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Suppressing Expression of SERPINE1/PAI1 Through Activation of GPER1 Reduces Progression of Vulvar Carcinoma

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Abstract. *Background/Aim: The serine proteinase inhibitor 1 (SERPINE1) gene codes for the plasminogen activator inhibitor 1 (PAI1) protein and is thought to play a tumor supportive role in various cancers. In this work we aimed to uncover the role PAI1 plays in the proliferation, migration, and invasion of vulvar cancer (VC), and define the protein's function as an oncogene or tumor suppressor. Materials and Methods: Through treatment with an agonist (G1) and antagonist (G36) of G-coupled estrogen receptor 1 (GPER1), an upstream regulator of SERPINE1 expression, and a forward transfection knockdown protocol, the expression of SERPINE1/PAI1 in VC cells was altered. The effects these altered SERPINE1/PAI1 levels had on tumor cell functions were then examined. Proliferation was analyzed using the resazurin assay, while migration was studied via the gap closure assay. Through colony- and tumor sphere- formation assays clonogenicity was tested, and western blots showed protein expression. Results: In A431 VC cells, when the levels of PAI1 were reduced via knockdown or treatment with G1, migration, proliferation, and colony growth was reduced. Treatment with G36 increased expression of PAI1 and increased migration and colony size in CAL39 cells. Conclusion: Based on the findings in this study, suppressing PAI1 expression in VC cells appears to reduce their progression and tumorigenic potential. Therefore, PAI1 could possibly function as an oncogene in VC. GPER1 appears to be a suitable target for suppressing PAI1 in VC.*

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Vulva cancer (VC) is an uncommon yet relevant gynecological cancer, diagnosed in roughly 45,000 new patients worldwide and resulting in 17,000 deaths every year (1). Most VC are squamous cell carcinomas (SCC) in older women, unassociated with human papillomavirus (HPV) infection. In younger women diagnosed with SCC, the tumors are more frequently related to a high-risk type of HPV (2, 3). Other, rarer forms of VCs include adenocarcinoma, melanoma, and sarcoma although they account for a far less significant number of cancers (2, 3). The established vulvar SCC (VSCC) cell lines A431 and CAL39 are both HPV negative and as the most common form of VC, were selected as the focus of this study. Surgical excision is currently the treatment standard for VC. Adjuvant radiochemotherapy or chemoradiotherapy is indicated in cases where clear margins cannot be achieved with surgery alone, or in tumors that have metastasized to regional or distant lymph nodes (4). With an increasing number of diagnoses in younger women, an aging population contributing to a rise in incidence, and VC patients experiencing the longest delay in diagnosis of all gynecological malignancies, now more than ever, an essential point of research is understanding the pathways involved in the aggressive features of VC at all stages of the disease (5, 6). Finding genes and proteins that play relevant roles in the development and progression of vulvar malignancies is a crucial step on the way to finding new potential therapeutic targets, developing drugs, and ultimately, successfully treating not only local, but also disseminated disease.

The serine proteinase inhibitor 1 (SERPINE1) gene codes for the plasminogen activator inhibitor 1 (PAI1) protein, a serpin responsible for blocking the activating factors of fibrinolysis, *i.e.*, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), and therefore, inhibiting the breakdown of blood clots (7). As part of the coagulation system, PAI1 plays an important role in normal pregnancy (8) and many different acute and chronic illnesses including cardiovascular disease (9), tissue fibrosis, various cancers and other age related diseases (10), as well as reproductive illnesses (8). Generally, in cancer tissues, PAI1 has been associated with angiogenesis, migration, growth, and invasion of tumors and linked to poor prognosis (10, 11).

However, in some breast, ovarian, and endometrial cancer cell lines, overexpression of PAI1 also reduced the invasion and migration (11). Filling a notable gap in the available literature, in this study we set out to uncover what effect the level of PAI1 had on VSCC cell lines. We hypothesized PAI1 is a downstream protein whose expression is thought to be regulated by the G-Protein-coupled estrogen receptor 1 (GPER1/GPR30). At low expression levels, GPER1 has a tumor-suppressive effect in some types of gynecological cancer (12, 13), while in other neoplasms and at high expression levels it can also act as an oncogene (13). Specifically, in A431 and CAL39 cell lines, GPER1 has been found to act as a tumor suppressor (14). The variability of the functions of GPER1 is mirrored in the plethora of complex signal cascades that it activates, triggering rapid estrogen effects in target cells. Manipulating GPER1 activity in VC cell lines with an agonist (G1) or an antagonist (G36) therefore can affect many pathways including one involved in PAI1 expression.

The first goal of this study was to assess the VC cell behavior with respect to migration and invasion capabilities as well as clonogenicity at varying levels of PAI1 expression. The PAI1 expression levels were manipulated by treating the cell lines with agonists and antagonists of the hypothesized upstream regulator of PAI1, GPER1. The second goal was to alter the PAI1 expression *via* siRNA knockdown of SERPINE1/PAI1 in the cell lines directly and compare their function with control cells. With these objectives in mind, we conducted a comprehensive analysis to address the role of PAI1 in VC and how this protein relates to GPER1.

Materials and Methods

Cell culture. The human SCC cell lines A431 and CAL39 were purchased from the American Type Cell Collection (ATCC; Manassas, VA, USA) and cultured in minimal essential medium (MEM; L0416-500 Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (FBS; South American origin S181B-500, Biowest), 1% penicillin/streptomycin (P/S; Sigma-Aldrich Co., St. Louis, MO, USA), 0.1% transferrin (Sigma-Aldrich Co., St. Louis, MO, USA), and 0.052% insulin (Sanofi, Frankfurt, Germany). To retain the identity of cell lines, purchased cells were expanded and aliquots were frozen in liquid nitrogen. A new frozen stock was used every half year and mycoplasma testing of cultured cell lines was performed routinely using the PCR Mycoplasma Test Kit I/C (PromoCell GmbH, Heidelberg, Germany). Cells were cultured under consistent conditions, in a humidified atmosphere at 37˚C with 5% CO_2.

Small interfering RNA (siRNA) knockdown. Following the forward transfection protocol, 1×10^6 cells for each cell line were seeded in a selection medium (SM; MEM + 10% FBS, 0.1% transferrin, and 0.052% insulin) 24 h before the transfection. After the incubation period, SM was removed and replaced with OptiMEM (31985-047, Gibco, Thermo Fischer Scientific, Rochester, NY, USA) on the adherent cells in the culture flask. The cells were then incubated for 6 h in a transfection mix, consisting of 1 ml of OptiMEM mixed well with 4 μl of diluted siRNA (sc-36179) targeting the SERPINE1/PAI1 gene or siControl (sc-37007) and 4 μl of siRNA transfection reagent (sc-29528, Santa Cruz Biotechnology Inc., Dallas, TX, USA). After the transfection period, MEM+ containing 20% FBS and 2% P/S, 0.1% transferrin, and 0.052% insulin was added to the transfected cells and incubated for an additional 18 h. The medium was changed to the standard culture medium 24 h after transfection. Cells were harvested for experimentation 48 h after transfection in both cell lines.

Cell viability assay. A431 and CAL39 cells were harvested and resuspended, in medium lacking phenol red, Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fischer Scientific) supplemented with 10% FBS, 1% P/S, 0.1% transferrin, and 0.052% insulin. The cell lines were plated on a standard 96 well plate (Sarstedt, Nümbrecht, Germany) with 1.4×10^3 cells per well. 24 hours after seeding, cells were treated with GPER1 agonist G1, GPER1 antagonist G36, DMEM with ethanol, or plain DMEM in the case of knockdown experiments. Cells were allowed an incubation period of 72 h to proliferate under the treatment conditions before the addition of 20 μl of a redox indicator, the fluorogenic dye, resazurin (Thermo Fischer Scientific). The cells were left to react with resazurin in the incubator for 4 h in the A431 cell line, and for 10 h in the CAL39 cell line. In the knockdown experiments both cell lines were given 10 h to react. The relative resazurin reduction was analyzed with a microplate reader (Synergy HT Microplae Reader, BioTek, Winooski, VT, USA) at λ=570 nm and λ=630 nm in the Gen 51.08 program (BioTek).

