

Simultaneous Treatment with Soluble Forms of GAS1 and PTEN Reduces Invasiveness and Induces Death of Pancreatic Cancer Cells

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Introduction: Pancreatic carcinoma cells exhibit a pronounced tendency to invade along and through intra and extrapancreatic nerves, even during the early stages of the disease, a phenomenon called perineural invasion (PNI). Thus, we sought to determine the effects of the simultaneous expression of soluble forms of GAS1 and PTEN (tGAS1 and PTEN-L) inhibiting tumor growth and invasiveness.

Materials and Methods: We employed a lentiviral system to simultaneously express tGAS1 and PTEN-L; in order to determine the effects of the treatments, cell viability and apoptosis as well as the expression of the transgenes by ELISA and intracellular signaling as ascertained by the activation of AKT and ERK1/2 were measured; cell invasiveness was determined using a Boyden chamber assay; and the effects of the treatment were measured in vivo in a mouse model.

Results: In the present work, we show that the combined treatment with tGAS1 and PTEN-L inhibits the growth of pancreatic cancer cells, by reducing the activities of both AKT and ERK 1/2, decreases cell invasiveness, and restrains tumor growth in a mouse model.

Conclusion: The combined administration of tGAS1 and PTEN-L could be a valuable adjunct therapy for the treatment of pancreatic cancer.

Keywords: growth arrest specific 1, PTEN-L, pancreatic cancer, perineural invasion, artemin, GFR α 3

Introduction

Pancreatic cancer is a very aggressive disease with a poor prognosis and it is expected to become the second leading cause of cancer death.¹ A crucial process of pancreatic tumor spread is Perineural Invasion (PNI), a form of cancer progression in which tumor cells invade (around or through) nerves and indicates a poor clinical outcome associated with pain, paresthesia, augmented cancer recurrence, and reduced patient survival.²⁻⁴ PNI is a common occurrence in various cancer types including prostate, head and neck, breast, colon and gastric.⁵⁻⁹ Glial Cell Derived Neurotrophic Factor (GDNF) is found in intrapancreatic nerves as well as in normal pancreas and it is highly expressed in pancreatic cancer together with RET and the GFR α 1 receptor, and the expression of both molecules correlates with cell invasion, survival, proliferation and decreased survival after surgical resection.¹⁰ On the other hand, the phosphorylation of RET induced by the GDNF-GFR α 1 complex activates both ERK and AKT, which in turn induces PNI and proliferation of pancreatic adenocarcinoma cells (PDAC).¹¹ GDNF and Artemin

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(ARTN), a ligand of the GDNF family, are present in the pancreatic cancer environment, as well as the GFR α 1 and GFR α 3 receptors, which are released by the PDACs themselves, and also by intra and extrapancreatic nerves into the perineural space.¹² Soluble forms of GFR α 1 released from nerves stimulate the movement of RET-expressing cancer cells along a chemotactic gradient of members of the GDNF family of ligands (GFLs), to invade the perineural space and move along nerve fibers toward the central nervous system.¹² This invasive process, as previously mentioned, is linked to poor prognosis, neuropathy and tumor-related pain. Artemin promotes invasiveness and neurotrophic activity of pancreatic adenocarcinoma. Moreover, the presence of ARTN has also been observed in other types of cancer such as endometrial, breast, and non-small cell lung carcinoma and it is related to proliferation and metastasis.¹³

On one hand, Growth Arrest Specific 1 (GAS1) is a 37 kDa protein anchored to the external cell membrane by a glycosylphosphatidylinositol molecule (GPI) capable of inducing cell arrest and apoptosis of different tumors including glioma, and neuroblastoma.^{14,15} GAS1 inhibits the GDNF-induced phosphorylation of RET on Tyr 1062 and thus causes an important decrease in AKT phosphorylation; this process facilitates the dephosphorylation of BAD that then translocates to the mitochondria and promotes the release of Cytochrome-C into the cytosol which activates caspase-9 and caspase-3, which in turn induces apoptosis.¹⁶ We previously demonstrated the effect of tGAS1, a soluble form of GAS1, inhibiting the growth of MDA-MB-231 human breast cancer cells.¹⁷ Interestingly, the MDA-MB-231 cells lack both RET and GFR α 1¹⁷ but express ARTN and GFR α 3. Although RET is not present in MDA-MB-231 cells, GFR α -GFL complexes can initiate signaling in a RET-independent manner, probably through the adhesion of molecules such as NCAM, which induces Fyn that in turn inhibits cell adhesion.^{18,19}

On the other hand, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a molecule with a central function in counteracting phosphatidylinositol 3-kinase (PI3K) signaling.^{20,21} The fact that PI3K and its mediator AKT are constitutively activated in pancreatic cancer, might be the result of the aberrant expression of their natural antagonist PTEN, which can also regulate ERK1/2.²² PTEN decreases AKT activity and survival-proliferative pathways, making PTEN one of the most important tumor suppressors. Moreover, PTEN acts as a tumor suppressor in pancreatic cancer²³ and a soluble

form of PTEN (PTEN-L) that is released from producer cells and enters into tumor cells reduces tumor growth.²⁴ Furthermore, we recently determined an additive effect of the simultaneous effect of tGAS1 and PTEN-L inhibiting glioma growth.²⁵

This information prompted us to study whether the combined administration of tGAS1-PTEN-L could inhibit cancer cell invasion in vitro and tumor growth in a murine pancreatic cancer model. Interestingly, HPAF-II pancreatic cancer cells do not express GDNF, RET, PTEN, or GFR α 1. However, they express ARTN and GFR α 3, and the application of tGAS1-PTEN-L inhibits the activation of ERK1/2 and AKT in an RET-independent manner. Furthermore, tGAS1 and PTEN-L inhibited the invasion of pancreatic cancer cells, and the lentiviral-mediated delivery of tGAS1-PTEN-L prevented pancreatic tumor growth in a murine model.

