ORIGINAL RESEARCH ARTICLE

PDGFB as a vascular normalization agent in an ovarian cancer model treated with a gamma-secretase inhibitor†

Running head: PDGFB and DAPT in an ovarian cancer model

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Abstract

Ovarian cancer is the fifth leading cause of cancer-related deaths in women. In the past 20 years, the canonical types of drugs used to treat ovarian cancer have not been replaced and the survival rates have not changed. These facts show the clear need to find new therapeutic strategies for this illness. Thus, the aim of the present study was to investigate the effect of a gamma-secretase inhibitor (DAPT) in combination with the Platelet-derived growth factor B (PDGFB) on an ovarian cancer xenograft model. To achieve this goal, we analyzed the effect of the administration of DAPT alone and the co-administration of DAPT and recombinant PDGFB on parameters associated with tumour growth and angiogenesis in an orthotopic experimental model of ovarian cancer. We observed that the dose of DAPT used was ineffective to reduce ovarian tumour growth, but showed anticancer activity when co-administered with recombinant PDGFB. The administration of PDGFB alone normalized tumour vasculature by increasing periendothelial coverage and vascular functionality. Interestingly, this effect exerted by PDGFB was also observed in the presence of DAPT. Our findings suggest that PDGFB is able to improve tumor vascularity and allows the anticancer action of DAPT in the tumor. We propose that this therapeutic strategy could be a new tool for ovarian cancer treatment and deserves further studies.

Keywords: Ovarian cancer, Angiogenesis, Notch system, PDGFB.

Introduction

In 2012, an estimated 239,000 women were diagnosed with ovarian cancer worldwide and 152,000 of them died of the disease (Ferlay J, 2013). Ovarian cancer has a high mortality rate and 69% of women with this illness will succumb to the disease (Lengyel, 2010). This is mainly due to a late detection because of the lack of symptoms, which implies that, at the time of the diagnosis, this cancer is in an advanced stage. The first step in treating most stages of ovarian cancer are surgery and chemotherapy, and a cure is expected if no cancer cells remain after initial therapy is completed. By contrast, in most patients, the recurrence is common if residual cancer cells are present after initial treatment (Narod, 2016).

In the last forty years, the mortality rates for ovarian cancer have declined only slightly. In addition, in the last two decades, the two canonical types of drugs used to treat ovarian cancer (a cisplatin and a carboplatin, and a taxane) have not been replaced. Thus, many new therapies for the treatment of ovarian cancer are currently under study. Some of these involve Poly ADP ribose polymerase (PARP) inhibitors and molecules targeting the Her2 or PIP3/AKT/mTOR pathway. However, although the newest drugs such as bevacizumab (a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody) or olaparib (a PARP-1 inhibitor) delay cancer progression, they are not able to extend the lives of patients (Oza *et al*, 2015; Perren *et al*, 2011; Pujade-Lauraine *et al*, 2014). These observations show the need of a better understanding of the disease and its diagnosis, and the development of new therapeutic strategies.

The tumor vasculature is structurally and functionally abnormal and is considered a hallmark of cancer. Thus, it is an attractive target for tumor therapy. However, as aforementioned, antiangiogenic treatments have failed to show significant response or to prolong survival in patients with solid tumors (Goel *et al*, 2012). This resistance of tumors to antiangiogenic therapies can be explained by the fact that tumor cells develop other mechanisms of vascularization that are not affected by the antiangiogenic compounds and produce several

proangiogenic factors that are not targeted by these drugs (Carmeliet and Jain, 2011). Although clinical data also suggest that anti-VEGF therapy cannot induce sustained shrinkage of human tumors, such as breast or colorectal tumors, patients who have received a combination of anti-VEGF and chemotherapeutics have shown to have a better response than to chemotherapy alone (Goel *et al*, 2012). This observation led to the hypothesis of "vascular normalization", which states that the judicious use of an antiangiogenic therapy may reverse the abnormal structure and function of the pathological vasculature. This would result in an improvement in the delivery of the drugs and oxygen to the tumor cells (Jain, 2001).

In the tumor vasculature, pericytes, which are the cells that provide support for endothelial cells of the vessels, are loosely attached or absent, and the basement membrane, if present, is often thick. As a result, tumor vessels are leaky, tortuous and dilated. This alteration in the vasculature contributes to tumor development and spreading by different mechanism. It also interferes with the delivery of therapeutics to solid tumors and the resulting hypoxia renders the tumor resistant to radiation and to several cytotoxic drugs (Jain, 2005). Interestingly, one of the features of vascular normalization includes vessel perivascular cell coverage. Several molecules are responsible for the recruitment of perivascular cells to vessels during angiogenesis, being PDGFB an essential player in this process. Angiogenic endothelial cells release PDGFB to chemoattract PDGF receptor-β^+ pericytes to stabilize endothelial cell channels. Accordingly, periendothelial coverage deficiency after PDGFB ablation causes vessel leakage, tortuosity and bleeding, features similar to those of the tumor vasculature (Quaegebeur *et al*, 2010). In addition, PDGFB stimulates the proliferation of vascular smooth muscle cells and induces mural cell fate in undifferentiated mesenchymal cells (Gaengel *et al*, 2009). Pericytes play an important role in the maintenance of ovarian cancer vasculature (Lu *et al*, 2010; Lu *et al*, 2008). Sood *et al*. (2008) demonstrated that PDGFB is predominantly expressed in endothelial and ovarian cancer cells, whereas PDGFRβ is present in pericyte-like cells (Lu *et al*, 2008).Several studies have found interesting results about the blockade of PDGF in tumor development and the effects of combined therapies

with VEGF inhibitors (Choi *et al*, 2015). However, no studies have analyzed PDGFB as a normalizing agent of the tumor vasculature.

Notch signaling is also an important player in angiogenesis. Beyond its role in regulating vessel branching (Hellstrom *et al*, 2007), the Notch system is involved in tumor development and metastasis (Li *et al*, 2013; Noguera-Troise *et al*, 2006; Pazos *et al*, 2017). In ovarian cancer, the DLL4 notch ligand has been suggested as a predictor of poor prognosis and its inhibition has been shown to potentiate the decrease in tumor growth caused by bevacizumab in an ovarian xenograft model (Hu *et al*, 2011). (Steg *et al*, 2011) demonstrated that the JAG1 notch ligand plays a role in tumor angiogenesis and chemoresistance. In addition, MK-0752, an inhibitor of Notch signaling, has been shown to induce a decrease in ovarian tumor growth, an effect significantly enhanced by cisplatin (Chen *et al*, 2016). In our lab, we have recently demonstrated that DAPT, an inhibitor of the Notch system, impairedalters the epithelial-to-mesenchymal transition induced by transforming growth factor beta (TGFβ) in ovarian cancer cell lines. Due to PDGFB function in vessel stabilisation and to the antitumor effect of Notch inhibition on ovarian cancer, in the present study, we sought to investigate the effect of DAPT treatment in combination with PDGFB in an ovarian cancer xenograft model.

Chemical compounds

Recombinant human PDGF-BB was kindly donated by Denver Farma S.A. (Buenos Aires, Argentina). Gamma-Secretase Inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)-S-phenylglycine t-butyl ester), Dimethyl sulfoxide (DMSO), NaCl, proteinase K, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), methanol, Na2HPO4, NaH2PO4, NP-40, glycerol and Tween-20 were from Sigma-Aldrich (St. Louis, MO, USA), and 3,3-diaminobenzidine (Aaboud and others) was from Roche Applied Science (Mannheim, Germany). The Fluorescein-labelled Lycopersicon (Tomato) Lectin was purchased from Vector Laboratories

(Burlingame, CA, USA). The details, suppliers and dilution of antibodies used in this study are reported in Table 1 (additional sheet). All other chemicals were of reagent grade and were obtained from standard commercial sources.

