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Platelets and Anti-Angiogenic Resistance in Ovarian Carcinoma

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PLATELETS AND ANTI-ANGIOGENIC RESISTANCE IN OVARIAN CARCINOMA

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
In Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

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Houston, Texas

August, 2012

PLATELETS AND ANTI-ANGIOGENIC RESISTANCE IN OVARIAN CARCINOMA

Publication No._____

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Background: Resistance to targeted anti-angiogenic therapy is a growing clinical concern given the disappointing clinical impact of anti-angiogenic. Platelets represent a component of the tumor microenvironment that are implicated in metastasis and represent a significant reservoir of angiogenic regulators. Thrombocytosis has been shown to be caused by malignancy and associated with adverse clinical outcomes, however the causal connections between these associations remain to be identified.

Materials and Methods: Following IRB approval, patient data were collected on patients from four U.S. centers and platelet levels through and after therapy were considered as indicators of recurrence of disease. *In vitro* effects of platelets on cancer cell proliferation, apoptosis, and migration were examined. RNA interference was used to query signaling pathways mediating these effects. The necessity of platelet activation for *in vitro* effect was analyzed. *In vivo* orthotopic models were used to query the impact of thrombocytosis and thrombocytopenia on the efficacy of cytotoxic chemotherapy, the effect of aspirin on thrombocytosis and cancer, and platelet effect on anti-angiogenic therapy.

Results: Platelets were found to increase at the time of diagnosis of ovarian cancer recurrence in a pattern comparable to CA-125. Platelet co-culture increased proliferation, increased migration, and decreased apoptosis in all cell lines tested. RNA interference implicated platelet derived growth factor alpha (PDGFRA) and

transforming growth factor beta-receptor 1 (TGFBR1) signaling. Biodistribution studies suggested minimal platelet sequestration of taxanes. Blockade of platelet activation blocked *in vitro* effects. *In vivo*, thrombocytosis blocked chemotherapeutic efficacy, thrombocytopenia increased chemotherapeutic efficacy, and aspirin therapy partially blocked the effects of thrombocytosis. In vivo, withdrawal of anti-angiogenic therapy caused loss of therapeutic benefit with evidence of accelerated disease growth. This effect was blocked by use of a small-molecule inhibitor of Focal Adhesion Kinase. Anti-angiogenic therapy was also associated with increased platelet infiltration into tumor that was not seen to the same degree in the control or FAK-inhibitor-treated mice.

Conclusions: Platelets are active participants in the growth and metastasis of tumor, both directly and via facilitation of angiogenesis. Blocking platelets, blocking platelet activation, and blocking platelet trafficking into tumor are novel therapeutic avenues supported by this data.

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Background and Introduction

Angiogenesis

Angiogenesis is necessary for tumor growth and progression (1). Tumors acquire a blood supply by varied means including vasculogenesis, co-option of established vasculature, and vascular mimicry (2, 3). Vasculogenesis refers to production of new blood vessels via infiltration of new endothelial cells in to the tumor. Co-option of established vasculature refers to tumor cells surrounding and invading established, otherwise normal and healthy vascular structures. Vascular mimicry refers to the phenomenon in which tumor cells take on the phenotypic and functional aspects of endothelial cells necessary to facilitate blood flow. Efforts to therapeutically target tumor angiogenesis have focused on the vascular endothelial growth factor (VEGF) pathway (4). VEGF targeting has shown promise, but it has not proven as efficacious as hoped (5, 6). Evidence supports the hypothesis that members of the tumor microenvironment mediate some mechanisms of escape from anti-angiogenesis therapy; it is thus hypothesized that simultaneous targeting of tumor and microenvironment may lead to improved clinical outcomes in the treatment of solid tumors (7, 8).

Targeting Angiogenesis via VEGF

Forty years ago, driven by the discovery that tumor-associated endothelial cells are foundational in tumor neovascularization, these endothelial cells were proposed as a therapeutic target. VEGFA was identified as a central promoter of

angiogenesis in general and endothelial cell survival in particular (9). Based on these findings, the VEGF signaling system has been targeted using both small molecule inhibitors and antibodies to components of the system. Bevacizumab (monoclonal antibody against VEGFA165) was among the earliest anti-angiogenic agents developed, and it is currently the most advanced of VEGF-targeted agents (10). This agent has provided clinical benefit in several types of solid tumors, including colorectal, renal, non-squamous/non-small-cell lung cancer, and glioblastoma (11). While bevacizumab was approved for metastatic breast cancer in 2008, the Food and Drug Administration ultimately withdrew approval over concerns about efficacy and toxicity (11). In ovarian cancer, Gynecologic Oncology Group (GOG) 218 and the Gynecologic Cancer InterGroup (GCIG) trial ICON7, both conducted in the front-line adjuvant setting following surgical tumor cytoreduction, demonstrated modest improvements in progression free survival (PFS) in the groups receiving maintenance bevacizumab. Overall survival (OS) data are not anticipated to be positive, and while interval to progression is improved, there appears to be no improvement in the total number of patients who progress (12, 13). Small molecule inhibitor data are less mature, but early data suggest similar concerns with respect to sorafenib and sunitinib (14, 15).