Materials and Methods

Cell Culture

HPAF-II human pancreatic cancer cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco by Life Technologies), 100 U/mL of Penicillin, 100 μ g/mL of Streptomycin (Gibco by Life Technologies), and maintained at 37°C in a 95% air, 5% CO₂ atmosphere. SH-SY5Y human neuroblastoma cells and U-87 MG human glioblastoma cells were cultured in DMEM-HG (Dulbecco's Modified Eagle Medium High-Glucose) (Gibco by Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Gibco by Life Technologies), 2 mM L-glutamine (Sigma-Aldrich), 100 U/mL of Penicillin, 100 μ g/mL of Streptomycin (Gibco by Life Technologies), and maintained at 37°C in a 95% air, 5% CO₂ atmosphere. Cells lines were obtained from ATCC.

Reverse Transcription PCR

Total RNA extraction was performed using the Trizol Reagent (Invitrogen), according to the manufacturer's instructions. Subsequently, 2 μ g of RNA was treated with DNase (Invitrogen) and retro-transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen), to perform the PCR. To amplify human *gas1*, the following primers were used: forward 5'-CTGTGCCTGATGGCGCTGCTGC-3' and reverse 5'-ATGAGGGCCGAGATGCAGTGA-3', for human β -*actin*: forward 5'-TGGCACCACACCTTCTACA-3' and reverse 5'-TCACGCACGATTTCCC-3'. To

amplify human *gdnf*, the following primers were used: forward 5'-GAAGTTATGGGATGTCGTGGC-3' and reverse 5'-GACAGGTCATCATCAAAGGCG-3'. To amplify human *artemin*, the following primers were used: forward 5'-CAGCCTAAAAGACACCAGAGAC-3' and reverse 5'-GCACTTTCAACCAAGCAACTGC-3'. To amplify human *gfra1*, the following primers were used: forward 5'-CTGCAGCACCAAGTACCGCA-3' and reverse 5'-ACCTTGACTCTGGCTGGCAGT-3'. To amplify human *gfra3*, the following primers were used: forward 5'-CTCTGCCTCAAGTTTGCCATG-3' and reverse 5'-CAGGGTTTTTCATTCTGGTGTGC-3'. To amplify human *ret*, the following primers were used: forward 5'-GGGATCACCAGGAATTCTCC-3' and reverse 5'-GGGAGGCGTTCTCTTTCAGC-3'. PCR products were obtained after 40 cycles of amplification with an annealing temperature of 55°C–66°C.

Transfection of Expression Vectors

For all treatments, 1×10^6 HPAF-II cells were seeded in 60 mm dishes and grown until they reached a confluence of approximately 80–90%. After 24 h, cells were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol, with each of the following expression plasmids: tGAS1 (pLenti6.3/TO/V5-tGAS1), and PTEN-L (JpExpress404 PTEN-Long); or combinations: tGAS1 and PTEN-LONG (PTEN-L) (pLenti6.3/TO/V5-tGAS1 and JpExpress404 PTEN-Long), previously described.³¹ As a control, pDsRed2-N1 (Red Fluorescent Protein) was transfected, Lipofectamine only treated cells and non-treated cells were also used as controls. All transfections were carried out under the same conditions: 10 µg of plasmids and 24 µL of Lipofectamine 3000 (Invitrogen, Cat. # L3000001).

Cellular Viability Assays

Cell viability was determined by the Trypan Blue exclusion technique (Sigma Aldrich), at different times after exposure to the treatments.

Western Blot Analysis

Protein extraction was performed using a lysis buffer containing a protease inhibitor cocktail (Complete; Sigma-Aldrich), and protein quantification was performed using the bicinchoninic acid method (Pierce; Rockford, IL). 50 µg of protein per condition was run on SDS-PAGE gels and then transferred onto PVDF membranes (BioRad), which were subsequently blocked

for 1 h with a solution of 5% skim milk/in TBS/Tween 0.1%. Membranes were incubated overnight with primary antibodies against PTEN (A2B1) (diluted 1:250; Cat No. 7974, Santa Cruz Biotechnology), AKT (diluted 1:5000; Cat No. 4691, Cell Signaling Technology), phospho-AKT (diluted 1:1000; Cat No. 9271, Cell Signaling Technology), β-Actin (diluted 1:10,000; Cat No. A3854, Sigma Aldrich), ERK1/2 (diluted 1:5000; Cat No. 9102S, Cell Signaling Technology) and pERK1/2 (diluted 1:500; Cat No. 9106S, Cell Signaling Technology). Subsequent washes were performed with TBS/Tween 0.1% and then membranes were incubated for 1 h with anti-rabbit and anti-mouse secondary antibodies diluted 1:3000 (Invitrogen), followed by washes with TBS/0.1% Tween. The presence of the proteins was revealed by chemiluminescence (Perkin-Elmer). Images were captured and analyzed using a UVP BioImaging System and LabWorks Image Acquisition and Analysis Software (UVP, Inc. Upland, CA).

Determination of Apoptosis

Proteins were obtained and quantified from cell cultures and tumors as described in the precedent section. 50 µg of protein per condition was run on SDS-PAGE gels and then transferred into PVDF membranes (BioRad) which were subsequently blocked for 1 h with a solution of 5% skim milk/in TBS/Tween 0.1%. Membranes were incubated overnight with primary antibodies against human Caspase-3 (diluted 1:300; Cat No. 9662, Cell Signaling Technology). Subsequent washes were performed with TBS/Tween 0.1% and then membranes were incubated for 1 h with anti-rabbit diluted 1:500 (Invitrogen), followed by washes with TBS/0.1% Tween. The presence of proteins was revealed by chemiluminescence (Perkin-Elmer). Images were captured and analyzed as previously described.

Primary Culture of Dorsal Root Ganglion Cells

Dorsal root ganglia (DRG), from postnatal 2–7 day-old CD1 mice were used for in vitro invasiveness assays. All animal procedures were performed according to current Mexican legislation NOM-062-ZOO-1999 (SAGARPA) and in agreement with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and approved by the Institutional Committee (CICUAL del CINVESTAV). DRG were incubated at

37°C in a 95% air and 5% CO₂ atmosphere for 30 min and mechanically dissociated with a pasteur pipette. The product of this procedure was centrifuged for 2 min in RPMI and washed with cold Balanced Hank's Saline Solution (HBSS) (Gibco by Life Technologies); this procedure was performed three times. After the last wash, the supernatant was removed, and the cell suspension was placed in a 35 mm culture dish (Corning) with 12 x 10 mm coverslips (Corning) pretreated with poly-L-lysine (Sigma-Aldrich). Cells were incubated in Neurobasal medium with supplements: B27 (Gibco B-27 supplement 50X, Cat No. 17504-044) N2 (Gibco N-2 supplement 100X, Cat No. 17502-048), Pyruvate, 2mM L-glutamine (Sigma-Aldrich), 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (Gibco by Life Technologies), and maintained at 37°C in a 95% air and 5% CO₂ atmosphere.

