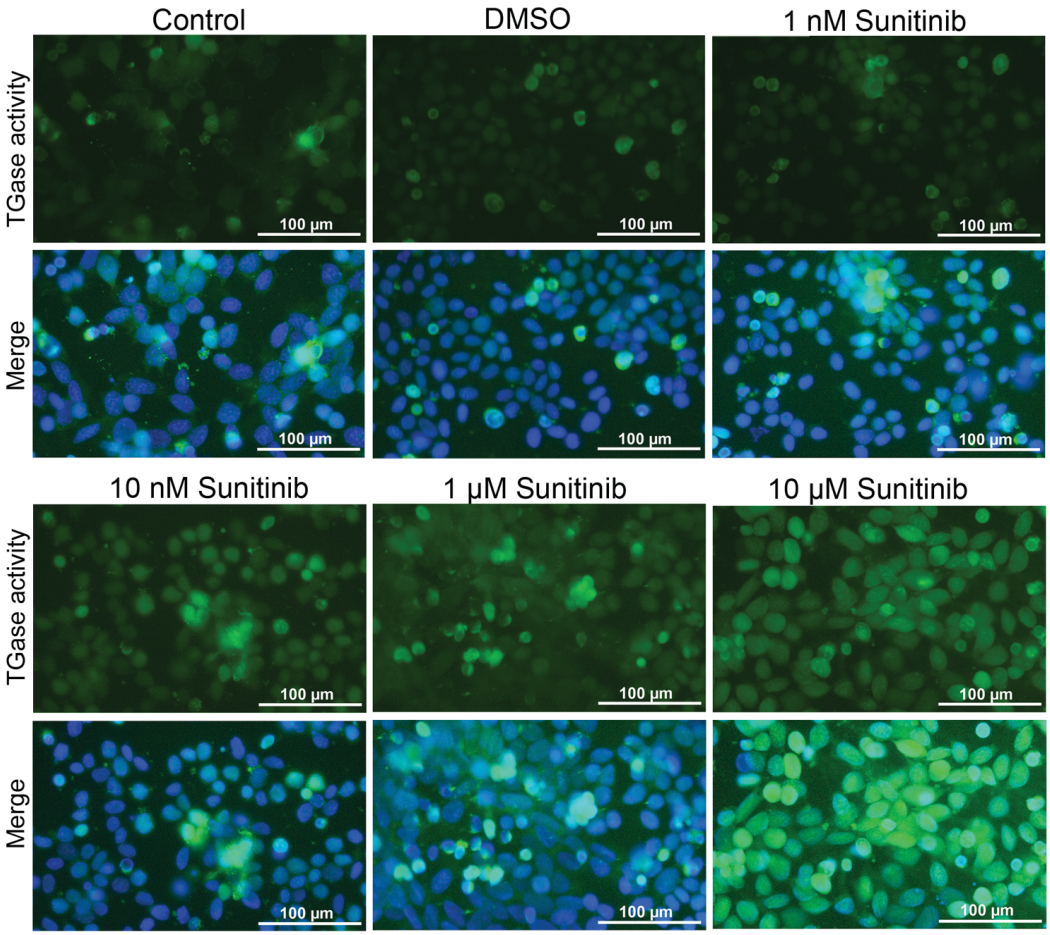
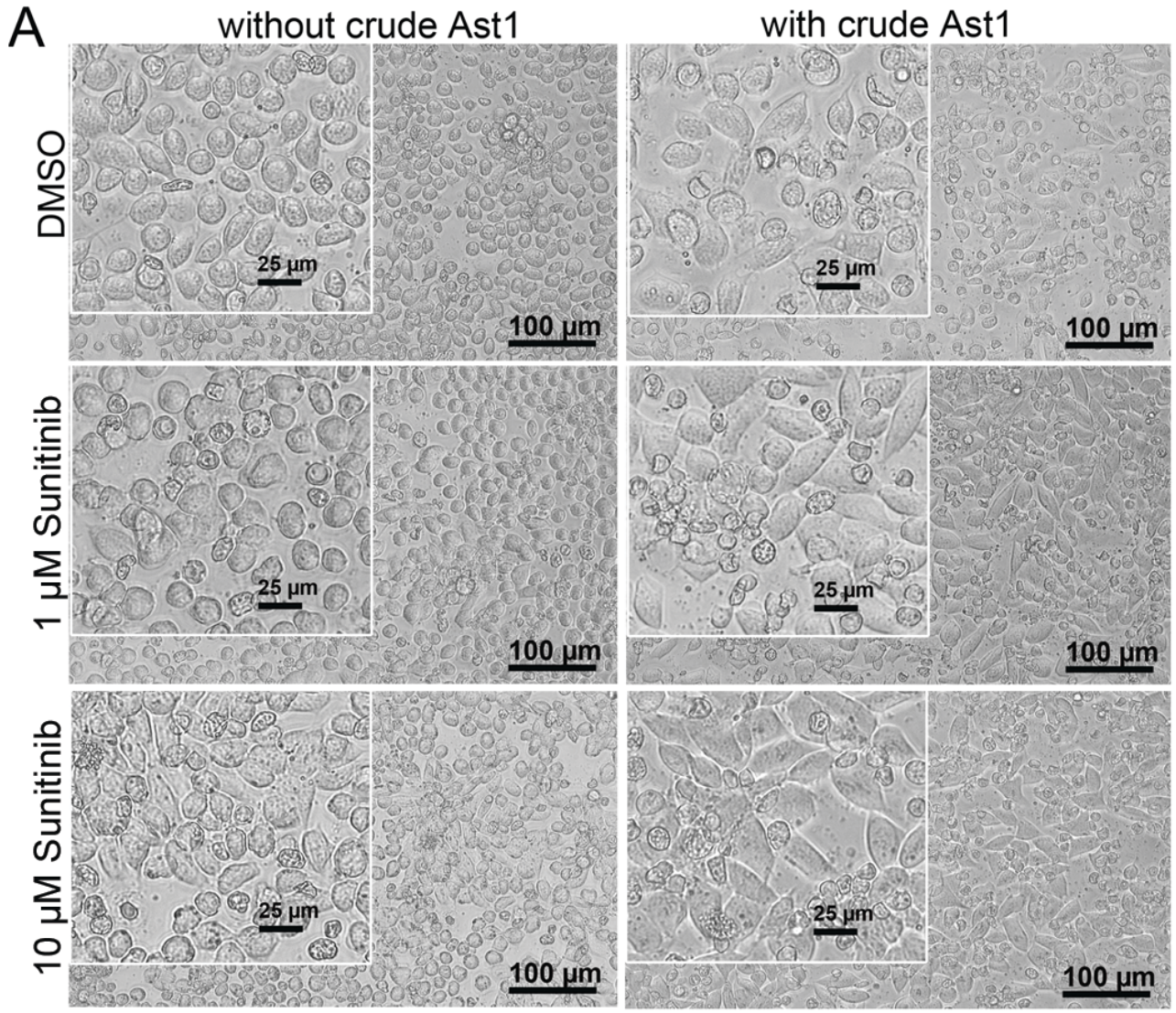
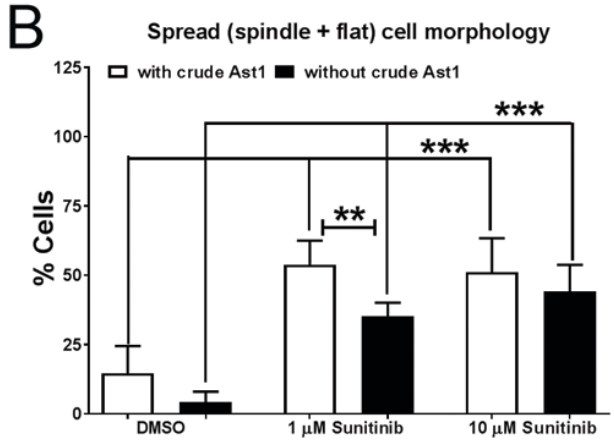