Gap closure/wound healing assay. VSCC cells were seeded in culture medium on a 24 well plate, with 7×10^4 cells on each side of a 2 well silicone insert (Ibidi, Gräfelfing, Germany) in each well. Twenty-four hours after seeding, the inserts were removed and the cells were treated with G1, G36, ethanol, or culture medium and photographed at 0, 10, and 20h. Using Adobe Photoshop (CS2 Version 9.0, Adobe Systems, San Jose, Ca, USA) and ImageJ (Version 1.53t, Wayne Rasband, National Institutes of Health, USA), gap areas were measured and compared between the various treatment groups.

Colony formation assay. A431 and CAL39 cells were seeded in 2ml of culture medium with 1×10^3 cells per well in a standard 6 well plate (83.3920, Sarstedt AG & Co.). In experimentation with treatments G1 and G36, 24 h after seeding, the medium was exchanged for culture medium containing the appropriate treatment. In the knockdown experiments this step was skipped. A431 cells were allowed to proliferate for 7 days while CAL39 cells for 10 days. Just before the colonies became confluent, the medium was replaced with pure methanol (Chemsolute, Th. Geyer GmbH & Co., Renningen, Germany) for 20 min to arrest colony expansion. Reusable crystal violet dye (Sigma-Aldrich Co., St. Louis, MO, USA) was then added to the wells for 20 minutes. The plates were scanned (Epson V850 Pro, Epson Europe B.V., Amsterdam Zuidoost, the Netherlands) and ImageJ was used to measure the number and size of the colonies.

Tumor sphere formation assay. Both cell lines were resuspended in culture medium and plated with 1×10^3 cells per well on a 96 well, ultra-low attachment surface plate (3474, Corning Inc. Life Sciences, Corning, NY, USA). Twenty-four hours after seeding, the wells were treated with 100 μl of medium containing either G1,

G36, or the ethanol control. For the knockdown wells, the cells were harvested 48 h after transfection and treated with regular culture medium 24 h after seeding. The plates were photographed (Celigo Cyntellect; Celigo, Redwood, CA, USA) every 72 h for 21 days. ImageJ was used to analyze the number and size of the tumor spheres after 15 days.

Western blot analysis. A431 and CAL39 cells were lysed with the use of Celllytic M cell lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.1% protease (Sigma-Aldrich) and 0.1% phosphatase inhibitors (Sigma-Aldrich). 40 μg of the isolated proteins were separated *via* SDS PAGE with 10% sodium dodecyl sulfate (SDS; PanReac AppliChem, Chicago, IL, USA) and transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Merck-Millipore, Burlington, MA, USA) *via* electro-transfer. Primary antibodies targeted PAI1 at a 1:1,000 dilution (13801-1-AP, Proteintech, Planegg-Martinsried, Germany) and GAPDH at 1:2,000 dilution (5174S, Cell Signaling, Danvers, MA, USA). The PVDF membrane containing the proteins was washed and incubated in horseradish peroxidase-conjugated secondary antibody (Donkey anti-rabbit IgG, 711-035-152; Dianova, Hamburg, Germany). Protein bands bound to the antibodies were assayed using a chemiluminescent luminol enhancer solution (PAI1, Westar Supernova: XLS3,0100, Cyanagen, Bologna, Italy; GAPDH: Westar Antares, XLS142,0250, Cyanagen).

Statistical analysis. All experiments in this study were performed in biological triplicates and technical duplicates at minimum. One-way ANOVA, two-way ANOVA, and unpaired *t*-tests were performed in GraphPad Prism (v. 8.0.1, GraphPad Software, Inc., San Diego, CA, USA). The one-way ANOVA analysis was followed by Dunnetts's or Tukey's multiple comparisons test. Unpaired, two-tailed, parametric *t*-tests were used under the assumption that both of the compared groups (knockdown and control) had the same standard deviation. Results were considered statistically significant when $p<0.05$.

Results

PAI1 expression is impacted by treating A431 and CAL39 cells with GPER1 agonist G1 and antagonist G36. Relative to control cells, PAI1 protein expression diminished when A431 cells were treated with G1 (Figure 1A). When the G1 concentration was increased to 1 μM the reduction in PAI1 expression was found to be statistically significant (28.34%±4.845% $p<0.01$ using one-way ANOVA followed by Dunnett's multiple comparisons test). Treating A431 cells with G36 (Figure 1B) caused a visible increase in PAI1 expression in the Western blots. However, upon quantification, none of the tested concentrations yielded statistically significant results.

Concerning the CAL39 cell line, treatment with G1 (Figure 1C) did not produce statistically significant changes in PAI1 expression at any tested concentration. At the same time, no tendencies in the cell behavior were discovered under this treatment either. G36 treatment also did not yield statistically significant results upon one-way ANOVA analysis. However, in both the blots and the quantification, a strong tendency of a dose dependent increase in PAI1 expression was observed under these conditions (Figure 1D).

Cell proliferation of VSCC cell lines is impacted by treatment with GPER agonist G1. A431 cells (Figure 1E) and CAL39 cells (Figure 1F) treated with the ethanol control, in comparison to those treated only with culture medium, showed no significant reduction in cell proliferation. This finding was determined through statistical analysis with an ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. Therefore, the ethanol control was set to 100% proliferation for further statistical analysis and all treatment concentrations were considered relative to this group as the control.

The cell proliferation in A431 cells treated with G1 showed a dose dependent reduction at the tested concentrations. At 1 μM of G1, 51.53%±5.548% proliferation was measured relative to the control cells ($p<0.001$). At 2.5 μM of G1 the A431 cells continued to show a significant reduction in proliferation (M=17.85%±1.589% *vs*. control; $p<0.0001$). At the highest G1 concentration of 5 μM, proliferation of A431 cells averaged 11.30%±2.084% relative to the ethanol control ($p<0.0001$). Treatment with the G36 antagonist showed a slightly increased proliferation and viability; however, in the statistical analysis, the changes were not found to be significant at any of the tested concentrations.

CAL39 cells treated with medium containing 1 μM of G1 were not significantly impacted by the treatment. However, when the G1 concentration was increased to 2.5 μM, a significant reduction in proliferation was observed (M=65.44%±9.844% *vs*. control; $p<0.01$). At the highest concentration of the G1 treatment (5 μM), CAL39 cells also showed a significant reduction in proliferation (M=36.28%±6.525% *vs*. control; $p<0.0001$). CAL39 cells treated with G36 in all tested concentrations showed a higher average proliferation, but not at a statistically significant level.

Migration is reduced when VSCC cell lines are treated with GPER1 agonist G1. The changes in the remaining gap area were used as a measure of cell migration in the tested cell lines. A two-way ANOVA statistical test was performed comparing the remaining gap areas in treatment versus control cells at each of the specified time intervals. A431 cells (Figure 2A) treated with 1 μM of agonist G1 showed no significant difference in gap area compared to control cells at 10h or 20h. When the concentration of G1 treatment was increased to 2.5 μM at 10h, the relative gap area remained insignificant compared to control; however, at 20h, significantly more gap area remained open compared to control cells (M=0.470±0.075 *vs*. control= 0.183±0.039; $p<0.05$). At the highest concentration of G1 treatment (5 μM), A431 cells showed significantly more unclosed gap area at both 10h and