Immunofluorescence

Cultures of DRG were fixed with 4% p-formaldehyde (Sigma-Aldrich) prepared in PBS (pH 7.4) and incubated with 1% BSA (Jackson ImmunoResearch) and the antibody against β-Tubulin-III (TUJ-1) (diluted 1:500, Cat No. MCA-1B12, Encor Biotechnology). The secondary antibody was a Fluorescein (FITC)-Affinipure Goat-Anti-Mouse IgG (H+L) (diluted 1:300 Cat No. 115-095-003, Jackson ImmunoResearch). Cells were counterstained with DAPI to reveal the nuclei. Images were obtained with an Olympus BX51 epifluorescence microscope coupled to a CoolSNAP-Pro_{cf} Color camera with the Image-Pro PLUS software (Media Cybernetics, Inc.).

Effect of Soluble Forms of tGAS1, PTEN-L and tGAS1-PTEN-L on Neural Invasion

Conditioned media from cells transfected with the expression plasmids tGAS1, PTEN and tGAS1-PTEN-L were obtained from HPAF-II cells transfected with Lipofectamine 3000 (Invitrogen). The conditioned media were centrifuged and filtered before their application.

In vitro Invasion Assay

Invasion assays were performed using a modified Boyden chamber method in 24-well plates containing 12 cell culture inserts with 8 µm pore size membranes (Costar, Corning Inc.). Briefly, 50 µL of Matrigel (Falcon) was added into culture inserts and kept for 1 h at 37°C.

Cancer cells were seeded onto the upper chamber of 24-well plates at a density of 3×10^4 cells in serum free culture medium. The lower chambers were seeded with DRG cells (see Materials and Methods) and cells incubated with the media obtained from cultures containing tGAS1, PTEN-L and tGAS1-PTEN-L, and control media containing 10% FBS. Cells were allowed to invade through the Matrigel-coated membrane for 48 h and non-invading cells were removed from the top membrane. Cells were stained with 0.1% crystal violet and cells that crossed the filters counted.

Lentiviral Production

Lentiviral vectors were obtained as previously described.²⁵ Briefly, pLenti6.3/TO/V5-tGAS1-p2A-PTEN-L and pLenti6.3/TO/V5-EGFP were produced according to the manufacturer's protocol (ViraPower™ HiPerform™ T-REx™ Gateway™ Expression System, Invitrogen) and independently transfected into HEK 293FT cells using Lipofectamine 3000 (Invitrogen, Cat No. L3000001). After 48 h, culture media containing the lentivirus were collected, spin-dried, filtered and concentrated. The viral title was subsequently determined according to the manufacturer's protocol.

Determination of GAS1 and PTEN Levels

ELISA was performed using commercial kits for human Gas1 DuoSet ELISA (R&D Systems, USA; Cat No. DY2636), and for human PTEN (Cell Signaling Technology, USA; Cat No. #7882), according to the manufacturer's instructions.

In vivo Tumor Model

Experiments were performed according to current Mexican legislation NOM-062-ZOO-1999 (SAGARPA) and in agreement with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Committee (CICUAL del CINVESTAV). Animals (*nu/nu* male mice) were maintained with ad libitum access to food and water in aseptic conditions with climate and light-controlled systems. 1×10^6 HPAF-II cells were subcutaneously inoculated into the flanks of 6–8-week-old male nude mice (*nu/nu*) and allowed to grow until the tumor volume reached approximately 30 mm³. Animals were then injected intratumorally for 3 days (days 0, 2 and 4) with 1.5×10^6 vp (viral particles) of tGAS1-p2A-PTEN-L virus, EGFP lentivirus, or culture medium without serum, at a final volume of

6 μ L. Tumor growth was monitored and tumor volume was calculated using the following formula: $V = (\text{length} \times \text{width}^2)/2$. Four weeks after the initiation of the treatments, animals were sacrificed by cervical dislocation, tumors dissected, weighted, homogenized, and processed for protein extraction with a lysis buffer containing a protease inhibitor cocktail (Complete; Sigma-Aldrich) to determine the expression of AKT, ERK1/2, and their phosphorylated forms and caspase-3 by Western blot assays, as previously described.

Statistical Analysis

For cell viability, protein levels determined by Western blot analysis, invasion assay, tumor weight and levels of GAS1, PTEN by ELISA in tumors we used one-way ANOVA followed by Duncan's or Tukey's multiple comparison test. For the analysis of tumor growth, in vivo data of tumor volume with independent observations through time on the same experimental unit were analyzed using a split-plot method, according to the following model for tumor volume: $V_{i,j,k} = \mu + \text{Tr}_i + \varepsilon_{ij} + t_k + [\text{Tr} * t]_{i,k} + \delta_{i,j,k}$; where $\varepsilon_{ij} \sim N[0, \sigma^2_{fp}]$ and $\delta_{i,j,k} \sim N[0, \sigma^2_{sp}]$ (where fp is full plot and sp is split plot), treatments (Tr) $i = 1, 2$ and 3 stand for vehicle, EGFP, and tGAS1-PTEN-L, repetitions $j = 1, 2$, and 3 , time $k = 0, 2, 4, 6, 8, 10, 13, 16, 19$ and 28 days, Duncan's post-hoc tests were performed to show differences among the treatments and treatments' time interaction. Tests were performed using either the NCSS or the SPSS software. $p < 0.05$ was considered significant.