Angiogenesis Resistance and Escape

As our concept of angiogenesis has matured and expanded, the complexity of angiogenesis with its balance of redundant and oppositional

systems has become more apparent (3). Evolutionary biology offers the perspective that disruptions in an ecologic system generated by a new selection pressure produce reactions that tend to stabilize the entire system; heterogeneity within a system will provide resilience against selection pressure. Cancer cells are heterogeneous, as is their microenvironment, facilitating systemic resilience within the whole of the tumor to selection pressures (16). Anti-angiogenic therapy decreases blood flow and increases hypoxia within the tumor microenvironment, triggering tumor cell-driven compensatory responses that have myriad downstream effects (17). Otherwise normal cells in the tumor microenvironment, e.g. endothelial cells, pericytes, platelets, fibroblasts, and white blood cells, are known to have healthy, regulatory functions that are co-opted to support the tumor. Potential mechanisms of resistance to anti-angiogenic therapy may be mediated by tumor cell selection directly, but microenvironmental selection pressure and modification is likely to play a significant role in resistance to these therapies.

Ovarian Cancer

Across the spectrum of human malignancy, over the past thirty years, SEER data suggest little progress in curbing mortality. Specifically, considering all mortality in the United States from 1975-2009, age-adjusted mortality with respect to cancer has only decreased from 200 deaths per 100,000 people to 175 deaths per 100,000 people over those thirty years.

Ovarian cancer remains the fifth leading cause of cancer death among women. Approximately 21,000 American women will receive the diagnosis of ovarian cancer this year, and about 15,000 American women will die this year from this disease (18). Recurrence rates have changed little over the past thirty years, and recurrence remains an essentially terminal event, and as a consequence, disease-specific mortality has changed little over the last thirty years. In contrast, improvements have been seen in median survival for women with advanced ovarian cancer, which has increased over 18 months during the same thirty-year period (19). However, the absence of reliable, prospective screening biomarkers, it remains that disease onset is insidious, and late diagnosis of advanced disease remains common (20).

Typical care for primary ovarian cancer involves some combination of tumor reductive surgery and 6-8 cycles of cytotoxic chemotherapy, most typically combined carboplatin and paclitaxel (21). While epithelial ovarian cancer tends to be chemo-responsive, with an induction-chemo response rate of 70-80%, responders have a 40-50% rate of relapse within 2 years (22). In the recurrent setting, salvage chemotherapy provides a 15-20% response rate with essentially zero cures (23-25). Considering large, modern clinical trials, women with ovarian cancer are estimated to have a median progression-free interval in the range of 11-18 months and median survival duration in the range of 24-40 months, depending on stage of disease at diagnosis and the success of the tumor reductive surgery (26-28).

Recent prospective clinical studies have suggested that the addition of more cytotoxic agents the current standard of paclitaxel and carboplatin does not improve progression or survival outcomes (29). This disappointing outcome has opened the floor to alternative targets and therapeutic regimens. One compelling strategy includes “metronomic” dosing of chemotherapy. By this strategy, lower doses of cytotoxic agents are given at greater frequency. One hypothesis is that this dosing regimen targets more than merely tumor cells, but targets systems-level biologic functions, including the development and maintenance of tumor vasculature (30, 31). Clinically, semi-metronomic, or “dose-dense,” regimens have shown intriguing superiority in early clinical trials to traditional dosing regimens (32-35). Mechanisms remain poorly characterized but suggest effects other than those typically considered, including microenvironmental targeting that may effect treatment efficacy and inform concepts of appropriate/effective dosing.

The fact that we have seen limited improvement in survival outcomes for ovarian cancer over the past 30 years should give pause, and it raises the question that we may be ignoring a significant biological component of cancer growth and development that strongly influences treatment efficacy. Given recent trends toward more comprehensive consideration of the totality of tumor biology and interaction with its environment, we considered the question of whether micro-environmental factors, for example platelets, could contribute to the pathogenicity of cancer.

Platelet Development

Megakaryopoiesis is a complex and evolutionarily conserved process. Nearly one billion megakaryocytes turn over in the bone marrow every day (36). The cell of origin is the committed myeloid progenitor cell, from which the colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) emerges. From the population of CFU-GEMM, some will become burst-forming unit-megakaryoctes (BFU-Meg) that are ultimately and irrevocably committed to megakaryocyte differentiation. IL-3 supports early stages of megakaryocyte development. Thrombopoietin (TPO) primarily regulates thrombopoiesis. IL-6, IL-11, and stem cell factor (SCF) are also stimulatory, but they will only act in coordination with TPO and IL-3. (Figure 1)

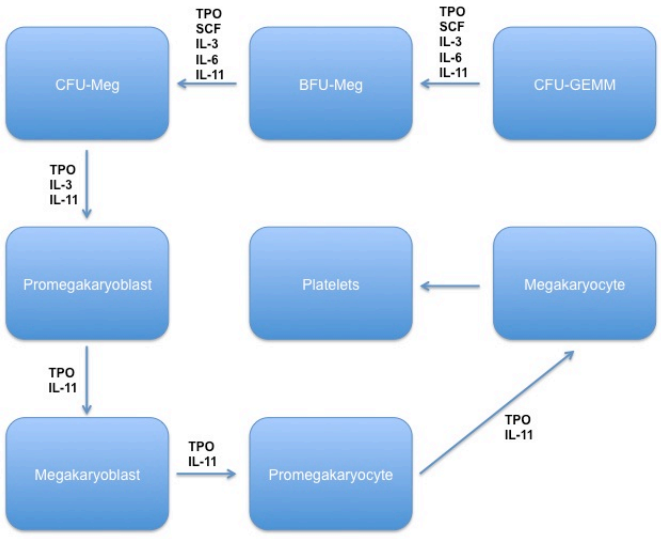


Figure 1: Schematic diagram of platelet development. The process is primarily driven by IL-3 and TPO and is augmented by other cytokines.