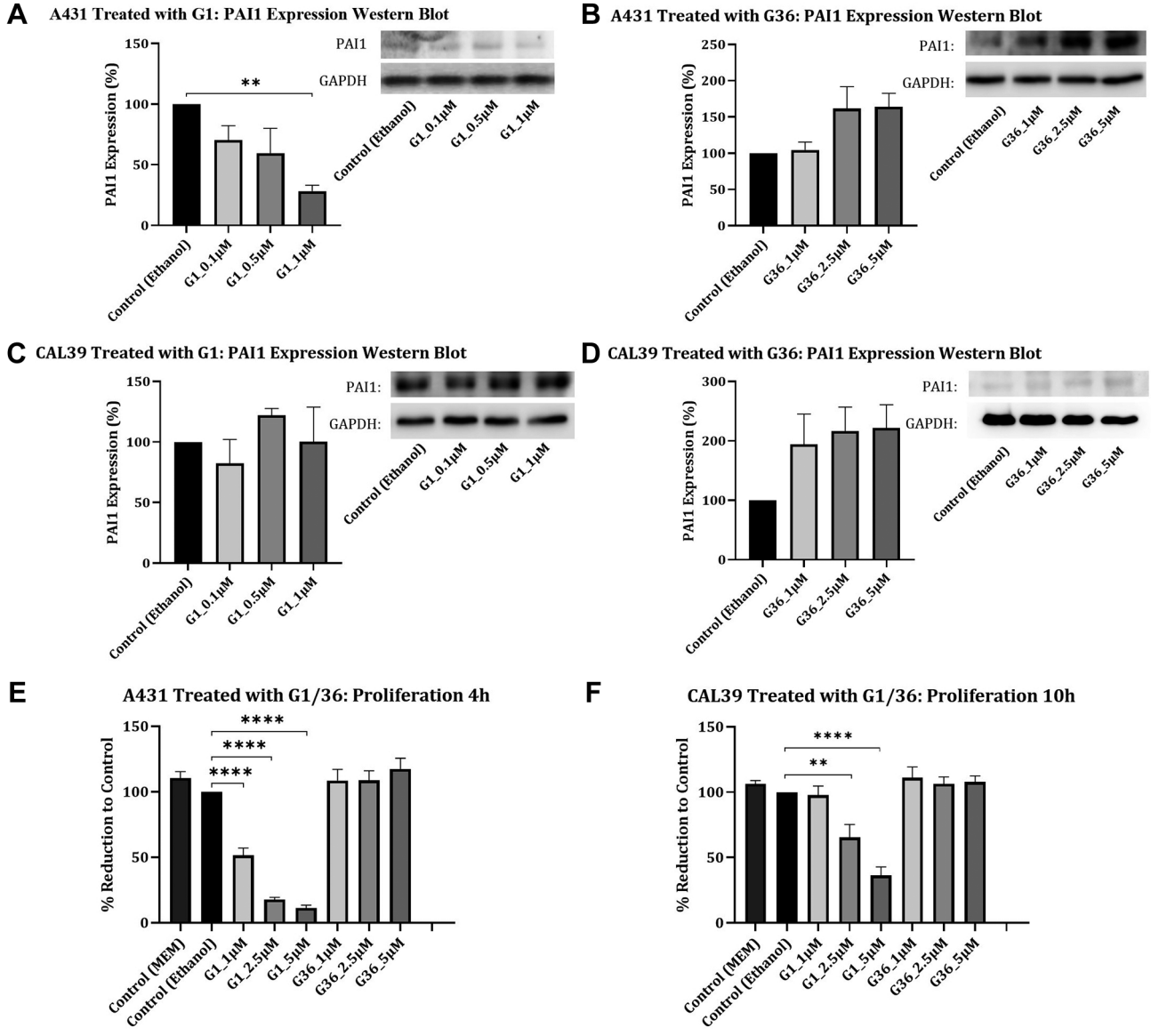

Figure 1. *A-D: Plasminogen activator inhibitor 1 (PAI1) expression is impacted by treating A431 and CAL39 cells with G-coupled estrogen receptor 1 (GPER1) agonist G1 and antagonist G36 (quantification via western blot). Expression of PAI1 in A431 (A, B) and CAL39 (C, D) cells treated with GPER1 agonist G1 (A, C) and GPER1 antagonist G36 (B, D) relative to ethanol treated control cells. Protein isolation was performed 72 h after treatment. E-F: Cell proliferation in vulvar squamous cell carcinoma (VSCC) cell lines is impacted by treatment with GPER agonist G1. A431 (E) and CAL39 (F) cells treated with ethanol control, GPER1 agonist G1 and GPER1 antagonist G36. Results measured relative to ethanol control after 4h in A431 and 10h in CAL39 cell line. Ordinary one-way ANOVA with Dunnett's multiple comparisons test, mean with standard error of the mean (SEM); (A-C) n=3, (E) n=4, (F) n=11; **p<0.01 and ****p<0.0001.*

20h (10h M=0.828±0.046 *vs*. control=0.574±0.038; $p<0.01$; 20h M=0.782±0.055 *vs*. control=0.183±0.039; $p<0.0001$).

Treating CAL39 cells (Figure 2B) with low doses of the GPER1 agonist G1 showed no effect on the gap area. Treatment with 1 μM and 2.5 μM showed no statistically relevant changes in gap area relative to the cells treated with the control at 10h or at 20h. At 10h, the highest concentration of G1 treatment also lacked an effect on CAL39 cells, however, after 20h of treatment, the gap area was significantly greater than the gap left between control cells (M=0.465±0.079 *vs*. control=0.202±0.053; $p<0.05$).

CAL39 VSCC cell lines treated with G36 display heightened migration capability. Changes in the gap area of A431 cells treated with an antagonist of GPER1, G36 (Figure 2E), relative to the area in the control cell group, were not found

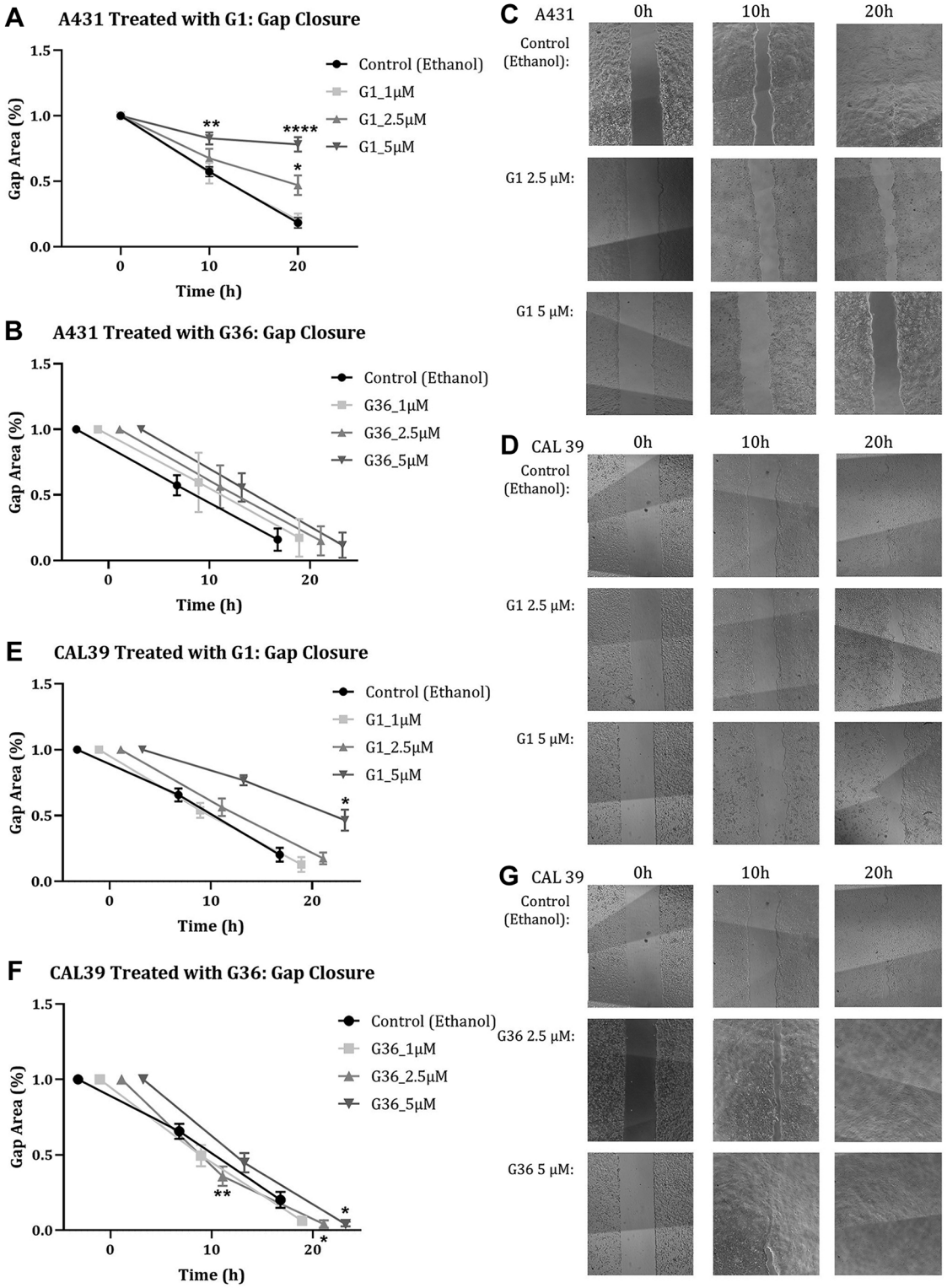

Figure 2. *Migration of VSCC cells is reduced when cell lines are treated with GPER1 agonist G1. CAL39 VSCC cells treated with G36 display heightened migration capability. Gap closure in A431 (A, C) and CAL39 (B, D) cell lines after treatment with GPER1 agonist G1 compared to ethanol control. Photos of representative gaps in A431 (C) and CAL39 (D). Gap closure in A431 (E) and CAL39 (F, G) cell lines after treatment with GPER1 antagonist G36 compared to ethanol control. (G) Photos of the gaps in CAL39 cells following treatment with G36 at higher concentrations photographed at 10h intervals; magnification ×4. 2-way ANOVA with Dunnett's multiple comparisons test, mean with standard error of the mean (SEM); (A) n=6, (B) n=9, (E, F) n=9; *p<0.05, **p<0.01 and ****p<0.0001. The asterisks refer to significant differences between the corresponding graph line and the ethanol control.*

to be statistically significant on two-way ANOVA analysis, no matter the treatment concentration.