Results

Characterization of HPAF-II Pancreatic Cancer Cells

The goal of this study was to evaluate the effect of the administration of tGAS1-PTEN-L on pancreatic cancer cell growth, survival and invasiveness. Thus, we first determined the phenotypic characteristics of the HPAF-II human pancreatic cancer cells used in this work. We performed RT-PCR assays to assess the expression of *gas1*, *gdnf*, *artemin*, *gfra1*, *gfra3*, and *ret* mRNAs utilizing confluent SH-SY5Y and U87 cells as positive controls. The cells express *artemin* and *gfra3*, but do not express *gas1*, *gdnf*, *gfra1* or *ret* (Figure 1A). Moreover, they do not express PTEN, as demonstrated by Western blot analysis (Figure 1B). Therefore, these cells are a good model to study the effects of GAS1 and PTEN inhibiting cancer growth and invasiveness.

Effects of tGAS1, PTEN and tGAS1-PTEN-L on the Viability of Pancreatic Cancer Cells

To compare the effect of soluble forms of tGAS1 and PTEN on cell viability, we transfected the plasmids coding for tGAS1, PTEN-L and tGAS1-PTEN-L together, and assessed their effects on the viability of HPAF-II cells for 24, 48, 72 and 96 h. As controls, we used cells treated only with Lipofectamine, cells transfected with a vector expressing red fluorescent protein (RFP) and cells without treatment. It was observed that both tGAS1 and PTEN-L had a significant effect on reducing the viability of HPAF-II cells; however, we also observed that the simultaneous

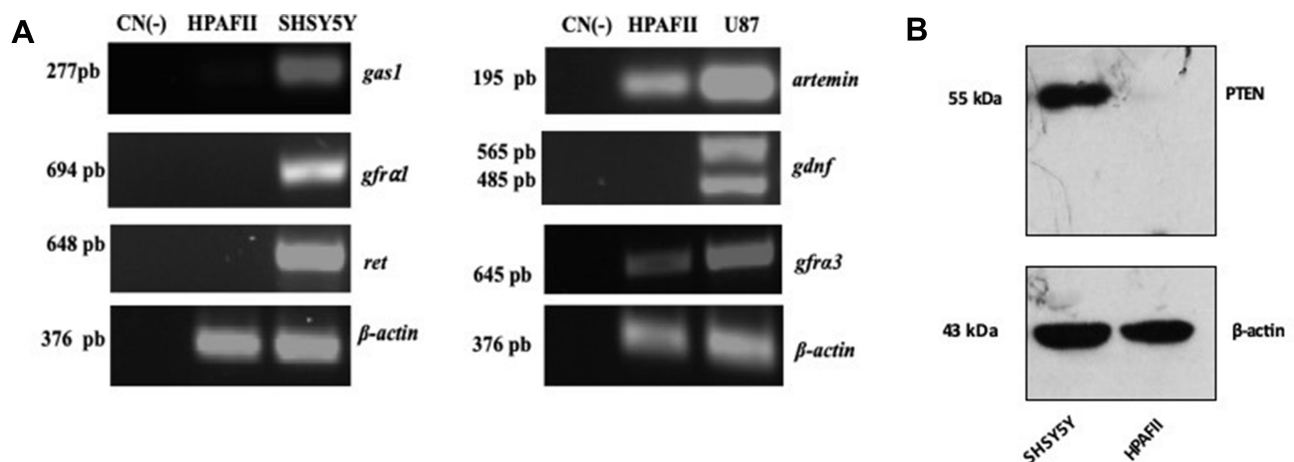


Figure 1 Characterization of HPAF-II pancreatic cancer cells. (A) HPAF-II cells do not express *gas1*, *gfra1*, *ret*, or *gdnf* but express both *artemin* and its receptor *gfra3*. SHSY5Y and U87-MG cells were used as positive controls. (B) HPAF-II cells do not express PTEN, as assessed by Western blot analysis; SH-SY5Y cells were used as positive control; β -actin is the loading control.

transfection of tGAS1 and PTEN-L had a better effect reducing cell viability than when the factors were independently used (Figure 2).

Effects of Soluble tGAS1 and PTEN-L and tGAS1-PTEN-L on the Activities of ERK and AKT and Apoptosis of HPAF-II Cancer Cells

To evaluate the molecular mechanism underlying the effect of tGAS1, PTEN-L and the simultaneous application of tGAS1-PTEN-L on cell viability, we determined the activity of signaling pathways associated with these proteins: AKT and ERK1/2. HPAF-II cells were transfected independently with tGAS1, PTEN-L and tGAS1-PTEN-L together, as controls, we used cells treated only with Lipofectamine, with RFP and control non-treated cells. Forty-eight hours after transfection, it was observed that PTEN-L induced a significant decrease in AKT activity (Figure 3A). Although a decrease was observed, tGAS1 did not significantly reduce AKT phosphorylation (Figure 3A). On the other hand, both tGAS1 and PTEN-L induced significant decreases in ERK 1/2 phosphorylation, an effect that was even stronger when they were simultaneously transfected (Figure 3B). To determine whether cell death was associated with the reduction of cell survival, we assessed the activation of caspase-3 as a marker of apoptosis. We observed a discrete activation of caspase-3, after exposure to tGAS1, but a more intense effect after the treatment with PTEN-L, or the simultaneous expression of both agents (Figure 3C). These data show that the

combined treatment with tGAS1 and PTEN-L induces an apoptotic process in HPAF-II cells.