Figure 7


A
without crude Ast1
with crude Ast1
DMSO
1 μM Sunitinib
10 μM Sunitinib
25 μm
100 μm


B
Spread (spindle + flat) cell morphology
with crude Ast1
without crude Ast1
% Cells
125
100
75
50
25
0
***
***
**
DMSO
1 μM Sunitinib
10 μM Sunitinib

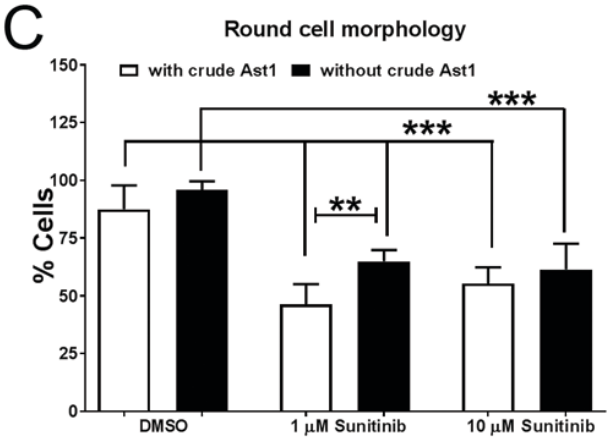

C
Round cell morphology
with crude Ast1
without crude Ast1
% Cells
150
125
100
75
50
25
0
***
***
**
DMSO
1 μM Sunitinib
10 μM Sunitinib

Figure 8

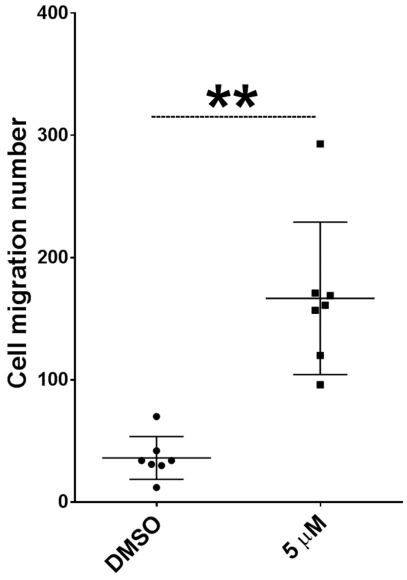
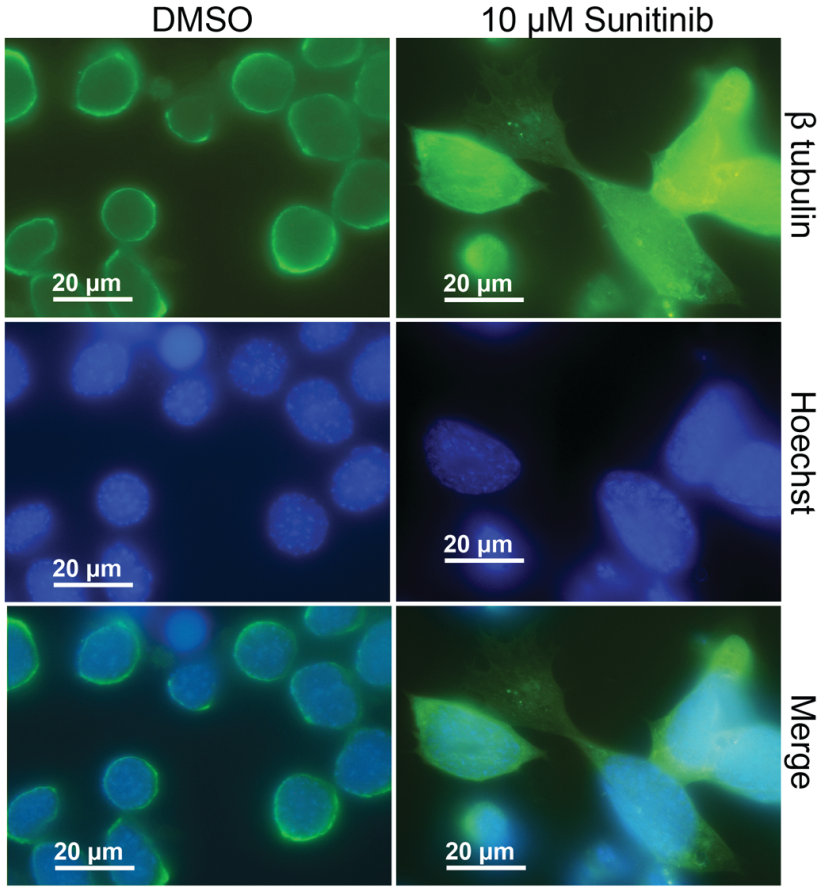


Figure 9



Supplementary Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')
Pl_PVR1_Fw	AGCCTTCGGGCGAGTGTACCG
Pl_PVR1_Rv	TGGAACAGAAGGCAGCAGCGTG
40S_Fw	CCAGGACCCCCAAACTTCTTAG
40S-Rv	GAAAACTGCCACAGCCGTTG

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