In vivo xenograft model

Human SKOV3 cells ($1x10^6$) suspended in 100 μL PBS were subcutaneously inoculated into two-month-old virgin female BALB/c mice (animal facility of the Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina), nude mice (*nu/nu*, University of La Plata, Buenos Aires, Argentina). First, we performed a pilot experiment to examine the effect of recombinant PDGFB (rec-PDGFB) administered to mice bearing ovarian tumor xenografts. Once the tumors grew to an average size of $80mm^2$, mice were randomized in two groups: control and rec-PDGFB. The control group consisted of mice intravenously injected with saline, and the rec-PDGFB group included mice injected with rec-PDGFB (250 ng/mouse) for 4 consecutive days. After 4 days of the last injection, all animals were weighed and euthanized, and the tumors were weighed and excised for western blot and immunohistochemistry techniques. Once that the rec-PDGFB dosis produced the desidered effect on the vasculature, we conducted an experiment that consisted of three groups of animals: control (injected with 20% DMSO solution), DAPT (injected with DAPT (0.1 mg/mouse)), and DAPT+rec-PDGFB (injected with DAPT and rec-PDGFB (250 ng/mouse)) for 4 consecutive days. We followed the same procedure as described above.

Vascular functionality assay

In tumor-bearing mice of the control, DAPT, rec-PDGFB and DAPT+rec-PDGFB groups, we studied vascular functionality by using Fluorescein-labelled Lycopersicon lectin administration. Before the tumors were excised, the animals were perfused with a FITC-lectin solution (20 mg/mL) followed by 4% paraformaldehyde. The tumors were excised and fixed in cold 4% paraformaldehyde overnight. Tissues were embedded in OCT (Tissued-Tek OCT Compound) and frozen tumors were cut (15 μm) with a cryostat and,

finally the nuclei were counterstained with propidium iodide. Endogenous fluorescence was analyzed using a Nikon Eclipse E800 confocal Microscope. Images were taken with a Nikon DS-U1 camera with ACT-2U software.

Western Blot analysis

Tumors were suspended in 500 mL of lysis buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% Nonidet P-40, and 10% glycerol) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N-p-tosyl-lysine chloromethyl ketone, and 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone), and phosphatase inhibitors (25 mM sodium fluoride, 0.2 mM sodium orthovanadate, and 10 mM b-glycerophosphate) and homogenized using an Ultra-Turrax (IKA-Werke GmbH & Co., Staufen, Germany) homogenizer. The samples were centrifuged at 4-8ºC for 10 min at 10 000 g and the resulting pellets were discarded. The protein concentration in the supernatant was measured by the Bradford assay. After boiling for 5 min, 20 mg of proteins was applied to an SDS–polyacrylamide gel (10–15%) and electrophoresis was carried out at 25 mA for 1.5 h. The resolved proteins were transferred onto nitrocellulose membranes for 2 h. The blot was reincubated in a blocking buffer (5% nonfat milk, 0.05% Tween-20 in 20 mM TBS pH 8 for 1 h at room temperature, and then incubated with appropriate primary antibodies (Table 1) in 0.05% Tween-20 in 20 mM TBS pH 8 overnight at 4- 8ºC. The blots were then incubated with anti-rabbit or anti-mouse secondary antibodies conjugated with HRP (Table 1), detected by chemiluminescence and autoradiography using X-ray film. Protein loading was normalized by reprobing the same blots with antibody against β-Actin or GAPDH (Table 1). Protein expression was quantified by densitometric analysis using Scion Image Software for Windows (Scion Corporation, Worman's Mill, CT, USA).

Immunohistochemistry

Tumors were deparaffinized in xylene and rehydrated by graduated ethanol washes. Endogenous peroxidase activity was blocked with hydrogen peroxide in PBS, and nonspecific binding was blocked with 2% BSA for 20 min at room temperature. The sections were incubated with appropriate primary antibodies (Table 1) overnight at 4-8 ºC. After washing, the slides were incubated with biotinylated anti-rabbit or anti-mouse IgG (Table 1) and after 30 min with avidin-biotinylated HRP complex (Vectastain ABC system; Vector Laboratories, Burlingame, CA, USA) for 30 min. Protein expression was visualised using (Aaboud and others) staining. The negative controls were obtained in the absence of primary antibody. The reaction was stopped using distilled water, stained with haematoxylin, and dehydrated before mounting with mounting medium (Canada Balsam Synthetic; Biopack, Argentina). The images were digitised using a camera (Nikon, Melville, NY, USA) mounted on a conventional light microscope (Nelipovich and others). Finally, the images were converted to a TIFF format (bi-level scale) for their analysis. Negative controls were obtained in the absence of primary antibodies. We determined the number of Ki67 and cleaved-caspase 3 positively stained nuclei. A total of 2000-3000 nuclei per experimental group were counted from five randomly selected fields per tumor and the number of positive cells were processed using Image J (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD, USA; Rasband).

To determine vascular stability, we measured the periendothelial area in tumor sections. For the quantification, the area occupied by vessels was manually limited, considering as such all immunopositive cells, isolated or in groups, with or without lumen. No immunostained vessel was excluded from the analysis. The total area of the image was measured and the periendothelial area was calculated dividing the absolute periendothelial area by the total image area.

Data analysis

When the experiment involved two experimental groups, the variables were analyzed using Student's t-test. For more than two groups, One-way analysis of variance (ANOVA) followed by Tukey test was used. Tumor growth curves were studied with Two-way repeated-measures ANOVA. Quantitative variables were expressed as means±standard error and, in all statistical tests, $p<0.05$ was considered statistically significant. GraphPad Prism (version 5.0, CA, USA) was used for statistical analysis.

Results

PDGFB administration increases periendothelial coverage of the tumor vasculature

Based on the aberrant architecture of the tumor vasculature and the PDGFB role in the recruitment of smooth muscle cells for vascular maturation, we performed a pilot experiment to examine the effect of recombinant PDGFB (rec-PDGFB) administered to mice bearing ovarian tumor xenografts generated with the SKOV3 cell line. The mice treated with rec-PDGFB for four consecutive days showed no significant differences in tumor growth compared to control animals (Figure 1, Panel A). PDGFB administration caused no changes in the cell proliferation or apoptosis indices, as concluded from Ki67 staining analysis and cleaved-caspase 3 quantification (Figure 2, Panels A and B, respectively). However, alpha smooth muscle actin (α-SMA) and PDGF receptor β (PDGFRβ) staining of tumor sections from mice treated with rec-PDGFB was significantly increased as compared to control animals (Figure 2, Panels C and D, respectively). The increased percentage of positive area for both proteins showed higher periendothelial coverage in the vasculature of these tumors. In our experimental model, this experiment demonstrated that the dose of rec-PDGFB chosen was efficient to chemoattract $PDGFR\beta^+$ pericytes to the tumor vasculature. In these conditions, PDGFB did not alter other tumor parameters. It is important to note that neither α-SMA nor PDGFRβ staining was detected in tumor cells or stroma, indicating no expression of either protein in non-vasculature-related cells (Figure 2, Panel D.).