Platelets average 2-5 mcm diameter and 0.5 mcm thickness. They have an average lifespan of 7-10 days, requiring production of 1.0×10^{11} cells per day to maintain normal blood levels in humans (37). This baseline production level

will fluctuate substantially in response to contextual cues, including humoral factors resulting from malignancy (37).

Platelet structure

Electron microscopy of the platelet plasma membrane suggests a folded surface that would accommodate significant spreading and shape change. It also shows small openings in the plasma membrane connected to the canalicular system. In the submembrane zone, there are no formal organelles, but cytoskeletal structures are seen that appear to have contractile function dedicated to the movement and localization of receptors on the platelet surface; these elements appear vital to adhesion and spreading.

Within the interior of the platelet, imaging suggests a matrix within which several organelle structures have been identified. What were thought to be circumferential coils of microtubules actually represent a single microtubule, coiled on itself many times like a cowboy's lasso. Experimental evidence suggests that this single, coiled microtubule provides cytoskeletal support and facilitates pseudopod extension for platelet migration. The effect of paclitaxel on platelet function has been studied in this context. Specifically, platelets were chilled to 4^{o}C, causing loss of disc shape, increased pseudopod extension, disassembly of microtubule coil, and assembly of new actin filaments, all consistent with migration and activation activities. Re-warming to room temperature reversed these changes. Paclitaxel blocked the cold-induced

changes and specifically limited pseudopod formation, suggesting that in theory paclitaxel may block platelet migration (38).

Microfilaments participate in platelet activation and the release of platelet granule contents into the environment around the platelet. They are composed of actin filaments and make up the preponderance of the cytoskeleton. When platelet activation occurs, microfilaments participate in microtubule constriction and mobilize granules to facilitate secretion of contents via the open canalicular system. The open canalicular system connects the surface of the platelet to the entire interior of the platelet, facilitating efficient material exchange and protein transfer (39-42). It is this release of granule contents in which may be found an underappreciated nexus of angiogenesis regulation.

Alpha-granules are the most abundant organelles. There are typically 40-80 alpha granules per platelet. Alpha-granules have been documented to contain a significant number of compounds, many of which are growth factors and angiogenesis modulators and are listed in Table 1.

Alpha-Granule Contents(43-50)	
Adhesion Molecules	**P-Selectin** **Von Willebrand Factor** **Thrombospondin** **Fibrinogen** **Integrin alpha-2b-beta-3** **Integrin alpha-5-beta-3** **Fibronectin**
Chemokines	**Platelet basic protein** **Platelet Factor 4** **CXCL4** **Beta-thromboglobulin** **MIP-1-alpha** **RANTES** **MCP-3** **CCL17** **CXCL1** **CXCL5** **IL-8**
Coagulation Pathway	**Factor V** **Multimerin** **Factor VIII**
Fibrinolytic Pathway	**Alpha-2-Macroglobulin** **Plasminogen** **Plasminogen activator inhibitor 1**
Growth Factors and Angiogenesis	**Basic fibroblast growth factor** **Epidermal growth factor** **Hepatocyte growth factor** **Insulin-like growth factor-1** **Transforming growth factor-beta** **Vascular endothelial growth factor-A** **Vascular endothelial growth factor-C** **Platelet-derived growth factor** **Gas6**
Immunologic Factors	**Beta-1-H globulin** **Factor D** **C1 inhibitor** **IgG**
Other	**Albumin** **Alpha-1-antitrypsin** **Histidine-rich-glycoprotein** **High molecular weight kininogen** **Osteonectin** **Protease nexin-2**

Table 1: Alpha granule contents identified to date.

Dense granules are smaller and less numerous than alpha-granules. Their contents are primarily ions and adenine nucleotides and are detailed in Table 2.

Dense Granule Contents(51, 52)	
Ions	**Calcium** **Magnesium** **Phosphate** **Pyrophosphate**
Nucleotides	**ATP** **GTP** **ADP** **GDP**
Membrane Proteins	**CD63** **LAMP2**
Transmitters	**Serotonin**

Table 2: Dense granule contents identified to date.

Platelets and Angiogenesis

As described earlier, the regulation of angiogenesis in tumors has been an active area of basic and translational research for forty years (53). As early as the 1960's, experimental models associated platelets with endothelial cell stability (54-56). Platelet co-culture was found to promote endothelial cell growth, and early descriptive studies suggest changes in the shape and migration of endothelial cells in response to platelet co-culture (57-59).

It has since been determined that platelets sequester and release an array of angiogenesis-regulating agents, underscoring the reasonable hypothesis that

platelets are involved in angiogenic regulation and the difficulty in determining a total effect given such a large and diverse set of angiogenic regulators. These regulators are detailed in Table 3.