Effect of the Administration of tGAS1-PTEN-L on Pancreatic Cancer Invasiveness

To explore the potential effects of tGAS1 and PTEN-L inhibiting the invasiveness of HPAF-II pancreatic cancer cells, we used an in vitro model employing Boyden chamber assays. To perform the assay, DRG neurons were seeded on the lower chamber, and HPAF-II cells on the upper chamber, on Matrigel-coated filters with 8.0 μm pores. We first determined the levels of both tGAS1 and PTEN-L in the conditioned media from producer HPAF-II cells transfected with the plasmids of tGAS1 and PTEN independently (Figure 4A). Interestingly, and in contrast to the effect on tumor cells, we observed that neither tGAS1 nor PTEN-L, or the combined treatment had any effect on the survival of DRG neurons, suggesting that these treatments do not affect healthy primary non-tumor cells (Figure 4B). We also showed that the primary cultures of DRG were enriched with neurons as shown by immunofluorescence against β -tubulin III. In these cultures over 95% of the cells were positive for this neuronal marker (Figure 4C). Next, we determined the effects of the treatments on the invasiveness of HPAF-II cells. As can be observed in Figure 4D, incubation with media containing tGAS1 or PTEN-L significantly reduced the migration of tumor cells. However, the combined application of the two agents had an even stronger effect. These data demonstrate that the presence of soluble forms of GAS1 and PTEN-L

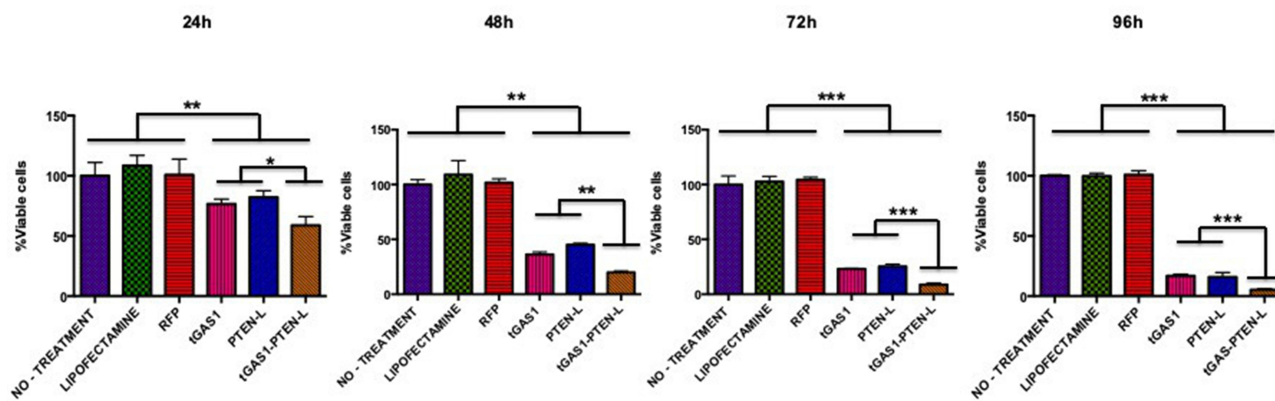


Figure 2 Comparison of the effect of soluble forms of GAS1 and PTEN on cell viability. The graphs show the percentage of viable HPAF-II cells, as determined by Trypan blue exclusion at different times after transfection with GAS1, PTEN, or both simultaneously transfected (24, 48, 72 and 96 h). Controls were non-treated HPAF-II cells, cells treated with Lipofectamine or transfected with the Red Fluorescent Protein plasmid (RFP). tGAS1 and PTEN-L were more effective together than when individually transfected. One-way ANOVA followed by Duncan's test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 3$.