Co-treatment with DAPT and PDGFB affects the structure and functionality of the tumor vasculature

We analyzed the histological characteristics of the four experimental groups with H&E stained sections of each tumor (Figure 1, Panels B-E). In control conditions, tumors consisted of sheets of big and poorly differentiated cells. These sheets were randomly separated by thin fibro vascular septa. Cytologically, two different types of cells stand out: A group of cells with big and eosinophilic cytoplasm, loose nucleus with prominent nucleoli ("A" cells); and another group of smaller cells, with clear cytoplasm and condensed nuclei ("B" cells). Prominent mitotic figures are present almost exclusively in "A" cells. When treated with PDGFB, there is a clear increase in the proportion of "A" cells comprising approximately 90% of the whole tumor. Moreover, the mitotic figures observed in these cells suggest an increase in tumor cell proliferation in this treatment. Regarding fibro vascular septa, an expansion in number and diameter is seen at the expense of capillaries and fibroblasts, which means high tissue remodeling. DAPT treated tumors show a 50:50 proportion of "A" and "B" cells which suggests a decrease in proliferating cell population. Also a slight increase in fibro vascular septa thickness can be observed, but still similar to control tumors.

Interestingly, for the combination of PDGFB and DAPT, vast areas of necrosis and haemorrhage predominate mainly in the central areas of the tumoral mass. Again, cytologically, a 50:50 proportion of "A" and "B" cells are seen, and a regular number of medium septa turns up between them.

We then examined the effect of DAPT and rec-PDGFB administration on the tumor vasculature. Mice bearing SKOV3 cell tumors were divided into three experimental groups, which received either no treatment (control group), DAPT (0.1 mg/animal) or DAPT and rec-PDGFB (DAPT+rec-PDGFB, 250 ng/mice). DAPT alone caused no changes in perivascular cell markers as α-SMA and PDGFRβ. However, the addition of rec-PDGFB to the DAPT treatment significantly increased periendothelial coverage (Figure 3). We then studied whether this increase in vessel coverage had a correlation with a better vessel perfusion. To analyze vessel

functionality, we injected tumor-bearing mice with fluorescent tomato lectin to label perfused vessels. Control animals showed a mix of leaky (Figure 4, Panel A *) and defined vessels (Figure 4, panel A arrow). In the DAPT-treated mice, DAPT increased the diffusion of the fluorescence out of the vessels, and tumors showed higher permeability of the vasculature. Also, more vessel lumens were observed, perhaps due to an increase in vasculogenesis, and in addition, DAPT admimistration caused more vessel sprouting (Fig 4, Panel A, asterisk). The increased lectin diffusion observed in the DAPT-treated mice was not observed in the DAPT+rec-PDGFB group. The tumors of co-treated animals showed a lack of leaky vasculature and, similar to that observed in the rec-PDGFB tumors, the vessels looked well defined perhaps due to vasculature stabilisation. This showed a controlled regulation of the vasculature and an improved tumor vessel functionality in the co-treated mice compared to the control and DAPT groups. As mentioned, lectin perfusion in tumors of mice treated with rec-PDGFB (pilot experiment) showed a discrete and defined tumor vasculature (Figure 4, panel rec-PDGFB). The tumor oxygen supply was assessed by western blot of HIF-1α accumulation, which is precisely regulated by cellular oxygen concentration. DAPT administration showed a not statistically significant tendency to decrease HIF-1α levels ($p=0.06$). However, the co-treatment significantly reduced HIF-1α compared to control animals, indicating less hypoxic tumors (Figure 4, panel B).

Co-treatment with DAPT and PDGFB alters tumor angiogenic factors

VEGF-A is a key player in tumor angiogenesis and a strong vascular permeability factor (Chen *et al*, 2016; Dvorak, 2002). In our model, DAPT treatment decreased tumor VEGF-A but caused no changes in its type 2 receptor, FLK-1, or its phosphorylated state (p-FLK-1-Tyr1175) (Figure 5, panel A, C and D). Similar to DAPT monotherapy, DAPT+rec-PDGFB treatment decreased tumor VEGF-A compared to untreated animals. Again, the co-treatment caused no changes in FLK-1, but significantly decreased p-FLK-1-Tyr1175 compared to the

control and DAPT groups. These results suggest a lower activation of the VEGF pathway in the co-treated animals.

The angiopoietin growth factor ligands (Ang 1-4) and their receptors (Tie 1 and 2) are essential in vascular maturation. Particularly, while Ang 1 stabilizes the quiescent endothelium, Ang 2 functions as a desestabilisation factor (Potente *et al*, 2011). When we examined the balance between Ang 1 and Ang 2 in the tumors, we observed that DAPT treatment caused no changes in the levels of either protein compared to control tumors (Figure 5, panel B and D). However, the co-treatment with DAPT+rec-PDGFB significantly increased Ang 1 and significantly decreased Ang 2, resulting in a higher Ang 1/Ang 2 ratio compared to the control or DAPT-treated animals (Figure 5, panel F). This showed that the DAPT+rec-PDGFB treatment induced a balance of Angiopoietins that favoured tumor vascular stabilisation.

Co-treatment with DAPT and PDGFB decreases ovarian tumor growth

Because PDGFB improved tumor vessel function in our experimental model, we sought to analyze whether DAPT treatment had an effect on this parameter, and if so, whether PDGFB caused any change on it. When the animals were treated with DAPT alone, tumor growth tended to decrease as early as from day 2 of administration, but the difference in tumor size was not statistically different compared to the control group until day 6 of the experiment (Figure 6, panel A). The co-administration of PDGFB and DAPT significantly reduced the tumor growth rate (nearly blocked the growth) compared to the control and DAPT groups and, during the whole treatment, the tumor size of the co-treated group remained close to the initial size (Figure 6, panel A). Besides, as early as day 2 of the co-treatment, the tumor size started to be significantly lower than that of DAPT tumors, which was the same until the end of the experiment (Figure 6, panel A, day 2) and had no differences with control tumors. In addition, the difference between DAPT and DAPT+rec-PDGFB treatment increased in significance throughout the whole treatment. Similarly, DAPT monotherapy had no

effect on tumor weight, while the co-treatment significantly decreased the values of this parameter (control: 1.54±0.09; DAPT: 1.55±0.11; DAPT+rec-PDGFB: 0.90±0.18, (gr) $p<0.05$). No signs of toxicity were observed and animal weight remained stable during the course of both treatments (data not shown).

We next evaluated tumor cell proliferation by analysing Ki67 staining by immunohistochemistry and PCNA expression by western blot. Both the DAPT and DAPT+rec-PDGFB treatments decreased tumor Ki67 expression compared to the control group, showing no differences between each other (Figure 3, panel Ki67). Similarly, the co-treatment decreased PCNA expression compared to the control group, but DAPT monotherapy caused no changes in this marker (Figure 6, panel B). This could be due to the different time points of expression of each protein during the cell cycle.

The DAPT and DAPT+ rec-PDGFB treatments also significantly decreased the phospho-AKT/AKT ratio ($p<0.01$ and $p<0.001$ vs control, respectively), showing no differences between each other (control: 0.81±0.02; DAPT: 0.45±0.05; DAPT+rec-PDGFB: 0.27±0.047, densitometric units) (Figure 6, Panel C). However, the co-treatment significantly increased the protein levels of phosphatase and tensin homolog (PTEN) (control: 0.39±0.03: DAPT+rec-PDGFB: 0.68±0.07, $p<0.01$) while DAPT monotherapy induced no changes in this parameter (DAPT: 0.40±0.03) (Figure 6, panel D).