Angiogenesis Regulators	
Vascular Endothelial Growth Factor (VEGF)	Angiogenesis agonists found in platelets as VEGFA (primarily $VEGFA_{165}$) and VEGFC.[71] Platelets have mRNA for $VEGF_{189}$ and $VEGF_{121}$.[72]
Platelet-Derived Growth Factor (PDGF)	Found in three dimer combinations of two chains (A and B).[73] Receptor binding results in proliferation and migration of fibroblasts and smooth muscle cells.[74]
Fibroblast Growth Factor (FGF)	Angiogenesis agonists FGF2 and bFGF recruit and induce proliferation of endothelial cells.[75,76]
Epidermal Growth Factor (EGF)	Angiogenesis agonist that induces proliferation and tube-formation in cell lines that express the appropriate receptor.[77,78,79]
Hepatocyte Growth Factor (HGF)	Angiogenesis agonist via the activation of the c-met receptor, which is highly expressed in smooth muscle and endothelial cell populations.[80]
Insulin-like Growth Factor (IGF)	Both isoforms (1 and 2) are angiogenesis agonists. Receptor activation is pro-angiogenic.[81,82]
Angiopoietin (APO)	APO-1 is a ligand for the tyrosine kinase receptor Tie2, and binding is pro-angiogenic.[83] Increased in platelets of breast cancer patients.[84]
Platelet Phospholipids	Molecular family with chemotactic, proliferation, and tube-formation effects on endothelial cells.[85,86]
CD40 Ligand and Platelet Activating Factor	Expressed on the platelet surface following activation. Binding to endothelial cells is pro-angiogenic and partially mediated by platelet-activating factor.[87]
Matrix Metalloproteinases	Of this family of endopepidases known to facilitate cellular migration with angiogenesis, MMP-1, MMP-2, and MMP-9 are contained in platelets.[88,89]
Heparanase	This endoglycosidase cleaves heparin sulfate, releasing angiogenic mediators and increasing bioavailability.[90,91]
Angiostatin	Potent angiogenesis inhibitor released on activation.[92]
Thrombospondin-1 (TSP1)	At low concentrations, TSP1 has anti-angiogenic effects. At high concentrations, it can mediate pro-angiogenic effects.[93,94]
Platelet Factor 4 (PF4)	Angiostatic effects are driven by interference with the binding of and function of growth factors, e.g. FGF.[95,96]

Table 3: Functions of angiogenesis regulators identified in platelets.

In the context of wound healing, angiogenesis regulation and function is increasingly understood include platelets, although the question of specific platelet function in wound healing remains controversial (60, 61). Platelets form plugs. Of more interest is the observation that VEGF concentration increases in the region of an *in vivo* thrombus, and VEGF derived from platelets accumulates within thrombus, presumably to be released as the thrombus resolves (62, 63). In an experimental wound model in rats, wound formation was correlated with increased serum VEGF and decreased endostatin. These changes in serum VEGF and endostatin did not occur when the animals were treated with ticlodipine (an inhibitor of platelet activation by ADP) or by platelet depletion (64). In other animal models, application of platelet-derived topical compounds to wounds stimulates formation of granulation tissue (65). In clinical trials, affirmative results have been obtained using the local, topical application of platelet-derived topical compounds to wounds (66-68).

Ultimately, most experimental work on platelets with respect to angiogenesis has focused on endothelial cell responses. Platelet co-culture promotes endothelial cell proliferation and survival when those cells are otherwise deprived of appropriate growth factors. These effects do not require direct contact, but activation does appear to be required, as aspirin pre-treatment and fixation with paraformaldehyde both appear to block the observed effects. Platelets have been shown promote tube formation in human umbilical vein endothelial cells in a dose-dependent manner (69). The observed effects appear to be mediated in part by VEGF and FGF2 (70).

Considering the breadth of regulatory molecules in platelets, consideration of the sum effect of effectors becomes important. In an *ex vivo* rat model, pro-angiogenic effects of platelets were substantially inhibited through blockade of the VEGF, bFGF, and PDGF signaling pathways (71). An *in vivo* mouse model using subcutaneous Matrigel suggested a similar spectrum of causal signaling and pointed to the importance of heparanase as a participant in the biology (71). Other investigators have specifically implicated VEGF and bFGF in platelet-driven angiogenesis (72). Separate studies have suggested that platelet involvement in angiogenesis and metastasis is at least partially mediated by the signaling of integrin alpha-5-beta-3 between platelets and the endothelium (73).

Platelets and Malignancy

Interactions between tumor cells and the tumor microenvironment are increasingly recognized as key context to understand the selection pressures that influence the survival and propagation of clonal cell populations that manifest particular phenotypes such as invasion and metastasis. For instance, a dynamic interaction between tumor cells, fibroblasts, inflammatory cells, and bone marrow derived precursors has been shown to facilitate tumor cell intravasation and metastasis (74).

With respect to platelets, the correlation between thrombocytosis (conventionally defined as >450,000 platelets per cubic millimeter of whole blood) and solid malignancy has been recognized for over a century and has been an area of intermittent research. Recently, it was shown that tumor production of IL-

6 caused increased hepatic production of thrombopoietin that caused increased megakaryocyte counts and production of platelets in the bone marrow (37). Correlative confirmation comes from a nude mouse model utilizing immortalized Chinese hamster ovarian cells, IL-6 transfection of the cells was associated with elevating circulating IL-6 in vivo, hypercalcemia, leukocytosis, thrombocytosis, and decrease in body weight compared to animals bearing non-transfected tumor (75).

The biological significance of this correlation has been more elusive, however several studies have suggested that elevated platelet counts in cancer are associated with adverse prognosis (37, 76, 77). As early as the 1960's, an experimental model supported a direct link between thrombocytosis and metastasis (78). It was first demonstrated that metastasis could be limited by platelet depletion; it was subsequently shown that metastasis of many tumor cell types (in the experimental setting) was facilitated by platelet interaction with tumor cells (78-82). The association between platelet aggregation and metastasis was later established (83, 84). Transgenic mouse models have since demonstrated that reduced platelet counts and function correlate with decreased metastasis formation (78, 82, 85, 86).