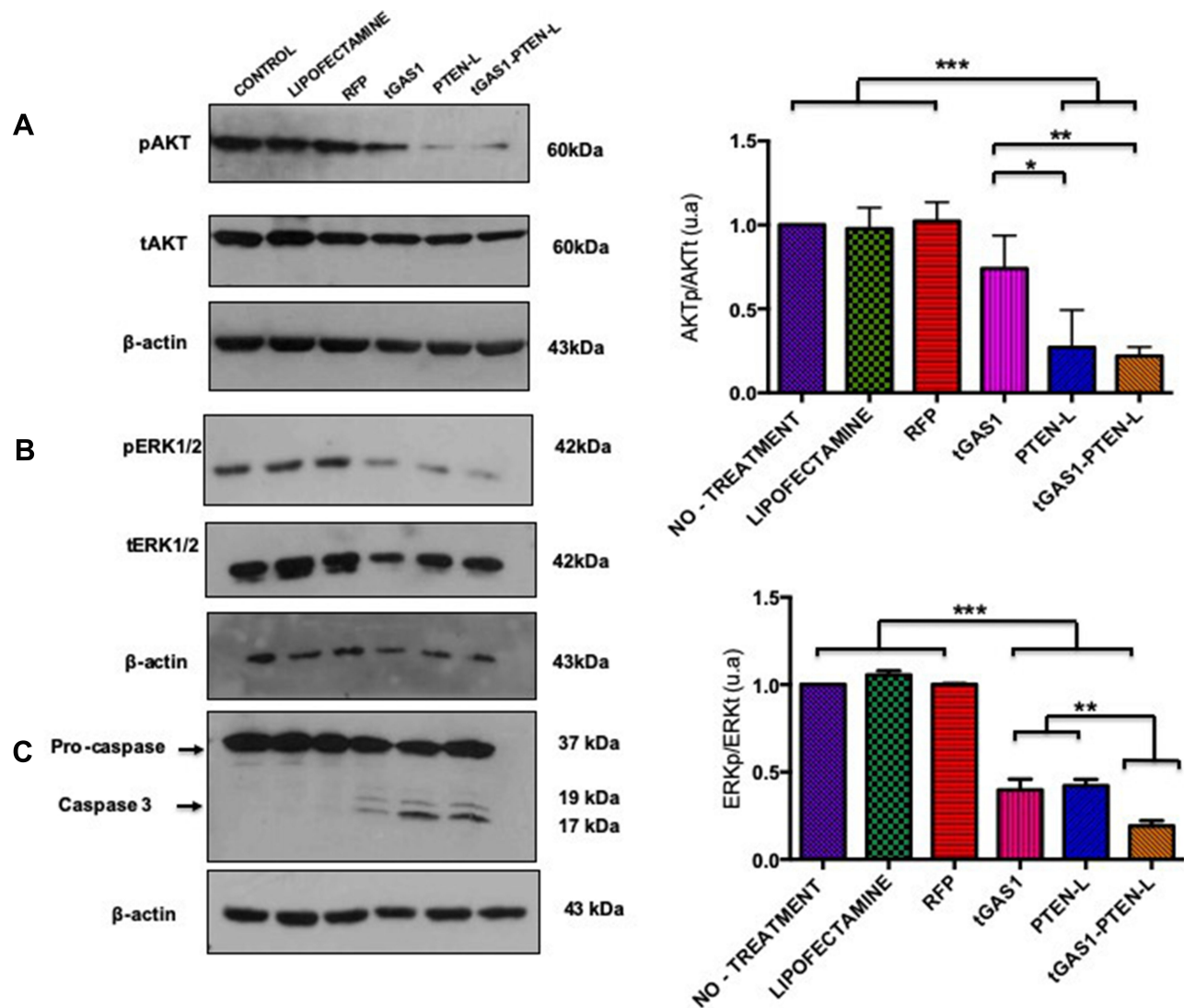


Figure 3 Effects of soluble forms of GAS1 and PTEN on intracellular signaling pathways and apoptosis. **(A)** Effects of the soluble forms of GAS1 and PTEN, and when transfected simultaneously on the activity of AKT (pAKT is the phosphorylated molecule and tAKT is the total); **(B)** effects of the soluble forms of GAS1 and PTEN, and transfected together on the activity of ERK1/2 (pERK1/2 is the phosphorylated molecule and tERK1/2 is the total); left panels show representative experiments and right panels, the statistical analysis of three independent experiments; **(C)** apoptosis demonstrated by the activation of caspase-3, β -Actin is the loading control. One-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 3$.

inhibits the invasiveness of pancreatic tumor cells to neurons.

In vivo Effects of the Lentiviral Vector Expressing Soluble Forms of tGAS1-PTEN-L

Since we had observed that the combined treatment with both tGAS1 and PTEN-L exerted the strongest effects inhibiting tumor cell growth and in vitro invasion of pancreatic cancer cells, we decided to test a lentiviral expression system that produces equimolar amounts of both proteins²⁵ in an in vivo mouse model. HPAF-II cells were subcutaneously inoculated into the flanks of male

nude mice. When the tumors reached a volume of approximately 30 mm³, they received tGAS1-p2A-PTEN-L-producing lentivirus, EGFP-producing lentivirus, or the same volume of vehicle on days 0, 2 and 4. Tumor growth was determined for 28 days after the start of the treatment when mice were sacrificed and a significant decrease in tumor growth was observed, as determined by tumor weight and volume in mice treated with the tGAS1-PTEN-L virus compared with controls (Figure 5).

To determine the molecular mechanisms associated with the inhibition of tumor growth, we measured the levels of both GAS1 and PTEN in treated tumors. As previously reported, the expression of both agents induced

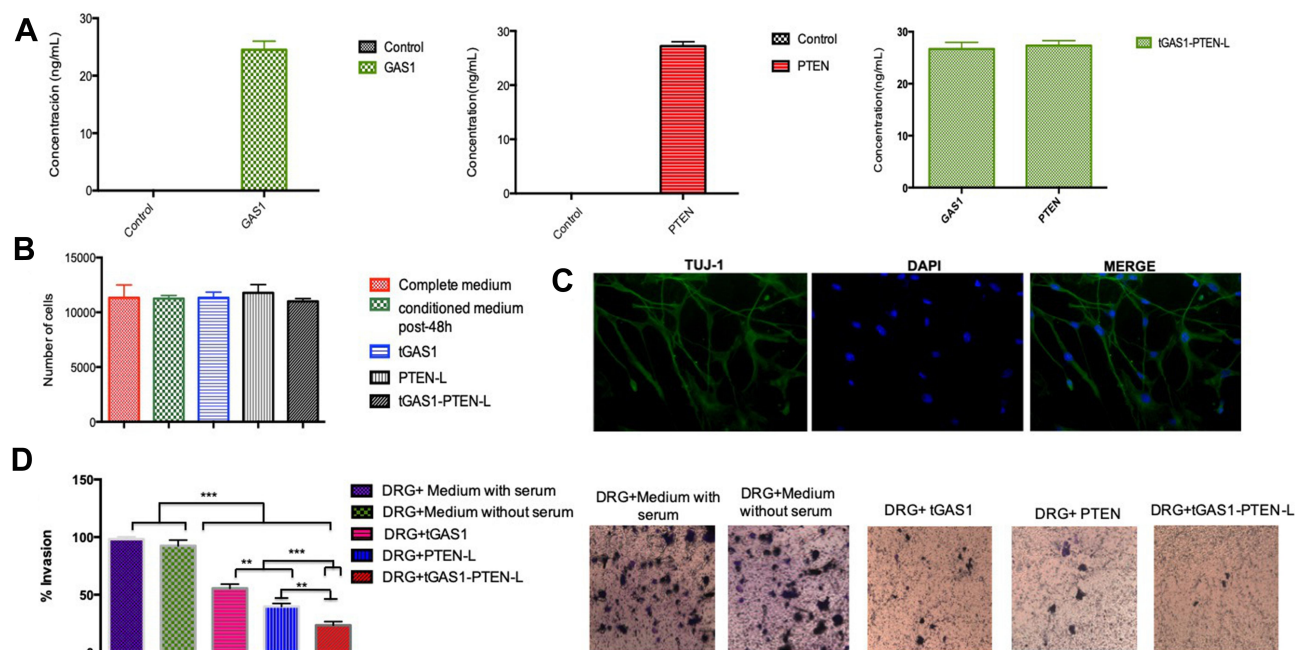


Figure 4 Effect of soluble forms of GAS1 and PTEN, and when simultaneously applied on pancreatic cancer cell invasiveness. **(A)** Levels of the soluble forms of GAS1 and PTEN in conditioned media from HPAF-II producer cells (left transfected with tGAS1; middle with PTEN-L; right with tGAS1 and PTEN-L); **(B)** number of DRG viable cells in the presence of the different media; **(C)** Immunofluorescence of DRG cultures against β -III Tubulin (left panel), nuclei revealed by DAPI (middle panel) and merge of the two channels (right panel). **(D)** Percentage of HPAF-II cancer cells that crossed the membrane towards DRG cells incubated in the presence of the different media; right panel shows a representative experiment. One-way ANOVA followed by Tukey's multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$; $n = 3$.