The DAPT treatment tended to increase the number of tumor apoptotic cells, as revealed by cleaved caspase 3 immunostaining (Figure 6, panel D). The addition of PDGFB made the trend significant, increasing tumor cell apoptosis in co-treated mice as compared to untreated ones.

Discussion

The novel finding of this study is that a dose of DAPT, a gamma-secretase inhibitor, which was ineffective to reduce ovarian tumor growth in mice, showed anticancer activity when co-administered with PDGFB. We

also demonstrated that the administration of PDGFB alone normalized the tumor vasculature by increasing periendothelial coverage and vascular functionality. Interestingly, in the presence of DAPT, PDGFB still altered the tumor milieu by improving vessel functionality and oxygenation. We also observed that the animals co-treated with DAPT and PDGFB were more sensitive to the DAPT anticancer effect than those which received DAPT alone. This sensitization could be due, at least in part, to an improvement of the tumor vasculature produced by PDGFB, which facilitates the delivery of the antitumor drug to the tumor.

The Notch and PDGF systems play pivotal roles in angiogenesis, and the Notch system has also been involved in cancer biology. Of the PDGF family members, PDGFB is involved in the recruitment of perivascular cells to vessels during angiogenesis. In this process, angiogenic endothelial cells release PDGFB to chemoattract PDGF receptor-β^+ pericytes. In this way, PDGFB is responsible for the stabilisation of the vasculature (Quaegebeur *et al*, 2010). Notch signaling is involved in vessel branching, regulating proliferation and migration of the tip and stalk cells. This system restricts branching by the downregulation of VEGF receptor 2 and generates perfused vessels (Phng and Gerhardt, 2009). Notch also participates in vessel maturation by upregulating PDGF receptor-β in Notch-positive mural cells (Benedito *et al*, 2009).

In search of new therapeutic strategies to improve ovarian cancer treatment, in the present study, we first evaluated the effect of PDGFB on the tumor vasculature, based on its role in physiological angiogenesis. As mentioned, the tumor vasculature has an aberrant structure, which causes high hypoxia and low pH in tumors, generating a promalignant microenvironment. These abnormalities facilitate the selection and dissemination of the most malignant cancer cells through the leaky vessels, preventing the correct delivery of anticancer drugs, and thus causing poor responses to therapies (Goel *et al*, 2012). In our experimental model, we observed that PDGFB increased the periendothelial area in tumors, as shown in the analysis of the expression of α-SMA and PDGFR-β. We did not observe PDGFR-β protein expression in tumor cells not related to the vasculature, and the dose of PDGFB used showed no effect on tumor cell proliferation or apoptosis. This was

in accordance with the lack of differences observed in the tumor growth curves between control and PDGFB-treated animals. Few reports have described the expression of PDGFR-β in ovarian tumor cells. (Apte *et al*, 2004) showed that SKOV3ip1, a variant of SKOV3 cells, stained weakly for PDGFR-β when implanted orthotopically in nude mice. This is similar to our results where we detected no staining for this receptor in tumor or stromal cells, unlike that observed in those stained with α-SMA. In the present study, we also found an improvement in vessel functionality and a reduction in the chaotic vascular structure by lectin perfusion in tumors of animals treated with PDGFB. The lack of effect of PDGFB on tumor growth is in agreement with the results of Chunhua L *et al*. 2010, who targeted pericytes with a PDGFB aptamer in human ovarian carcinoma models and observed no changes in tumor growth (Lu *et al*, 2010). Tumor vessel normalization often does not affect the size of primary tumors, but its benefits are related to a reduction of metastasis and to an improved drug delivery and efficiency of the chemotherapy (Carmeliet and Jain, 2011). The effect of PDGFB treatment could be an interesting strategy to be further studied because of the reported ability of vascular smooth muscle cells to inhibit endothelial cell proliferation. The increase in pericyte content in the tumor microenvironment inhibits the growth of angiogenesis-dependent tumors (McCarty *et al*, 2007).

Several lines of evidence have demonstrated the involvement of the Notch system in ovarian cancer biology. Some reports have shown an increased content of Dll4 and Jagged1 ligands as well as of Notch1 and Notch3 receptors, in ovarian tumor tissues (Hu *et al*, 2014; Hu *et al*, 2011; Jung *et al*, 2010; Xie *et al*, 2017). Moreover, in 2011, the Cancer Genome Atlas published a study performed in 316 cases of High Grade Serous Ovarian Cancer, 22% of which showed an alteration in the Notch signaling pathway among other deregulated signaling pathways (Bell, 2011). In addition, in a granulosa tumor cell line representative of the adult form of Granulosa Cell Tumors, we have previously demonstrated that JAGGED1, DLL4, NOTCH1, and NOTCH4 are highly expressed in tumor cells as compared to that observed in granulosa-lutein cells obtained from patients undergoing assisted reproductive techniques (Pazos *et al*, 2017). Notch signaling inhibitors have been

developed in recent years, and some are in current clinical trials. Gamma-secretase inhibitors, which targ*et al*l Notch receptors, are the most widely studied agents. Different gamma-secretase inhibitors have shown promising antitumor activity in early-phase clinical trials in advanced solid tumors and a correlation has been found between the expression of Notch members and the response to these therapies (Xie *et al*, 2017).

In the present study, we also detected an interesting antiproliferative and proapoptotic effect of the gamma-secretase inhibitor DAPT in an ovarian cancer xenograft model. It has been demonstrated that the inhibition of the Notch ligand JAGGED1 causes anti-angiogenic effects in ovarian tumor models and significantly decreases cell viability (Steg *et al*, 2011). Similar results have been obtained for DLL4, where the inhibition of this protein in tumor and tumor-associated endothelial cells has been shown to inhibit cell growth and angiogenesis simultaneously with an increase in hypoxia (Hu *et al*, 2011). In our experimental model, the blockade of the Notch system by means of DAPT showed a tendency to decrease tumor growth compared to control tumors. The complete blockade of the system independently of the type of ligand and receptor caused no alterations in most of the angiogenic parameters analyzed but there was a clear action of the inhibitor in vascular functionality and structure. This was easily observed in our experiment with tomato-lectin and the images of the morphology of the tumor vasculature shown in photographs of the tumors treated with DAPT. Both images showed numerous and disorganised vessels, which seem highly permeable as denoted by lectin pictures where diffused vessel walls due to lectin extravasation were observed. The high number of vessels and the chaotic structure observed in the pictures of DAPT-treated tumors compared to control tumors agree with the experiments in mouse retina performed by Hellstrom *et al*. (2007). These authors described an increase in vascular density and vascular abnormalities after DAPT administration. One of the first reports of Notch involvement in tumor angiogenesis was performed by Noguera-Troise *et al*., who defined DLL4 function in murine tumor models (Noguera-Troise *et al*, 2006). By the blockade of DLL4, these authors showed a decrease in tumor growth and a highly branched vasculature full of sprouts and filopodia. However,