The conventional view of the role of platelets in metastasis initially focused on the promotion of adhesion and the formation of a protective barrier around tumor cells. Platelet barriers have been shown to protect tumor cells from natural killer cells (87, 88). Platelets also have been shown to protect tumor cells from shear stress and promote endothelial adhesion (89-94). It was subsequently

hypothesized that platelet-tumor emboli cause ischemic damage to the endothelium, facilitating binding and arrest of platelet-tumor aggregates, but while this hypothesis has intuitive appeal, experimental evidence supporting this view is lacking (87, 88).

Tumor emboli are well-known to include aggregated platelets (95, 96). Correlations have been experimentally identified between the ability of tumor cells to induce platelet aggregation *in vitro* and the degree to which platelets facilitate metastasis for those tumor cell types *in vivo* (79, 81). Circulating tumor cells appear to have the capacity to facilitate platelet activation and promote platelet aggregation (97). It is now established that platelets interact directly with tumor cells, improving the ability of tumor cells to survive in circulation (95, 96).

Early attempts to translate this work into therapy focused on the question of platelet aggregation and the platelet-tumor embolus. Initial attempts to block experimental tumor metastasis with aspirin and ticlodipine were largely unsuccessful (83, 93, 98-100). In contrast, partial diminution of *in vivo* pulmonary metastasis was demonstrated using an anti-von Willebrand factor antibody and an anti-GP2b-3a antibody, supporting the role for platelets in metastasis via systems reliant on integrin GP2b-3a, P-selectin, fibronectin, fibrin, von Willebrand factor, and thrombin (93). There is further experimental support for involvement of laminin, vitronectin, type IV collagen, thrombospondin, and several integrin receptors (alpha3-beta1, alpha5-beta1, and alpha5-beta-3) in platelet-tumor interactions (101-108).

Recalling that tumors co-opt systems with normal functions, it should be recognized that some of the mechanisms through which platelets impact tumor biology are dysregulated mechanisms by which platelets otherwise would participate in healthy wound repair. Abnormal tumor vasculature functions poorly, reflected by chaotic, stagnant, even reversed flow (109, 110). The resulting hypoxic, acidic microenvironment is associated with protease-mediated matrix remodeling, anchorage-independent growth, and resistance to apoptosis (111, 112). It is intuitive that this environment also should permit increased platelet extravasation followed by a cascade of increased platelet activation, and a feed-forward loop should be anticipated, especially given that platelets are already known to increase adhesion to VEGF-activated endothelial cells (113). The impact of platelets on systemic, serum levels of angiogenic mediators is an area of on-going study (114). Platelets have elevated angiogpoietin-1 in cancer patients and sequester significant VEGF (115, 116). It has been hypothesized that platelets may nudge the balance of angiogenic regulators toward a net pro-angiogenic balance in cancer patients (117). Recent evidence has supported platelet sequestration and differential release of angiogenic mediators in the face of differential environmental stimuli, and prominent researchers have advocated this phenomenon as a possible screening mechanism for the early detection of solid malignancy (118-120). Recent evidence suggests that platelet release of alpha-granule contents is variable based on pH and that at relatively low pH, platelet-driven neutrophil chemotaxis becomes prominent (121).

Platelet provision of mitogenic signaling to cancer cells remains under-studied. Purinergic signaling from platelets has been shown to influence cell migration and proliferation (122). Dense granules release agents known to modulate cell growth and migration (123, 124). Alpha granules have emerged as mediators of pro- and anti-angiogenic effector molecules (118, 125, 126). Many of these proteins have mitogenic significance. For example, VEGF and PDGF signaling are known to be pro-proliferative, pro-migratory, and anti-apoptotic. A cancer with the capacity to co-opt to that system of platelet-driven angiogenesis regulation would be able to promote its own angiogenesis and impart upon itself a significant survival advantage, potentially decreasing responsiveness to traditional cytotoxic agents. In solid malignancy, soluble growth factors and cytokines secreted by stromal cells have been shown to contribute to the so-called epithelial-to-mesenchymal transition, a reversible alteration of phenotype in which increased motility and invasive capacity is seen (127, 128). TGF-beta is known to promote the epithelial-to-mesenchymal transition and invasive capacity of solid malignancies (129). Platelet-derived TGF-beta signaling through the Smad and NF-kB pathways via direct platelet interaction with tumor cells facilitated metastasis from a subcutaneous *in vivo* model to the lung (130).

Drugs and Platelet Function

Platelets clearly warrant attention as targetable mediators of angiogenesis. Aspirin is a commonly used inhibitor of platelet aggregation, acting via irreversible acetylation of components of cyclooxygenase (COX)-1 and COX-

2. COX-1 is widely expressed in platelets, and blockade COX-1 function results in failed prostaglandin synthesis and therefore failed Thromboxane A_2 synthesis, thus blocking thromboxane-dependent platelet activation and aggregation (131-134). Aspirin is a relatively weak inhibitor of platelet activation and aggregation because multiple other activating stimuli, (e.g. shear force, catecholamines, thrombin, and ADP) are capable of activating platelets in the face of aspirinization through non-thromboxane-dependent mechanisms (135). Recent interest has emerged regarding aspirin as a chemopreventative agent. A series of meta-analyses of secondary end-points of randomized and case-control studies have indicated a significant hazard benefit with respect the to the development of multiple malignancies in people allocated to even low-dose aspirin use (136-139). These investigations, while thought-provoking, should be interpreted with care in view of their retrospective (and therefore necessarily biased) point of view and reliance on secondary end-points.