CAL39 cells' migration behavior differed from the A431 cells when treated with G36 (Figure 2F). At the lowest concentration (1 μM), the gap area in the treated cells was not significantly impacted relative to the control group cells. CAL39 cells treated with 2.5 μM of G36, however, showed significantly less gap area at the measured time intervals compared to the ethanol control cells (10h: M=0.358±0.064 *vs*. control=0.657±0.050; $p<0.01$; 20h: M=0.041±0.025 *vs*. control=0.202±0.053; $p<0.05$). When treated with 5 μM of G36, the relative gap area at 10h was just shy of significance with a *p*-value of 0.0515 (10h: M=0.448±0.064 *vs*. control=0.657±0.050; p=0.0515), suggesting a treatment effect. At 20h, significantly more gap closure was observed compared to control cells (M=0.041±0.015 *vs*. control=0.202±0.053; $p<0.05$).

GPER1 agonist G1 inhibits colony formation and size, while GPER1 antagonist G36 promotes colony size. The colonies formed by each cell line showed different morphological features when observed with the naked eye. How they each reacted to the treatment, however, showed similar results on statistical analysis *via* ordinary one-way ANOVA. Of the 1,000 seeded A431 cells in the control group, an average of 353.8±26.51 colonies were formed over the course of 7 days (Figure 3A). Relative to this control, A431 cells treated with 1 μM of G1 showed a slight but insignificant reduction in the number of colonies formed. However, when the concentration of G1 was increased to 2.5 μM, the reduction in colonies was drastic, and on statistical analysis, became significant (M=3.222±1.392 colonies *vs*. control; $p<0.0001$). At the highest concentration of G1 (5 μM), these results were maintained (M=0.5556±0.4444 colonies *vs*. control; $p<0.0001$). Treatment with the antagonist G36 showed no significant effects on A431 colony formation at any concentration.

The size of each of the A431 colonies was also measured and the averages were analyzed with one-way ANOVA (Figure 3C). Control cells formed colonies with a mean size of 24.49±0.4704 px. A431 colonies treated with 1 μM G1, showed no significant impact on colony size. Treatment of A431 cells with 2.5 μM G1 (M=10.65±1.54 px *vs*. control; $p<0.0001$) and with 5 μM G1 (M=10.40±1.98 px *vs*. control; $p<0.001$) resulted in significant decrease in colony size compared to control colonies. A significant increase in colony size was observed in A431 cells treated with all tested concentrations of G36 relative to control colonies. At the lowest concentration of 1 μM, the average colony size increased to 28.70±0.7604 px (*vs*. control; $p<0.001$). When the treatment concentration was increased to 2.5 μM, the average colony size was found to be 31.40±0.9753 px (*vs*. control; $p<0.0001$). At the highest concentration of tested G36 treatment, colony size was increased to an average of 29.94±0.8332 px (*vs*. control; $p<0.0001$).

The number of colonies formed by CAL39 cells was significantly impacted by treatment with G1 relative to control cells (Figure 3B). In the control group, a mean number of 203.5±15.34 colonies were formed after treatment for 10 days. Treated cells were compared to this average in order to determine statistical significance in the ANOVA test. A dose dependent decrease in the number of colonies formed was observed in the seeded CAL39 cells treated with G1. At 1 μM, an average of 45.67±38.69 colonies were formed ($p<0.005$). As the concentration of treatment was increased to 2.5 μM, the average number of colonies was reduced to 1.833±0.9804 ($p<0.0001$). Treatment with a concentration of 5 μM significantly reduced the number of CAL39 cell colonies to 0.5000±0.2236 ($p<0.0001$). Treatment with G36 had no significant impact on the number of CAL39 colonies formed at any of the tested concentrations.

The size of the colonies formed by CAL39 cells was also impacted by treatment with both G1 and G36 (Figure 3D). The control cells had an average size of 45.28±3.352 px, and all treatment groups were compared to this average in one-way ANOVA analysis. Colonies treated with 1 μM G1 exhibited significantly reduced colony size relative to the control group (M=16.07±0.8998 px *vs*. control; $p<0.01$). At the higher concentrations of G1, the number of colonies was also significantly reduced. Although the average colony size was even further reduced compared to the 1 μM treatment group, the statistical analysis showed a slightly reduced significance level due to the lower number of colonies (2.5 μM G1, M =13.00±1.24 px *vs*. control; $p<0.05$; 5 μM G1, M=12.00±1.53 px *vs*. control; $p<0.05$). The treatment of CAL39 cells with the G36 antagonist also showed an increase in the average size of colonies formed; however, only at the highest treatment concentration (5 μM G36) did the increase relative to the control become statistically significant (M=61.30±4.068 px *vs*. control; $p<0.05$).

GPER1 agonist G1 inhibits tumor sphere formation and size, while GPER1 antagonist G36 promotes it. Only A431 cells formed tumor spheres during experimentation. The number and size of tumor spheres was analyzed at two separate time points under all treatment conditions. One-way ANOVA was performed on the average number of tumor spheres in treatment groups relative to the control group.

After 15 days of treatment, the same cells were analyzed again (Figure 3E). In the control group only 13.67±0.8819 tumor spheres remained. Treatment with 1 μM of G1 had no significant impact on the number of spheres formed. At the higher concentration of 2.5 μM of G1, a significant reduction in tumor spheres was observed relative to the control cells on day 15 (M=5.667±0.8819 *vs*. control; $p<0.05$). When the concentration of G1 was increased to 5 μM, again a significant reduction in the number of tumor spheres formed was observed (M=4.000±0.000 *vs*. control; $p<0.01$). The