they found an increase in hypoxia in tumors lacking DLL4 activity compared to control tumors, indicating that the vasculature was not efficiently delivering oxygen to the surrounding tumor. In our experiments, DAPT administration led to a slight but not significant decrease in HIF-1α levels. This suggests that although we observed more vessels in lectin images obtained from DAPT-treated mice, this vasculature could be non-functional, in agreement with previous reports. In our DAPT-treated tumors, HIF-1α levels were similar to those of control tumors, probably due to the short treatment period or the dose of DAPT administered. In addition, DAPT inhibits not only the activation of the system through DLL4 (directly involved in angiogenesis), but also the complete activation of the Notch system. This could dilute the effect of the inhibition of the Notch ligands and receptors strictly involved in angiogenesis with other members of the system that do not participate in the process in a predominant manner. Interestingly, the addition of rec-PDGFB to the DAPT treatment decreased tumor hypoxia (HIF-1α levels). This effect was equally observable in lectin studies, where the images of lectin-perfused tumors from animals treated with DAPT+rec-PDGFB showed that the number of vessels was similar to that in control tumors. However, the tumor vasculature of the co-treated animals was more defined as a result of the low permeability and also regularly distributed when compared to control and DAPT-treated tumors. At macroscopic and functional levels, these results showed the normalizing effect of PDFGB on tumor vasculature. The vascular normalization was corroborated with the pronounced increase in α-SMA and PDGFR-β staining in tumor sections of DAPT+rec-PDGFB-treated mice. Another evidence of the normalized vasculature in our model was the effect of the co-treatment on the VEGF and Angiopoietin systems. The fact that the administration of DAPT or DAPT+rec-PDGFB decreased tumor VEGF levels is important because, for the normal development and function of blood vessels, VEGF must be tightly regulated in endothelial cells. It is very well known that the production of VEGF and other growth factors by the tumor results in the 'angiogenic switch', where new structurally and functionally abnormal vasculature is formed in and around the tumor (Carmeliet, 2005). Thus, when designing

and testing a new and potential therapy for ovarian cancer, it is important to consider the action of the therapy in the tumor and serum VEGF levels. In the vasculature, VEGF is a potent and powerful vascular permeabilising agent and a potent vasodilator, features of the tumor vasculature which are due to, among other causes, the high levels of tumor and serum VEGF (Glass *et al*, 2006). Sixteen studies with 1111 patients analyzed performed in 2011 demonstrated that, in patients with early-stage ovarian cancer, the overexpression of VEGF in primary tumor and serum is associated with poor progression-free survival and overall survival (Yu *et al*, 2013).

Another interesting and beneficial effect of the treatment with DAPT and rec-PDGFB was the alteration of tumor Angiopoietins 1 and 2 (ANG1 and ANG2). In angiogenesis, ANG1 functions as an agonist of the receptor TIE2 and ANG2 functions as a competitive ANG1 antagonist. In our experimental model, the co-treatment increased tumor ANG1 and decreased tumor ANG2. It is well known that ANG1 stimulates mural coverage and basement membrane deposition, thereby promoting vessel tightness (Augustin *et al*, 2009). Therefore, with DAPT+rec-PDGFB treatment, the increase in tumor ANG1 could be cooperating in the vasculature normalization. Regarding ANG2, beyond its role as a vascular destabilization factor, it has been identified as a tumor biomarker of tumor progression in a number of different tumors. Hence, the decrease in the levels of this protein in our model is also a beneficial effect of our antitumor therapy. Although the exact roles of ANG1 and ANG2 in the tumor vasculature are not clear, the change in the balance between angiopoietins (high ANG2 relative to ANG1) may promote the growth and vascularization of tumors (Holash *et al*, 1999). Therefore, therapies that reverse this ratio may be useful to inhibit undesirable angiogenesis. Moreover, the benefit of diminishing both VEGF and ANG2 has also been described by Peterson *et al*. (2016) in a glioblastoma model where a dual therapy inhibiting both factors enhanced the morphological normalization of the vessels, in agreement with the modulation of vascular function.

AKT is a protein known to play a central role in many cellular processes that, when deregulated, can contribute to the development or progression of cancer. In ovarian cancer, the PI3K/AKT/mTOR pathway is frequently deregulated and has been identified as the pathway most commonly altered in this type of cancer (Huang *et al*, 2011). It is also known that VEGF exhibits multiple biological effects on endothelial cells that are mediated by VEGFR2-PI3K-AKT (Shiojima and Walsh, 2002). The DAPT treatment decreased tumor AKT pathway activation, an effect enhanced by the co-treatment, which led to an increase in the levels of PTEN. The less active state of this pathway in tumors could be due to different reasons, including a decrease in FLK-1 receptor phosphorylation and a decrease in tumor cell proliferation. Besides, DAPT administration increased the number of apoptotic cells, an effect intensified by the co-treatment with PDGFB. These molecular effects were in accordance with the action of the treatments on the tumor size.

Finally, among the Notch actions on tumor cells, several reports describe the consequences of blocking the Notch system on angiogenesis, suggesting a synergism effect of inhibition of tumor angiogenesis and tumor cell proliferation. Although Notch signaling is very context-dependent, in ovarian cancer it has been unequivocally demonstrated to be an oncogenic pathway (Nowell and Radtke, 2017). In the present study, we suggest the use of the gamma-secretase inhibitor DAPT as an antitumor therapy for ovarian cancer and the co-administration of the recombinant factor PDGFB as a tumor vasculature normalizing agent. Due to the promising observations in this work, our future studies will be focused in determining a suitable administration protocol and the vascular normalization window for this potential therapeutic strategy.

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LEGENDS

Figure 1. PDGFB treated tumour growth and inmunohistochemical analysis of ovarian xenograft tumours.

Panel A. SKOV3 cells were inoculated in female nude mice. When tumours were palpable the animals received rec-PDGFB as treatment or saline solution (control) as described in Material and methods section. Tumour size were measured both groups. The variable was analyzed using Student's t-test. No statistical difference was found between groups.

Panels B-E show H&E stained sections of each tumour belonging to all experimental groups (control, rec-PDGFB, DAPT and DAPY+rec-PDGFB). Scale bars: 20 μm in 10x magnification pictures and 50 μm in 40 x magnification insets.

Figure 2. Immunohistochemistry analysis of factors related to proliferation, apoptosis and angiogenesis in control and PDGFB treated tumours.

Ki67 proliferation marker immunostaining of tumour sections (Panel A) and cleaved caspase-3 staining (Panel B). Staining is observed in cell nuclei for both proteins and quantification histogram for each shows the ratio between the number of positively stained cell nuclei and the total number of the nucleous.. Panel C shows alpha smooth muscle actin (αSMA) and panel B depicts PDGF receptor β staining (PDGFRβ). The quantification histograms represent the percentage of positively stained area of the tumour sections. Asterisk indicates significant differences between groups. Unpaired t test.

Figure 3. Immunohistochemistry analysis of Ki67, cleaved caspase-3, αSMA and PDGFRβ in ovarian xenograft tumours.

Ki67 and cleaved caspase-3 quantification are expressed as the ratio between the number of positively stained cell nuclei and the total number of the nucleous. Staining is observed in cell nuclei for both proteins. αSMA and PDGFRβ quantification histograms show the percentage of positively stained area of the tumour sections. Panel A shows αSMA staining and Panel B depicts PDGFRβ of control, DAPT and DAPT±rec-PDGFB treated tumours. Quantification histograms show percentage of positively stained area of the tumour sections. Different letters indicate significant differences between groups. One way ANOVA followed by tukey multiple comparisions test.

Figure 4. Analysis of vessel funcionality with fluorescent tomato lectin staining and HIF-1α levels.