ADP is a biologically significant platelet activator. Platelets have at least two distinct receptors that interact with ADP, the $P2Y_1$ and $P2Y_{12}$ receptors (140). ADP signaling through these receptors is responsible for ADP-driven platelet activation. $P2Y_{12}$ is the more platelet-selective of the two receptors in its expression, and it is currently targeted in clinic by ticlodipine and clopidogrel. No $P2Y_1$ selective antagonists are currently used in the clinical setting. It is notable that while ADP is itself a relatively weak platelet activator, the amplification of platelet aggregation caused by release of ADP from dense granules is an important part of platelet aggregation; blockade of this of this activation-

amplification-effect by $P2Y_{12}$ antagonists secondarily tamps down the platelet activation effects of other platelet agonists such as thromboxane A_2, collagen, and thrombin (141, 142).

The final, common pathway for platelet activation involves integrin-driven signaling via the interaction of either fibrinogen or von Willebrand factor with integrin alpha-2b-beta-3 on the platelet surface (143-145). The success of aspirin and $P2Y_{12}$ inhibitors at improving cardiovascular mortality led to exploration of inhibitors of this pathway in the hope of achieving an improved means of clinical inhibition of platelet aggregation. Clinically available agents have been developed but remain outside the scope of common use as they did not offer significant clinical benefits over existing agents. In breast cancer, the GP2b-3a inhibitor eptifibatide was found to suppress metastasis (146).

Multiple alternative and experimental agents exist in the cardiovascular literatures that are outside the scope of the current discussion. However, within the context of integrin signaling, the cardiology literature has recently suggested the importance of focal adhesion kinase as a component of platelet function. In particular, where conventional wisdom had held that platelets tend to remain static, recent evidence suggests that platelets will migrate in response to shear stress, that migration is associated with redistribution of focal adhesion kinase, and that platelets from a mouse that is megakaryocyte-null for focal adhesion kinase have limited capacity to migrate in response to fibrinogen (147, 148). The use of a novel focal adhesion kinase-inhibitor on platelets decreased reactivity in a manner partially reversed by the addition of ADP and also caused defective

transition from filodipodia to lamellopodia (149). It is well-known that focal adhesion kinase over-expression is associated with adverse outcomes in solid malignancy, however the implications of findings in platelets have not been evaluated in the context of malignancy (150).

Hypotheses and Specific Aims

Given the extensive amount of work done to elucidate the normal platelet function and the participation of platelets in the regulation and management of normal wound healing and angiogenesis, and given the large volume of work currently being done on the subject of the therapeutic management of angiogenesis as it relates to the treatment of cancer, it remains that there is insubstantial literature on the relationship of platelets to tumor angiogenesis and aggressive biological behavior. Further, given the long-held relationship between platelets and tumor cell metastasis, very little work has been done to explain the relationship between platelets and metastasis.

Therefore, the unified hypothesis for this work is that platelets are a key component of the tumor microenvironment whose normal functions as angiogenic regulators are co-opted to promote the well-being, selection, promotion, and metastasis of cancer, thereby contributing to the phenomenon of resistance to anti-angiogenic therapy. This central hypothesis can be divided into four sub-hypotheses, with specific aims embedded, as follows:

- Hypothesis 1. Platelet levels can be used as a biomarker of prognosis and recurrence in ovarian cancer.
    - Specific Aim 1. Determine the clinical implications of elevated platelet counts at the time of cancer diagnosis, during induction therapy, in the post-therapy monitoring interval, and at the time of recurrence.

- Hypothesis 2. Platelets promote apoptosis resistance, proliferation, and migration of tumor cells *in vitro,* and platelets promote chemotherapeutic resistance *in vivo*.
    - Specific Aim 2a. Determine the biological effects *in vitro* of platelets on cancer cells, including effects on proliferation, migration, and apoptosis.
    - Specific Aim 2b. Determine the biological effects of platelet depletion and platelet transfusion *in vivo*.
- Hypothesis 3. Platelet mitogenicity is driven by platelet activation and subsequent signaling at least in part by the platelet-derived growth factor and transforming growth factor beta pathways.
    - Specific Aim 3a. Determine the relevant signaling pathways responsible for the observed biology *in vitro*.
    - Specific Aim 3b. Determine whether platelet activation is necessary for the observed biology in vitro.
    - Specific Aim 3c. Determine whether any component of apoptosis resistance may be reasonably explained by platelet uptake of taxanes.
    - Specific Aim 3d. Determine whether blockade of platelet activation *in vivo* abrogates the observed effects of platelet transfusion on tumor growth.

- Hypothesis 4. Platelet promotion of tumor cell apoptosis resistance, proliferation, and migration *in vivo* represents a mechanism of resistance to anti-angiogenic therapy.
    - Specific Aim 4a. Model resistance to anti-angiogenic therapy in an orthotopic model of ovarian cancer.
    - Specific Aim 4b. Determine whether the use of agents known to block platelet migration and/or activation can be used to abrogate the biological effects of platelet interaction with cancer.