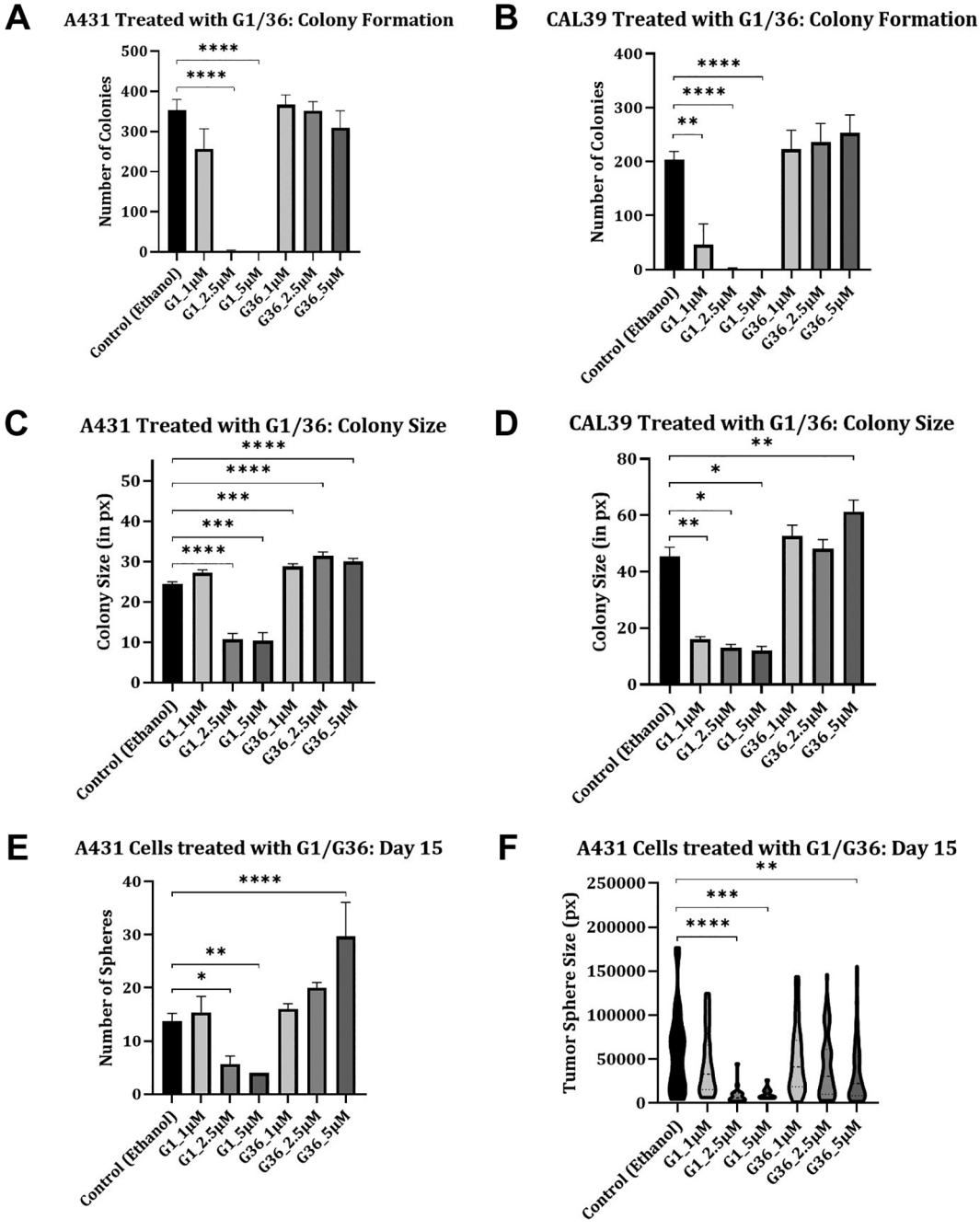

Figure 3. *A-D: Colony formation and size are inhibited under treatment with GPER1 agonist G1. In contrast, colony size is increased under treatment with GPER1 antagonist G36 in vulva carcinoma cell lines. A431 (A) and CAL39 (B) colony formation capability after treatment with G1 and G36 compared to ethanol treated control. A431 (C) and CAL39 (D) colony size after treatment with G1 and G36 compared to ethanol treated control. E, F: Formation (E) and size (F) of tumor spheres derived from A431 VSCC cells are inhibited after 15 days of treatment with GPER1 agonist G1 and increased after 15 days of treatment with GPER1 antagonist G36. Ordinary one-way ANOVA with Dunnett's multiple comparisons test, mean with standard error of the mean (SEM); (A, C) n=9, (B, D) n=6, (E, F) n=3; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.*

number of tumor spheres increased when the cells were treated with G36 over the course of 15 days. Treatment with 1 μM and 2.5 μM of G36 showed a slight but non-significant increase in the average number of tumor spheres. At 5 μM, the highest tested concentration, the increase in tumor sphere formation became significant (M=29.67±3.712, $p<0.0001$).

After 15 days of treatment, tumor sphere size analysis was performed again (Figure 3F), revealing that control spheres

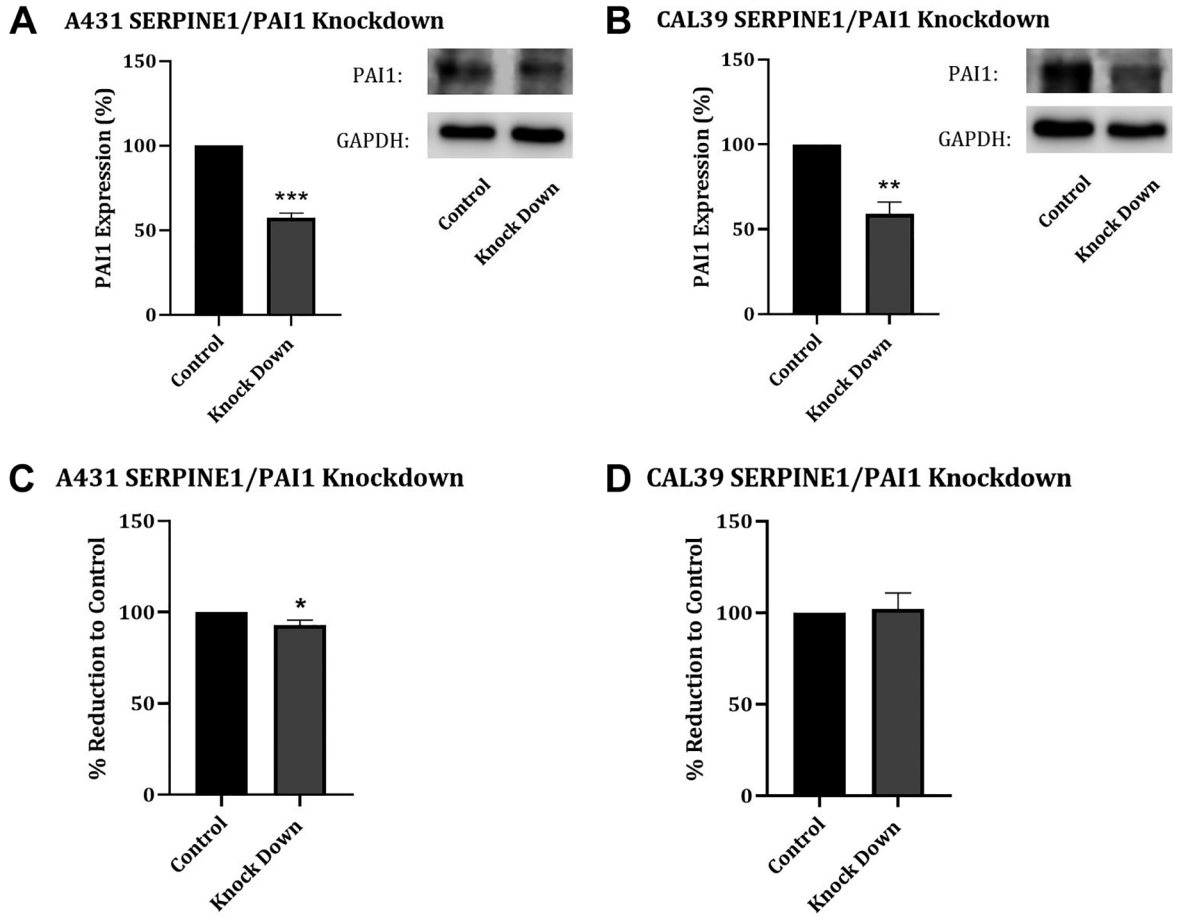

Figure 4. *Successful knockdown of PAI1 is confirmed by western blot (A, B) and the impact of SERPINE1/PAI1 knockdown on cell proliferation in A431 and CAL39 cells is shown (C, D). Relative gene expression of PAI1 in SERPINE1/PAI1 knockdown group compared to siRNA control in A431 (A) and CAL39 (B) cells 48 h after transfection. Cell viability in SERPINE1/PAI1 knockdown and siRNA control cells, in the A431 (C) and CAL39 (D) cell lines. Results measured relative to siRNA control after 10h in both cell lines. Unpaired t-test, two tailed, mean with standard error of the mean (SEM); (A, B) n=2, (C, D) n=9; *p<0.05, **p<0.01 and ***p<0.001.*

had an average size of 58,488±7,226 px. Cells treated with 1 μM of G1 exhibited a non-significant reduction in size. As the concentration of G1 was increased to 2.5 and 5 μM, the reduction in tumor sphere size became significant (2.5 μM: M=9,478±2,481 px *vs*. control; $p<0.0001$; 5 μM: M=10,931±1,815 px *vs*. control; $p<0.001$). Upon treatment with G36, a significant change in tumor sphere size was only observed in cells treated with the highest concentration of the antagonist. When treated with 5 μM of G36, the A431 tumor sphere size significantly decreased relative to control cells (M=34,672±3,645 px *vs*. control; $p<0.01$).