Panel A shows fluorecent tomato lectin staining of control, DAPT, rec-PDGFB and DAPT±rec-PDGFB treated tumours. In control, rec-PDGFB and co-treated tumour sections, asterisk indicates leaky vessels and arrow shows defined vessels. In DAPT tumour section, asterisk indicate lectin extravasation and arrow indicates vessel lumens and vessel sprouting. Panel B depicts quantification histogram of HIF-1**α** levels determined by western blot in tumour protein extracts and expressed as densitometric units. a vs b means significant differences with $p<0.05$. Scale Bar: 50 μm.

Figure 5. Protein levels of angiogenic factors in ovarian xenograft tumours.

Angiogenic factors levels were determined by western blot in ovarian xenograft tumours protein extracts and different panels show quantification histograms with densitometric units and expresed as mean±SEM. Different letters indicate significant differences between treatments. a vs b $p<0.05$.

Figure 6: Ovarian xenograft tumour growth of DAPT and PDGFB co-treated animals and levels of related proteins.

Panel A. SKOV3 cells were inoculated in female nude mice. When tumours were palpable the animals received the treatments (control, DAPT, rec-PDGFB and DAPT+rec-PDGFB). Tumour size were measured in the three groups. The data was analyzed with Two-way repeated-measures ANOVA. Significances were analyzed day by day and are shown as follows: *DAPT vs DAPT+rec-PDGFB; ** control vs DAPT+rec-PDGFB; a vs b, control vs DAPT $p<0.05$; a vs c, control vs DAPT $p<0.001$; b vs c, control vs DAPT, $p<0.01$. The other panels show protein levels of PCNA, phospho-AKT/AKT ration and PTEN in tumour protein extracts determined by western blot. a vs b, $p<0.05$ and a vs c, $p<0.01$.

Table 1. List of antibodies and their dilutions used for the different techniques.

Antibody Target	Host/type	Catalog Nº	Supplier	Technique	Dilution
Primary Antibodies					
AKT	Rabbit Polyclonal	9272	Cell Signaling Technology[a]	Western Blot	1/5000
Phospho-AKT (ser 473)	Rabbit Polyclonal	sc-7985	Santa Cruz Biotechnology, Inc.[b]	Western Blot	1/400
PTEN	Rabbit Monoclonal	MAB4037	Millipore[c]	Western Blot	1/500
PCNA (Fl-261)	Rabbit Polyclonal	Sc-7907	Santa Cruz Biotechnology, Inc.[b]	Western Blot	1/500
VEGF	Mouse Monoclonal	Sc-7269	Santa Cruz Biotechnology, Inc.[b]	Western Blot	1/100
VEGFR2/ FLK-1 /KDR	Rabbit Monoclonal	2479	Cell Signaling Technology[a]	Western Blot	1/500 5%BSA
Phospho-VEGFR2 (Tyr1175)	Rabbit Monoclonal	2478	Cell Signaling Technology[a]	Western Blot	1/500 5%BSA
Angiopoietin-1	Rabbit Polyclonal	10516	Millipore[c]	Western Blot	1/500
Angiopoietin-2	Rabbit Polyclonal	180820	Abcam[f]	Western Blot	1/500
HIF-1α	Mouse Monoclonal	MAB1536	R&D Systems[d]	Western Blot	1/500
β-Actin	Rabbit Polyclonal	sc-1616	Santa Cruz Biotechnology, Inc.[b]	Western Blot	1/10000
GAPDH	Rabbit Monoclonal	2118	Cell Signaling Technology[a]	Western Blot	1/10000
Ki67	Rabbit Polyclonal	Ab15580	Abcam[f]	IHC	1/500
Cleaved Caspase 3	Rabbit Polyclonal	Cp229	Biocare Medical[e]	IHC	1/100

α-SMA	Mouse Polyclonal	ab18147	Abcam[f]	IHC	1/100
PDRFRβ	Rabbit Polyclonal	Sc-432	Santa Cruz Biotechnology, Inc.[b]	IHC	1/200
Secondary Antibodies					
Rabbit IgG (Biotinylated)	Goat Polyclonal	BA-1000	Vector Laboratories[g]	IHC	1/400
Mouse IgG (Biotinylated)	Goat Polyclonal	BA-9200	Vector Laboratories[g]	IHC	1/400
Rabbit IgG (conjugated to HRP)	Goat Polyclonal	A-9414	Sigma-Aldrich[h]	Western Blot	1/1000
Mouse IgG (conjugated to HRP)	Goat Polyclonal	HAF007	R&D Systems[d]	Western Blot	1/1000

[a]Cell Signaling Technology (Beverly, MA, USA).

[b]Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA

[c]Millipore.

[d]R&D Systems (MN,USA)

[e]Biocare Medical (Concord, CA, USA).

[f]Abcam (Cambridge, Massachusetts, USA).

[g]Vector Laboratories, (Burlingame, CA, USA).

[h]Sigma-Aldrich (St. Louis, MO, USA).

IHC, immunohistochemistry; Ig, immunoglobulin; HRP, horse radish peroxidase.