Methods

Approvals

Approval for relevant studies was obtained from the University of Texas at M.D. Anderson Cancer Center Institutional Review Board (IRB). All animal experiments were approved and supervised by the MDACC Institutional Animal Care and Use Committee. Approval for clinical studies at collaborating institutions (University of Iowa, University of Maryland, and the University of Virginia) was obtained from the relevant review boards at those institutions.

Statistical Considerations

For mouse-driven experiments, sample size was estimated utilizing a two-way ANOVA model. For an effect size of 0.65, a sample size of 10 mice per group was considered sufficient to provide 80% power for a test utilizing a significance level of 0.05. A 2-sided long-rank statistic was used to compare Kaplan-Meier survival curves that were created within the computer program SPSS (IBM®, Armonk, New York). Variables estimated to have a normal distribution were compared using the Student's T-Test using Excel (Microsoft®, Redmond, Washington). The F-Test was used to compare variances where indicated. A *p*-value of <0.05 was considered statistically significant.

Clinical Analysis

After IRB approval was obtained, patients were identified at the University of Texas at M.D. Anderson Cancer Center (MDACC), the University of Iowa, the

University of Maryland, and the University of Virginia who were diagnosed with ovarian, primary peritoneal, or fallopian tube carcinoma. Patients were excluded if they did not receive primary therapy at the institution of record or did not have routine follow-up at that institution. Patients were further excluded for a history of other malignancy, myeloproliferative disease, inflammatory disease, splenectomy, or other confounding cause of thrombocytosis prior to their diagnosis of cancer. Patients were excluded if they did not have recurrence of disease. All patients were treated by surgical cytoreduction performed by a gynecologic oncologist in addition to adjuvant or neoadjuvant taxane- and/or platinum-based chemotherapy. Surgical staging was performed according to International Federation of Gynecology and Obstetrics guidelines. All treatments were administered at the institution of record. Specimens were reviewed by a gynecological pathologist at the institution of record. Clinical data collected included patient demographics, tumor characteristics, details of treatment, as well as outcomes data such as progression-free interval and overall survival. Optimal cytoreduction was defined by convention as residual disease less than 1 cm as reported by the surgeon of record. Platelet levels and CA-125 measurements were recorded at the time of primary evaluation, through therapy, after the completion of surgery and 6 cycles of cytotoxic chemotherapy, during the post-therapy monitoring period, and at the time of diagnosis of ovarian cancer recurrence. Thrombocytosis was defined as a platelet count greater than 450,000/mL (151). Patients who were known to be alive and/or progression-free at the time of last contact were censored accordingly.

Cell lines and culture conditions

The derivation of the human ovarian cancer cell lines 2774, A2780, HeyA8, SKOV3-IP1, and 2774 are previously reported (152-156). The cell line 2774 was maintained in MEM media supplemented with 5% fetal bovine serum (FBS), 1x L-glutamine, 1x sodium pyruvate (100mM stock, Invitrogen, Carlsbad, CA), and 1x non-essential amino acid. The cell lines A2780, HeyA8, and SKOV3-IP1 were maintained in RPMI-1640 medium with 15% FBS. HeyA8-MDR and SKOV3-TR are taxane-resistant derivations of HeyA8 and SKOV3ip1 (Dr. Isaiah J. Fidler, MDACC, Houston, TX). The taxane-resistant phenotype was maintained by adding 300 ng/mL (HeyA8-MDR) and 100 ng/mL (SKOV3-TR) of paclitaxel to the media used for HeyA8 and SKOV3-ip1 (above). The MDA-MB-231 metastatic human breast cancer cell line was established from pleural effusions (Dr. Janet Price, MDACC, Houston, TX) (157). MDA-MB-231 cells were maintained in RPMI-1640 media with 5% FBS. The cell line OVCAR5 was developed from human serous ovarian carcinoma (158). OVCAR5 cells were maintained in DMEM media with 10% FBS. The cell line RMUG-S was established from human mucinous ovarian carcinoma (159). RMUG-S cells were maintained in RPMI-1640 media with 10% FBS. The syngeneic mouse ovarian cancer cell line ID8 was developed from the surface epithelium of C57B6 mice (160). ID8 cells were maintained in DMEM media with 5% FBS and 1x Insulin-Transferrin-Sodium Selenite. 50-70% cell confluence was considered appropriate for *in vitro* experiments. Cell lines were routinely genotyped to confirm identity. Cell lines

were also routinely tested to confirm absence of Mycoplasma. Cells were maintained at 37°C in a humidified incubator infused with 20% O_2 and 5% CO_2, referred to as the normoxic condition. For hypoxia-based experiments, cells were placed in a hypoxia chamber (Bellow Glass, Inc., Vineland, NJ) infused with 0% O_2, 5%CO_2, and 95% N_2 also at 37^{o}C.

Orthotopic model of ovarian cancer in nude mice

Female athymic nude (NCr-*nu*) were purchased from Taconic Farms, Inc. (Rockville, MD). The development and characterization of the orthotopic mouse model of ovarian cancer has been previously described (161-163). Briefly, SKOV3-IP1 (1 x 10^6), A2780 (1 x 10^6), HeyA8 (0.25 x 10^6), or 2774 (2 x 10^6) human ovarian cancer cells lifted with trypsin/edta, washed with PBS, and resuspended in 200 mL of Hank's balanced salt solution (HBSS, Mediatech, Inc. Manassas, VA) and were injected into the peritoneal cavity of female nude mice. All cell lines have been shown to reliably form macroscopic tumor implants on peritoneal surfaces throughout the pelvis and abdominal cavity.