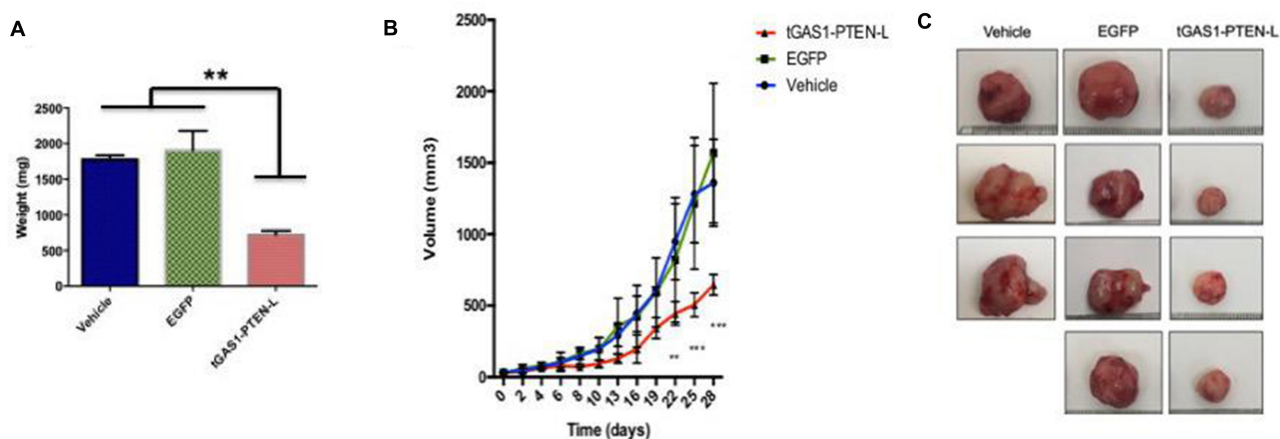


Figure 5 Effect of the expression of tGAS1-PTEN-L on tumor growth. HPAF-II cells were subcutaneously inoculated into the flanks of *nu/nu* mice, and when tumors reached approximately 30 mm³, they received the same volume of vehicle (culture medium without serum), EGFP-expressing virus, or tGAS1-PTEN-L-expressing virus. **(A)** Weight of tumors after the different treatments **(B)** Volume of tumors treated with tGAS1-PTEN-L compared with controls through time. **(C)** Size of the tumors after the treatments (ruler is in cm). ANOVA followed by Tukey's **(A)**; and Duncan's post-hoc test **(B)**. ** $p < 0.01$, *** $p < 0.001$; $n = 3$ for vehicle; $n = 4$ for EGFP and $n = 4$ for tGAS1-PTEN-L treatments.

high and equal levels of expression in treated tumors,²⁵ whereas low or undetectable expression of both GAS1 and PTEN-L were seen in control tumors (Figure 6A and B). Consistent with prior in vitro results, we observed large decreases in the active forms of both AKT and ERK 1/2 in treated tumors, compared with the control groups (Figure 6C and D). Moreover, we also showed activation of

caspase-3 in tGAS1-PTEN-L-treated tumors, compared to the controls, demonstrating an apoptotic process caused by the tGAS1-PTEN-L treatment (Figure 6E).

Altogether, the present data demonstrate that the simultaneous expression of soluble forms of GAS1 and PTEN induces inhibition of cell growth, apoptosis and, interestingly, reduces the invasiveness of pancreatic cancer cells.

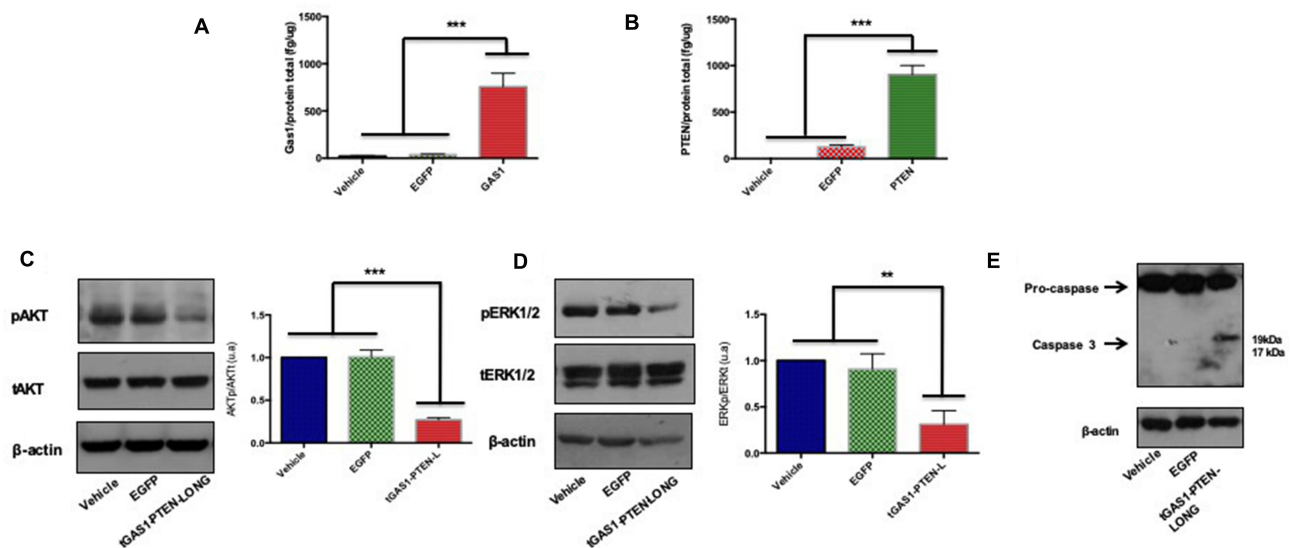


Figure 6 Effects of tGAS1-PTEN-L on tumor cells intracellular pathways and apoptosis. **(A and B)** Levels of Gas1 and PTEN in tumors; **(C)** activity of AKT in tumors, left representative experiment, right statistical analysis; **(D)** activity of ERK1/2 in tumors, left representative experiment, right statistical analysis; **(E)** apoptosis of tumor cells as shown by caspase-3 activity. One-way ANOVA followed by Tukey's multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$; $n = 3-4$ (**A-D**).

Thus, the combined treatment seems to strike pancreatic cancer by both killing the cells and reducing PNI, a feature that facilitates metastasis, a major factor associated with the malignancy of pancreatic cancer.