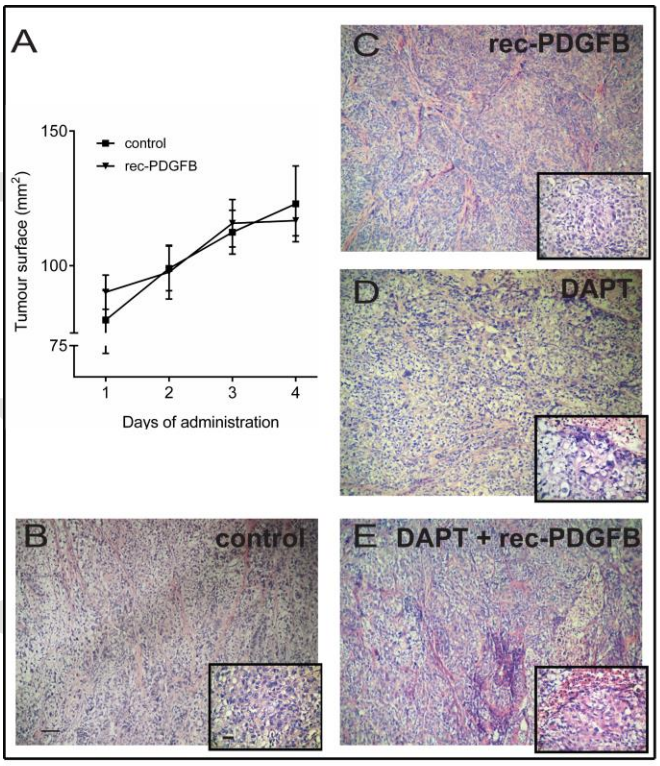

Figure 1

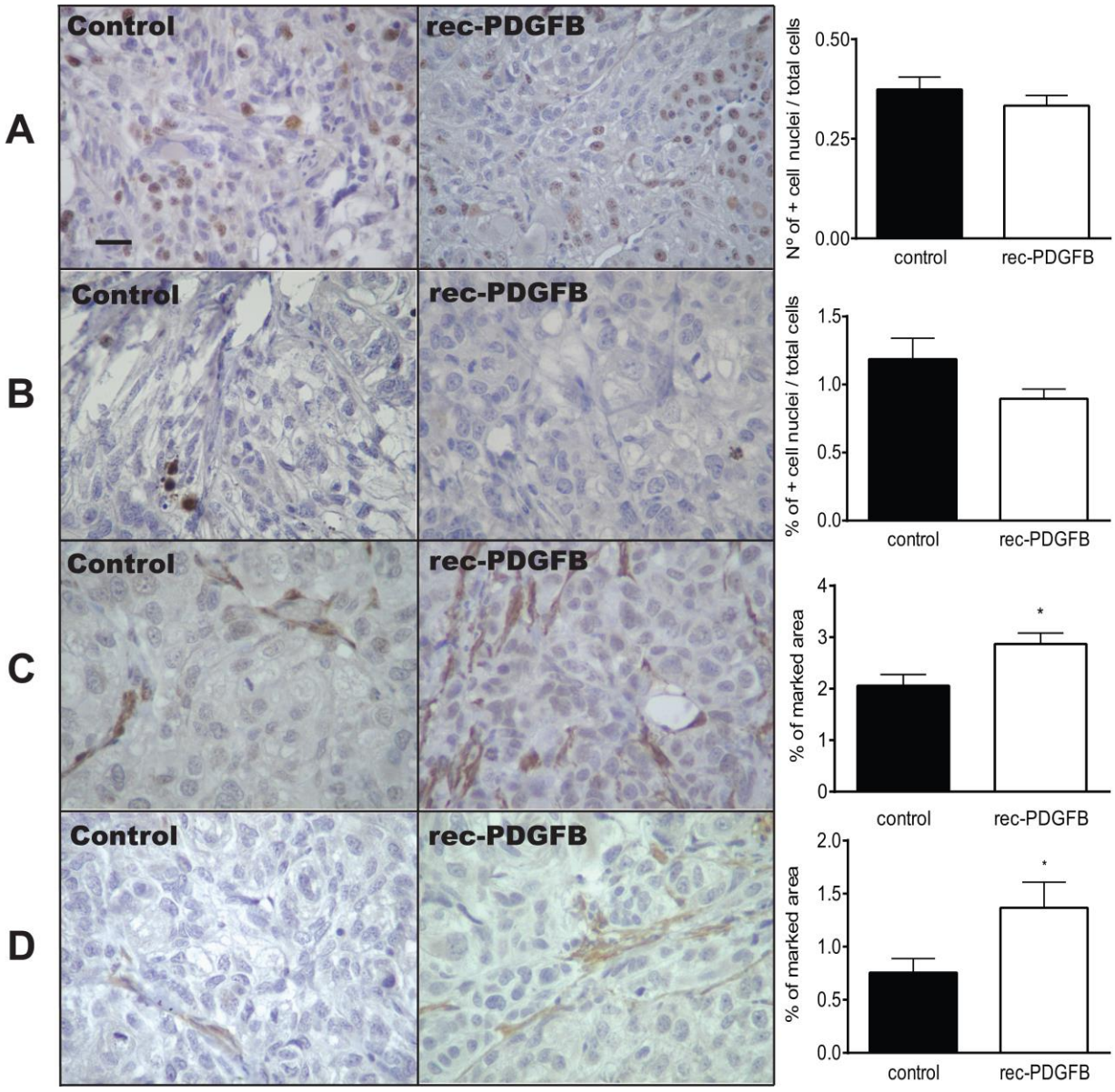

Figure 2

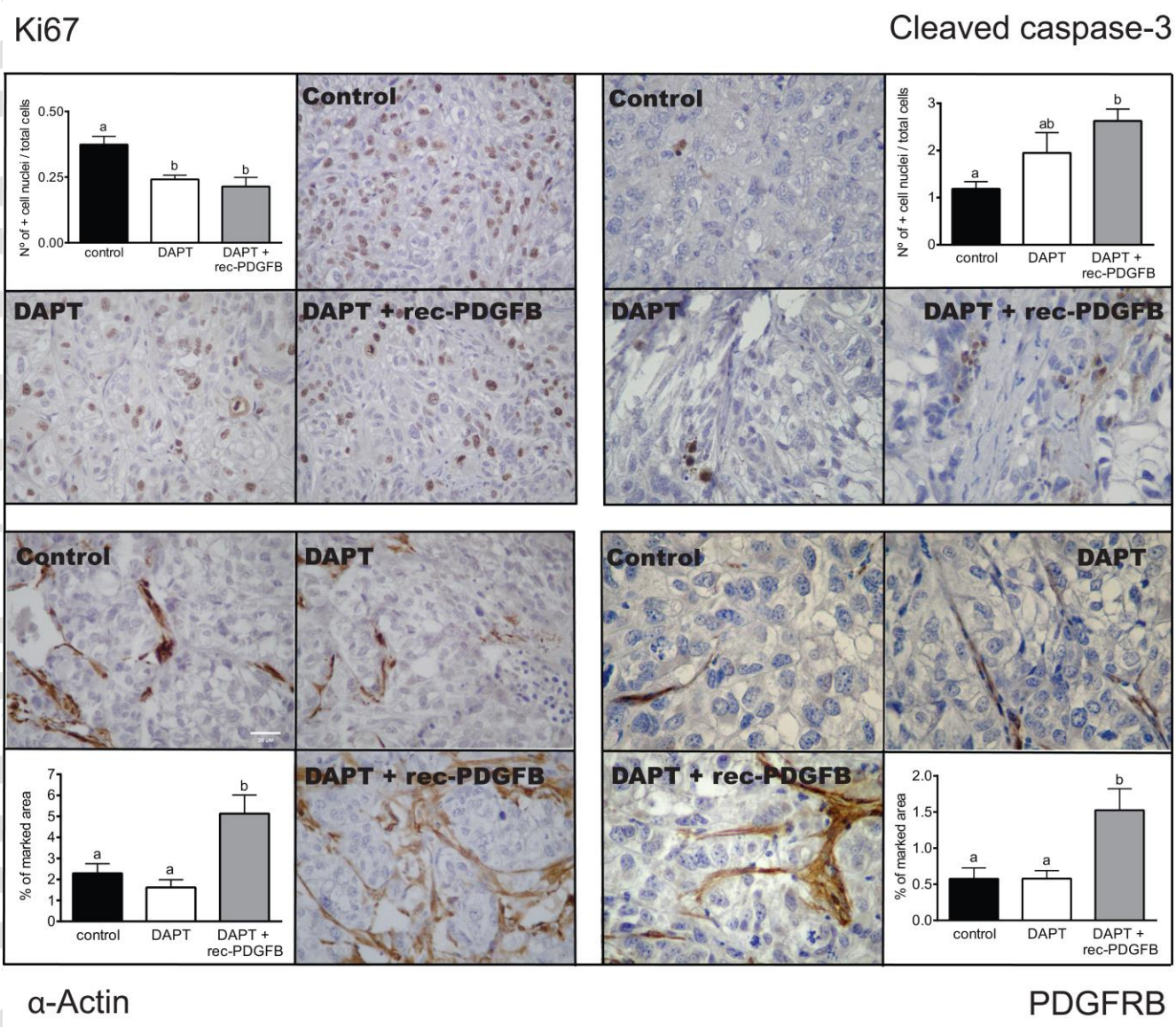

Figure 3