Successful knockdown of SERPINE1/PAI1 was confirmed via western blot. Via unpaired t-test, statistical analysis of quantified western blots was used to determine the relative protein expression of PAI1 in the SERPINE1/PAI1 knockdown group compared to the siRNA control in A431 cells (Figure 4A). The results showed a significant difference, with PAI1 expression in the knockdown group measured at M=57.25±2.892% compared to the control group (100%, $p<0.001$). Reduction of PAI1 expression *via* knockdown in CAL39 was also statistically significant (Figure 4B; M=58.89±7.233% *vs*. control; $p<0.01$).

SERPINE1/PAI1 knockdown impacts cell proliferation in A431 cells. SERPINE1/PAI1 knockdown using the forward transfection technique significantly decreased cell proliferation in the resazurin assay in A431 cells (Figure 4C) relative to the siRNA control group. The knockdown group showed 93.04±2.613% of the cells remained vital compared to the control group's 100% ($p<0.05$). In CAL39 cells (Figure 4D), cell proliferation was not significantly affected by SERPINE1/PAI1 knockdown.

Migration of A431 VSCC cells is reduced after SERPINE1/PAI1 knockdown. The wound healing assay compared the gap area in knockdown cells with siRNA control

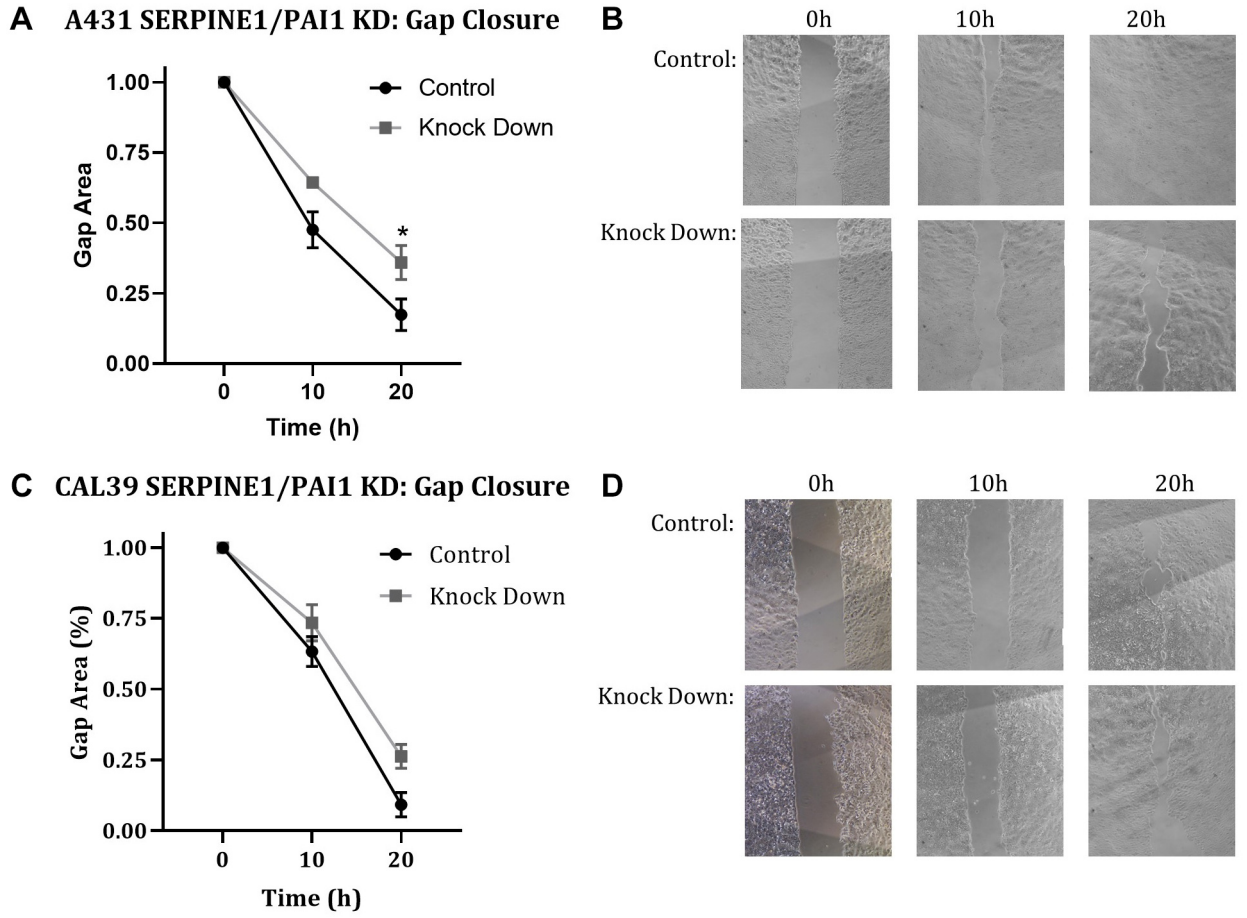

Figure 5. *Migration in A431 vulvar SCC cells is reduced in SERPINE1/PAI1 knockdown cells. Gap closure in A431 (A, B) and CAL39 (C, D) cell lines after a successful SERPINE1/PAI1 knockdown compared with siRNA control. (B, D) Photos of the gaps in knockdown and control at 10h intervals; magnification×4. Ordinary one-way ANOVA with Tukey's multiple comparisons test, mean with standard error of the mean (SEM); n=6; *p<0.05. The asterisks refer to significant differences between the corresponding graph line and the ethanol control.*

cells at established time intervals. The area of the gaps in both test groups at time 0h was normalized to 1.0. A431 knockdown cells (Figure 5A) showed no significant difference in gap area at 10 h relative to control cells. However, at 20 h, knockdown cells showed significantly more remaining gap area than their siRNA control counterparts (KD=0.359±0.060 *vs*. control=0.174±0.056; $p<0.05$).

In the CAL39 cell line (Figure 5C), knockdown cells showed more remaining gap area than control cells; however, in the results of the one-way ANOVA both at 10 and 20 h the change was not found to be statistically significant.

Tumor sphere formation and size are not significantly impacted in the A431 cell line in SERPINE1/PAI1 knockdown cells. On average, the tumor sphere formation at 15 days after transfection (Figure 6A) showed an average reduction in knockdown compared to control cells; however, the difference was not statistically significant. Tumor sphere size at this time point (Figure 6B) was also not significantly reduced in knockdown cells compared to the siRNA control cells.

Colony size is reduced after SERPINE1/PAI1 knockdown in VSCC. In the A431 cell line (Figure 6C), the reduction in the number of colonies formed in knockdown cells compared to control cells was not deemed statistically significant on *t*-test analysis. The area of each colony was measured, averaged, and a *t*-test was performed with the data (Figure 6D). In knockdown cells, colony size was significantly reduced compared to control cells (KD=23.26±0.5086 px *vs*. control=24.83±0.5512 px; $p<0.05$).

On *t*-test analysis, the SERPINE1/PAI1 knockdown in CAL39 cells did not lead to a statistically significant change in the number of colonies formed (Figure 6E). Similarly to the A431 cell line, the knockdown treatment of CAL39 cells led to a significant reduction in colony size (Figure 6F) compared to control cells (KD=38.35±1.852 px *vs*. control=46.80±2.790 px; $p<0.05$).