Discussion

It is known that there is an interaction between pancreatic innervation and pancreatic cancer cells that facilitates PNI.^{26,27} The presence of GDNF, ARTN and their cognate receptors in pancreatic cancer indicates the participation of the GDNF family in tumor progression and invasiveness.^{13,18,28} Also, a role for the GDNF-RET signaling pathway is recognized in cancer²⁸ as well as the capacity of ARTN to promote pancreatic cancer invasion.¹³ Moreover, the capacity of ARTN to induce migration and invasiveness of pancreatic cancer cells is mediated by CXCR4; this effect is mediated by the activation of AKT and ERK 1/2 signaling, that stimulates the accumulation of NF- κ B which in turn promotes the expression of CXCR4.²⁹ On the other hand, PTEN is frequently deleted in pancreatic ductal adenocarcinoma (PDA), but it acts as a tumor suppressor regulating the NF- κ B network, indicating the participation of the PTEN/PI3K pathway, inhibiting cancer growth.²²

Based on the previous information, we considered that manipulating both the GDNF-family signaling pathways and the PI3K pathway could be a relevant approach to inhibit both cell survival and the invasiveness of

pancreatic cancer cells. GAS1 is a protein that induces cell arrest and apoptosis of glioma and neuroblastoma cells by blocking the GDNF/RET pathway.¹⁴⁻¹⁶ Interestingly, we have also observed that GAS1 inhibits tumor growth and angiogenesis in a breast cancer model, through a RET-independent mechanism that involves interfering with the ARTN-GFR α 3-mediated signaling and the corresponding inhibition of ERK1/2.¹⁷ Moreover, we have generated a soluble, releasable form of GAS1 (tGAS1) that exerts both autocrine and paracrine effects eliminating tumor cells.³⁰ GAS1 released from producing cells can also potentially interact with soluble GFR α s, as occurs with nerve-released GFR α 1 that enhances PNI.³¹ PTEN-L is a soluble form of PTEN that decreases PI3K signaling in both producing cells and neighboring cells, inhibiting tumor growth.²⁴ We have previously employed a p2A lentiviral system that produces equimolar amounts of tGAS1 and PTEN-L and observed an additive effect inhibiting glioma growth.²⁵

In the present work, we used the pancreatic cancer cell line HPAF-II and determined that these cells neither express GAS1 nor PTEN, making them a good model to test the effects of the expression of these tumor suppressors. Moreover, the cells express Artemin and GFR α 3, but no GDNF, GFR α 1 or RET (Figure 1). We tested the effect of the independent treatments with GAS1, PTEN-L or both together on the viability of HPAF-II cells, and it was observed that both agents decreased the number of

viable cells, but that their combined effect was significantly more marked (Figure 2). To determine the molecular mechanisms underlying these effects, we ascertained the activities of AKT and ERK 1/2. As can be seen in Figure 3A, tGAS1 did not significantly reduce AKT phosphorylation, whereas PTEN-L had a significant effect on reducing AKT activity. On the other hand, both GAS1 and PTEN-L reduced the activity of ERK 1/2 and when both proteins were simultaneously expressed in HPAF-II cells, their effect was the best one (Figure 3B). These results are consistent with our previous data, showing that GAS1 does not reduce the activation of AKT, in cells lacking RET, but that it is capable of inhibiting ERK 1/2 activation in a RET-independent manner, through an Artemin-GFR α 3-dependent, non-canonical pathway.¹⁷

Taking into consideration previous reports that showed ARTN facilitates pancreatic cancer invasiveness,¹³ we assessed the effects of soluble forms of GAS1 and PTEN-L in an in vitro assay of tumor cell invasiveness toward a primary culture of DRG neurons. It was observed that both tGAS1 and PTEN-L reduced the invasiveness of HPAF-II cells, but that the effect was more pronounced when both proteins were expressed together, indicating an additive effect (Figure 4). It is conceivable that the effects of tGAS1 and PTEN-L could be caused, at least in part, by their capacity to kill primary neurons of the culture, thus reducing the production and release of attractant substances. However, neither tGAS1 nor PTEN-L had any effect reducing the viability of DRG neurons. All these data indicate that soluble GAS1 is capable of inhibiting the invasiveness of pancreatic cancer cells, likely by interfering with attractant molecules released by nerves, such as soluble GFR α s.

Considering that the effects of the combined application of tGAS1 and PTEN-L on pancreatic tumor cell viability and invasiveness were stronger than when used independently (Figures 2 and 4), we decided to employ a lentiviral p2A-enabled dual expression system, that allows the production of both proteins in equimolar amounts, to test its effects in a mouse cancer model. The viral vector was directly injected into tumors inoculated in mice and we observed a significant decrease in tumor growth, compared to control mice and with mice receiving a virus expressing a reporter gene (Figure 5). To ascertain the molecular mechanisms responsible for the inhibition of tumor growth, we determined that tGAS1 and PTEN-L were expressed in tumors injected with the tGAS1-PTEN-L-producing virus but with low or undetectable levels in the control groups (Figure 6). Moreover, in agreement

with the in vitro results presented in this paper, and our previous reports, we determined a significant reduction of the phosphorylation of both AKT and ERK 1/2 in tumors expressing tGAS1 and PTEN-L, which are also associated with apoptosis, as revealed by the activation of caspase-3 (Figure 6).

Conclusions

The results presented in this paper show that the simultaneous application of soluble forms of GAS1 and PTEN inhibits the growth and invasiveness of pancreatic cancer cells. These effects are caused by inhibiting both the AKT and ERK 1/2 intracellular signaling pathways. The advantage of using soluble forms of GAS1 and PTEN is that they have autocrine effects on producing cells but also paracrine effects on neighboring cells, thus augmenting the therapeutic range of the combined treatment. Moreover, it is also likely that soluble GAS1 interferes with nerve-secreted GFR α s, reducing PNI and the resulting formation of metastasis. We consider that the combined administration of tGAS1 and PTEN-L could be a valuable adjunct therapy for the treatment of pancreatic cancer.

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Disclosure

The authors declare that they have no conflict of interest.

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