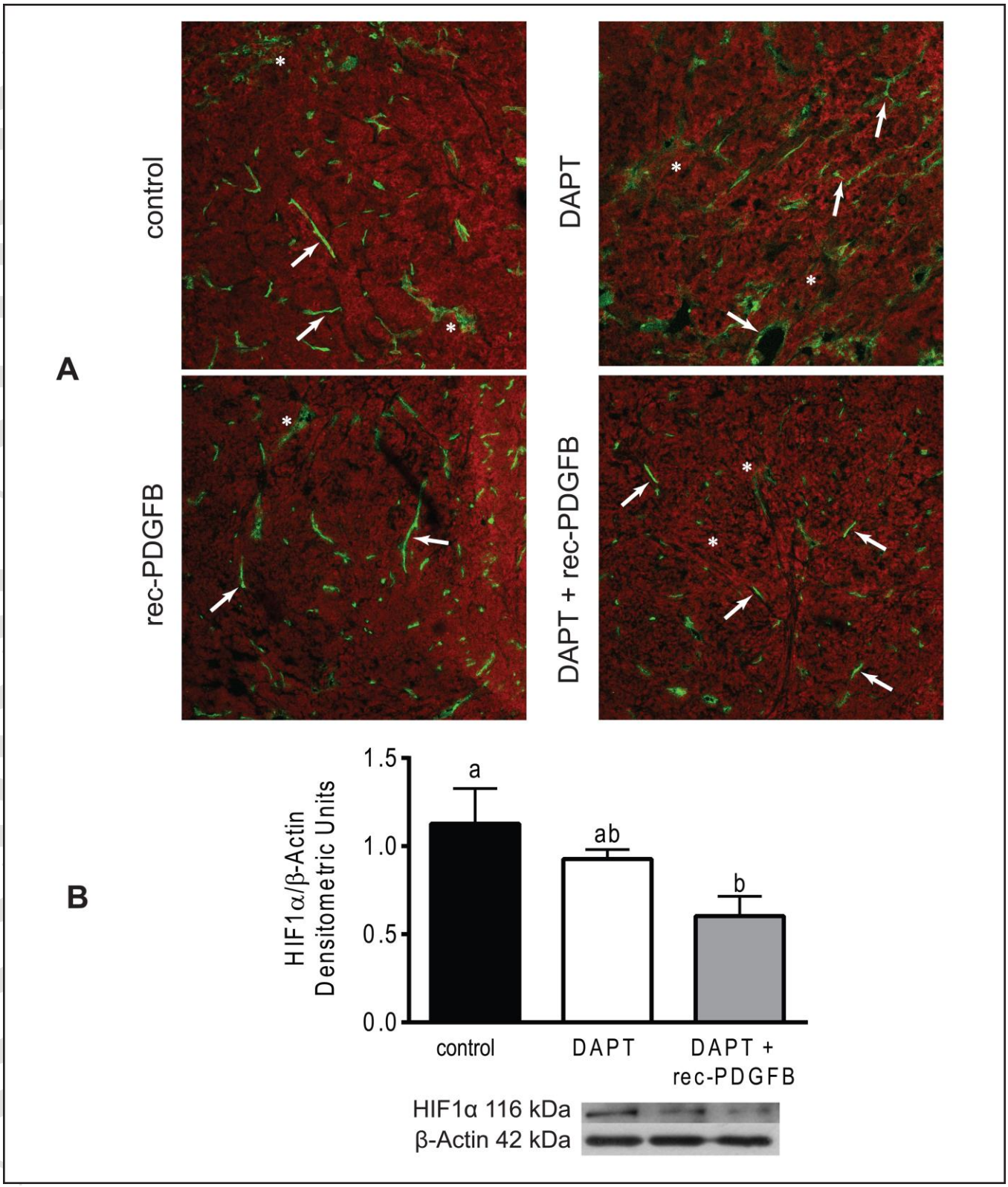

Figure 4

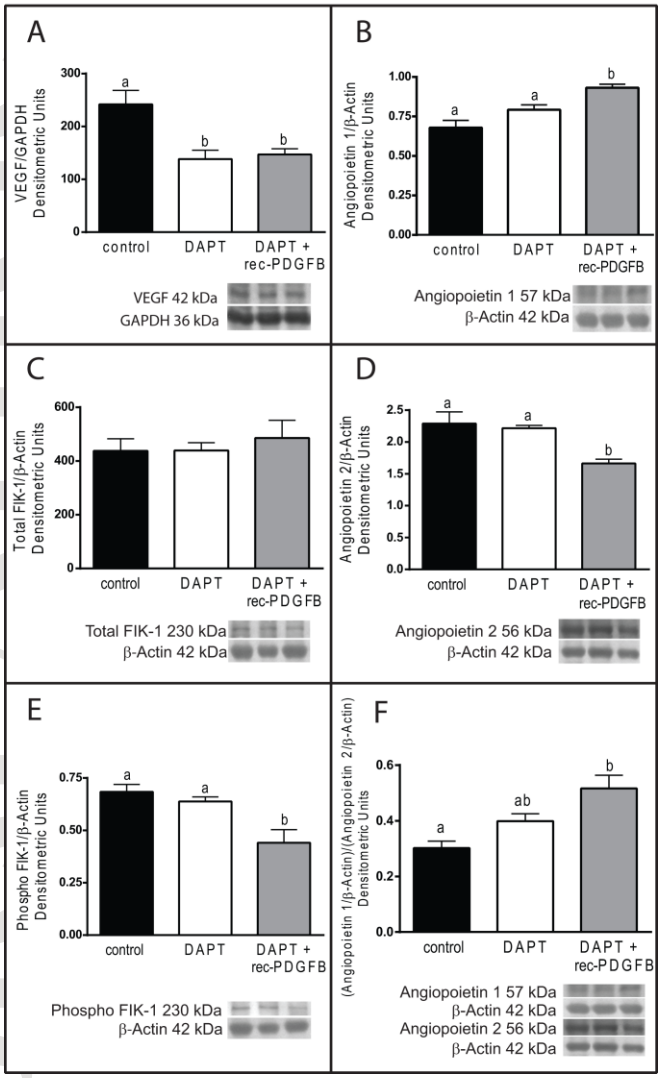

Figure 5

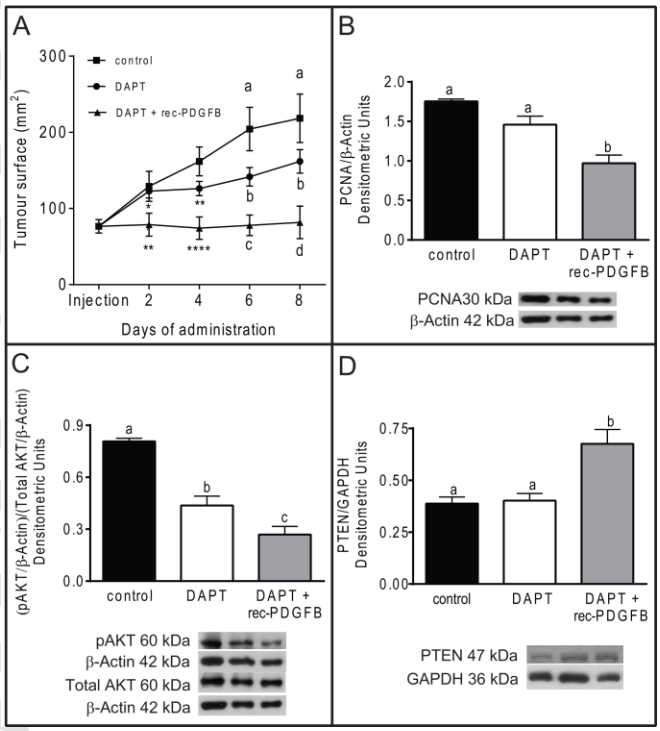

Figure 6