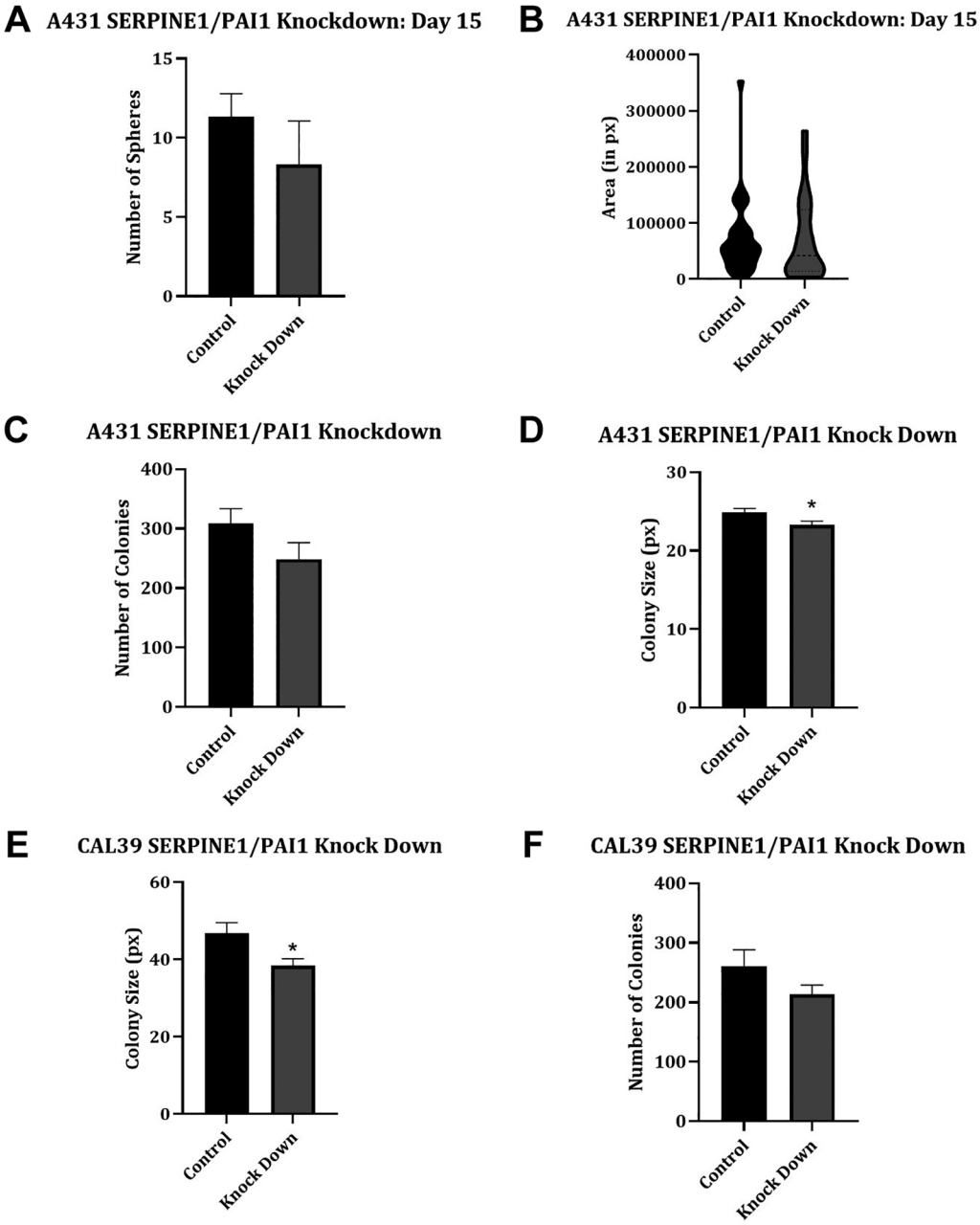

Figure 6. *(A, B) Tumor sphere formation and size are not significantly impacted in the A431 cell line in SERPINE1/PAI1 knockdown cells. 15 days after transfection, A431 tumor sphere formation (A) and size of spheres (B) are shown, relative to siRNA controls. (C-F) Colony size is reduced after SERPINE1/PAI1 knockdown in VSCC. Colony formation relative to siRNA control in A431 (C, D) and CAL39 cells (E, F). Number of colonies formed in A431 (C) and CAL39 (E) cell lines. Colony size in A431 (D) and CAL39 (F) cell lines compared to siRNA control. Unpaired t-test, two tailed, mean with standard error of the mean (SEM); (A, C) n=3, (C-F) n=6; *p<0.05.*

Discussion

Considering all aspects, the results of this study showed that PAI1 was an effective target for reducing the tumorigenic potential of the tested VC cell lines. Using the GPER1 as a target, we showed that activating this receptor suppressed some characteristics of malignancy in both cell lines. Proliferation, migration, and clonogenicity in the form of colony formation and tumor sphere formation were all significantly reduced when GPER1 was most active – when exposed to high concentrations of the receptor agonist. The outcomes of cell exposure to GPER1 antagonists yielded less definitive results.

Western blots linked the adjustment of GPER1 activity to changes in the expression of the PAI1 protein. This connection serves as further evidence that PAI1 is involved in the cascade of GPER1 and affected by estrogen signaling (15, 16). The knockdown of PAI1 demonstrated a slightly more pronounced effect in A431 compared to CAL39 cells. Specifically, in A431 cells, reductions in PAI1 expression significantly influenced proliferation, migration, and colony size. These results further highlight the significance of PAI1 as a pivotal protein involved in the functional aspects of malignant cells in VC.

Emerging research has found that PAI1 and GPER1 play a role in restricting proliferation in several malignancies of the female reproductive system. Although the role of SERPINE1/PAI1 is not undisputed, it has been found to be oncogenic in breast, gastric, ovarian and lung cancer and may serve as a prognostic factor (17, 18). High expression of PAI1 has been linked to poorer prognosis and patient outcomes (17). Our findings support this claim on a cellular level. In the proliferation assay we found a significant reduction in viability of the SERPINE1/PAI1 knockdown cells in the A431 cell line. This result suggests that in VC, PAI1 is in part responsible for the rapid proliferation of the tumor cells. Similarly, GPER1 activation *via* the agonist G1, also exerts a repressive effect on proliferation. Strong evidence of this function has been discovered in the realm of gynecological oncology, specifically in breast, ovarian, and most recently also in VCs (10, 14, 19-22). All of these studies showed highly significant results in line with our findings: that treating malignant cell lines with G1 had a dose dependent impact on the proliferation or cell viability of the tumor cells. Specifically Loris *et al*. also studied GPER1 in A431 and CAL39 cell lines and found complementary results (14). On the other hand, a study conducted by Lan *et al*. yielded contradictory results in A431 cells. They found a dose-dependent increase in cell viability at low doses of G1 (23). The discrepancy between their findings and ours, is possibly explained by A431 cells exhibiting varying responses to different doses of treatment, with lower doses promoting viability and higher doses restricting proliferation and inducing toxicity. Since growth restriction is a pillar of cancer treatment, the broader implications of these findings are that PAI1 and GPER1 may be considered as targets for clinical treatment someday. In the context of available research, the potential that these findings can be applied beyond VC to general cancer biology is substantial.

Another key feature of malignant cells is their ability to migrate and invade surrounding tissues. SERPINE1/PAI1 has been most studied in the literature with regard to its effect on migration and invasion in other cancers. Consistent with the literature, this investigation established SERPINE1/PAI1 as a relevant molecule involved in migration in VC. (10, 17, 24-27). In cancerous cervical cells and their associated fibroblasts, PAI1 was found as one of the four most relevant proteins associated with epithelial mesenchymal transition (EMT), a hallmark feature of malignancy that involves cell migration (27). Another study showed that cancer cells in PAI1 knockout mice were no longer able to migrate into surrounding healthy tissue (24). Our findings suggest that in some VSCC cell lines, PAI1 may exert a similar effect and play the same role in EMT and invasion. In contrast, Whitley *et al*. found that in a study of breast, ovarian, cervical, and endometrial cancer cell lines, various correlations showed cervical and endometrial cell lines were generally less reliant on the PAI1 system for their invasive qualities than breast and ovarian cancer cell lines (11). In breast and ovarian cancer cell lines, Whitley found that overexpression of wild type PAI1 *via* adenovirus transfection, led to decreased motility and invasive character of the cells (11). The juxtaposition of our results with those of Whitley *et al*. highlights the complexity and variability between different cancer entities and could be proof that PAI1 plays varying roles depending on protein expression and type of cancer.

Our study highlighted the significance of GPER1 in migration of malignant VC cell lines. When the A431 and CAL39 cells were exposed to G1, a significant reduction in migration was observed in both cell lines, aligning with Loris *et al*. findings (14). Breast and ovarian cancers, under GPER1 agonist targeting, have also shown reduced migration in similar conditions (19, 28, 29). This finding implies that GPER1 shows potential to be utilized as a target to slow the spread of multiple gynecological cancers. On the other hand, treatment with GPER1 antagonist, G36, revealed a notable disparity in cell behavior between the two cell lines. CAL39 cells responded to treatment with G36 while A431 cells did not. This finding suggests that the response to GPER1 antagonists is cell line specific and emphasizes the importance of considering cellular context when targeting GPER1 in cancer therapy. Every primary tumor consists of uniquely mutant cells along with a complex tumor microenvironment and personalizing treatment to individual cancer types, even within one diagnosis, is necessary to optimize therapeutic outcomes and improve patient prognosis. Additionally, these results raise questions about the underlying mechanisms driving the differential responses between the cell lines, presenting avenues for future research in the field.

Clonogenicity assays, such as colony formation and tumor sphere formation, quantify the proliferating fraction of cells with cancer stem cell (CSC) characteristics and can be conducted in both two dimensional (2D) and three dimensional (3D) experimental designs (30). Colony formation assays represent the 2D version of clonogenicity, while tumor sphere formation represents the 3D experimental design. In the 2D environment, cells adhere to the plate, introducing variables, such as cellular cooperation and the adherence itself, which can lead to the loss of some CSC characteristics (30). We did not observe loss of CSC in VC cell lines upon plate adherence. Colony growth and size can be used as a measure indicative of

aggressiveness, growth potential, and capacity for self-renewal (30, 31). Beyond GPER1's role in clonogenicity, our findings solidify PAI1 as a protein of interest in tumor expansion and therefore as a potential target influencing these characteristics of malignancy in VC.

In this study, PAI1 knockdown cells in both cell lines exhibited smaller colonies than in the siRNA control cells, expanding on what was previously known about these cell lines (14). The knockdown of PAI1 has a negative impact on both VC cell line's regeneration capability and aggressive growth potential, by slowing the expansion of individual colonies. This finding implies that targeting PAI1 in patients could help slow the expansion of disseminated cancer cells throughout the body and help prevent them from turning into life threatening, fast growing, metastases. Understanding the behavior of malignant cells in the 3D model *via* the tumor sphere assays, provides a meaningful perspective into their invasive properties. This assay serves as an effective *in vitro* model for predicting tumor behavior within its own microenvironment, and for developing drugs (31). Again mirroring the findings of Loris *et al*., the claim that GPER1 and its downstream elements play a critical role in invasion of VC *in vitro*, is significantly strengthened (14). Given the relevant predictive value of clonogenicity assays in cancer research, our investigation established that targeting GPER1 and PAI1 has the potential to prevent or slow the expansion of metastasized cells into full blown metastases.

The western blot analysis revealed a significant correlation between GPER1 and PAI1, a connection documented in a limited number of studies thus far. Research by Ruckriegl *et al*. demonstrated that GPER1 knockdown in fast-growing invasive cervical cancer cell lines led to increased PAI1 expression (12). Meanwhile, in a non-migratory cell line, lower expression levels of PAI1 were observed upon baseline and also upon GPER1 knockdown, associating PAI1 as a downstream element of GPER1 and implying PAI1 is relevant for migration in these cell lines (12). In our study, treating A431 and CAL39 cells with the GPER1 agonist G1 induced rapid estrogen effects within the cells. Under these conditions, a notable reduction in PAI1 expression was observed in western blots. This reduction acts as further evidence of a causal relationship between GPER1 and PAI1. Consequent to a successful knockdown, we have a strong argument claiming that PAI1 contributes to the restriction of migration, proliferation, and colony size growth since it was the only variable factor during experimentation. Our study contributes to the small, but growing body of evidence supporting the notion that PAI1 acts downstream of GPER1 and plays a significant role in influencing the behavior of VSCC cells.

The most relevant gap in the current literature is the biological mechanism linking GPER1 to PAI1. The work of Ruckriegl *et al*. initially revealed a correlation between the activation status of GPER1 and changes in the expression of PAI1 in cervical cancer cell lines (12). We hypothesize the cellular mechanism in cervical and VC cells may be *via* the Hippo pathway. GPER1 is able to regulate Hippo signaling (32). When the Hippo signaling pathway is switched on, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are phosphorylated and degraded. When the Hippo cascade is switched off, YAP and TAZ enter the nucleus and promote the expression of SERPINE1/PAI1, among other factors (33-35). This pathway is one of many complex estrogen-signaling cascades activated by GPER1 and may be the missing connection between cellular receptor and PAI1 transcription in VC and other gynecological cancers. The reduction in PAI1 expression following G1 treatment of A431 cells, along with the decrease in the proliferation, migration, and tumorigenicity under the same conditions, suggest this pathway is how PAI1 is involved in these elements of malignancy. Although the underlying mechanism connecting GPER1 and SERPINE1/PAI1 is not definitively proven, our findings reinforce the notion that these two cellular elements are related in gynecological cancers.

PAI1 also plays a part in a paradoxical phenomenon which makes it a clinically relevant marker of prognosis and a potential target for novel therapies. Urokinase-type plasminogen activator (uPAR) is a receptor which has been studied to be an attractive target of tumor therapy since it has high expression in malignant cells and low expression in healthy ones (36). The ligand uPA binds uPAR activating a proteolytic cascade involved in degrading the extracellular matrix (36). The uPA/uPAR complex has been associated with the progression of cancer, and since PAI1 inhibits the assembly of this complex, we would anticipate that PAI1 also functions as a tumor suppressor (37). However, the paradox of PAI1's function, described in the literature and supported by our own findings, is that cancer patients with high levels of PAI1 circulating systemically have been correlated with poorer prognosis (11, 37). Our findings showed a reduction of malignant characteristics when the tumor cells expressed lower levels of PAI1. However strong the association, the exact connection between PAI1 and poorer prognosis remains incompletely understood. Studying this relationship more closely may uncover other elements involved in this pathway that may become therapeutic targets down the line. As a marker of prognosis, PAI1 has great potential to assist clinicians in managing patients during staging or monitoring for tumor recurrence during remission.

Correlations between the expression of PAI1 and GPER1, as a possible regulator of PAI1, and clinical parameters must also be examined. Extensive clinical studies are necessary for this. In this context, the combination of imaging techniques (colposcopy, MRI, *etc*.) would be ideal in order to correlate sample collection and analysis of the expression levels of PAI1 and GPER1 in a stage-appropriate manner. Colposcopy-supported biopsy could facilitate stage-appropriate diagnosis

and thus the course-dependent expression analysis of PAI1 and GPER1 (38). The MRI procedure is suitable for the investigation of metastases that have arisen from VSCC. Leonhardi *et al*. were able to show several associations between MRI textures and immunohistochemical parameters in lymph node metastases of VSCC (39).

The literature surrounding SERPINE1/PAI1 in gynecological cancers is limited so far, opening a world of opportunity for further research in the future. A critical gap in the currently available literature is an explanation of how the GPER1 estrogen signaling pathway interplays with the SERPINE1 gene transcription and PAI1 expression. Finding the mechanism with which GPER1 and PAI1 are connected would offer insight to how estrogen signaling reduces the malignancy of VSCC cells. Our findings reinforce the notion that these two cellular elements affect one another (14). A relevant idea for the further study of PAI1 would be to overexpress the gene and observe cellular functions in malignant cells. Exploring the interplay between the GPER1 estrogen signaling pathway and PAI1 expression in gynecological cancers offers a promising avenue for future research, shedding light on their impact on cellular processes and to the potential discovery of new therapeutic targets.

Conclusion

To conclude, our study highlights the intricate interplay between GPER1 and SERPINE1/PAI1 in VSCC. We have demonstrated that SERPINE1/PAI1 functions as an oncogene, while an activated GPER1 acts as a tumor suppressor. Our findings support the notion that SERPINE1/PAI1 expression levels are influenced by GPER1 activation. To support this claim, our research revealed a significant decrease in proliferation, migration, and tumorigenic potential in both the A431 and CAL39 cell lines following treatment with a GPER1 agonist, or a knockdown of PAI1. This finding implicates both GPER1 and PAI1 as potential targets in future cancer therapy. The pathway by which GPER1 influences PAI1 expression has not yet been fully understood and warrants further investigation. Moving forward, discoveries in this pathway have the potential for uncovering novel therapeutic targets and refining treatment strategies for VSCC and other related malignancies.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

Conceptualization, C.G.; investigation, T.D.; writing, original draft preparation, T.D. and C.G.; writing, review and editing, J.G.; project administration, C.G. All Authors have read and agreed to the published version of the manuscript.

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