Platelet isolation for *in vitro* assays

Platelets were prepared for *in vitro* assays in a manner that would remove plasma contents and nucleated cells. Whole blood was drawn from the inferior vena cava of anesthetized nude mice into a syringe pre-loaded with 1:9 v/v 3.8% sodium citrate and then mixed 1:1 v/v with tyrodes buffer (140 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 6.45 mM NaH_2PO_4, 5.5 mM glucose in diH_20) lacking Mg^{2+}

and Ca^{2+}. Blood was centrifuged at 1100 rpm for 3 minutes, twice, at room temperature in order to separate platelet-rich plasma from red blood cells and leukocytes by room temperature centrifugation of blood at 1100 rpm for 3 minutes, performed twice. In order to separate larger cells and proteins, the platelet-rich plasma was then passed through a filtration column using Sepharose 2B beads (Sigma Aldrich, St Louis, MO) loaded into a siliconized glass column also containing a 10 mcm nylon net filter (Millipore, Billerica, MA). Prior to being placed in the column, Sepharose 2B beads were washed in acetone 1:1 v/v, followed by 0.9% NaCl 1:1 v/v, and "Buffer 1" 1:1 v/v (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose, 0.3g/100 mL bovine serum antigen, pH 7.4). After the beads were loaded onto the column with the filter, the were again washed with "Buffer 1." Platelet-rich plasma was loaded onto the column and allowed to enter the beads fully before additional buffer was added. The initial, clear eluent was discarded. Cloudy eluent was collected. Eluent was diluted 1:200 and platelets were counted with a hemocytometer by phase-contrast microscopy at 400x magnification.

In vitro apoptosis

To assess the effect of platelets on apoptosis, cells were plated in 6-well plates at 50,000 cells per plate, targeting approximately 50% confluence in serum-containing medium. After adequate starting confluence was confirmed, media was changed to serum-free for approximately 24-hours prior to the start of

treatment. After serum-starvation, platelets were isolated (as described above), and platelets were added to achieve a final dose of 1 x 10^8 platelets/mL. Docetaxel was used at previously published IC_{50} dose to induce apoptosis. Controls were established using an equivalent volume of the appropriate buffer. All treatments were performed in triplicate. In the case of transfection as treatment, double transfection was performed as described below. After 72 hours of platelet and docetaxel exposure, cell viability was assessed using Annexin V and 7-amino-actinomycin-D (7AAD) staining (BD Pharmingen™, Franklin Lakes, NJ) by flow cytometry. Briefly, cells were harvested, washed in PBS, and incubated with PE-Annexin V and 7AAD according to package insert instructions for 20 minutes prior to flow cytometric analysis. Indirect mediation of effect was considered by the use of an intervening cell culture insert with 0.4 mcm pores (BD Falcon™, Franklin Lakes, NJ) in which platelets and/or docetaxel and/or control buffer was placed during co-incubation experiments.

In vitro proliferation

To assess the effect of platelets on proliferation, cells were plated in 6-well plates at 50,000 cells per plate, targeting approximately 50% confluence in serum-containing medium. After adequate starting confluence was confirmed, media was changed to serum-free for approximately 24-hours prior to the start of treatment. After serum-starvation, platelets were isolated (as described above), and platelets were added to achieve a final dose of 1 x 10^8 platelets/mL. An equivalent volume of buffer was used for control, and all treatment groups were

performed in triplicate. In the case of transfection as a treatment, reverse transfection was performed as described below and other treatment (e.g. platelet co-culture) was started 48 hours after transfection. After 24 hours of treatment, the percentages of cells proliferating (defined as S-phase) were determined using the Click-iT EdU flow cytometry kit (Invitrogen, Carlsbad, CA). Cells were incubated with 10 mcM 5-ethynyl-2-deoxyuridine (EdU) for 2 hours, lifted, and washed with 1% bovine serum antigen in d-PBS. Cells were fixed with 4% paraformaldehyde in d-PBS for 15 minutes at room temperature and then maintained at 4^{o}C protected from light until the time of flow cytometric analysis, a duration not to exceed 7 days. On the day of analysis, cells were washed in 1% BSA in d-PBS and then permeabilized with 1x saponin-based reagent for 15 minutes at room temperature. The cells were then incubated with a solution of 1x reaction buffer, CuSO4, Alexa-Fluor 488 azide dye, and proprietary reaction buffer additive for 30 minutes at room temperature prior to flow cytometric analysis.

In vitro migration

To assess the effect of platelets on cancer cell migration, a modified Boyden chamber system was used. The wells separated by a 0.1% gelatin-coated, cell-permeable membrane. Platelets were isolated as described above and added to serum free media to a final concentration of 1 x 10^8 cell/mL, and this platelet solution was added to the bottom wells. An equivalent volume of buffer was used as the control. Cancer cells were added to the top wells at

approximately 100,000 cells/well. Incubation was at 37^{o}C for 6 hours. After incubation, cells in the bottom chambers were removed using 0.1% EDTA and were loaded onto a 3 mcm polycarbonate filter (Osmonics, Livermore, CA) with the S&S Minifold I Dot-Blot System (Schleicher & Schuell, Keene, NH). Cells were stained and scored by counting cells from five representative 100x fields.

ADP quantification

To assess the relative production of ADP in hypoxic compared to normoxic environments, cancer cells were cultured in serum free medium in hypoxic compared to normoxic environments, and the ADP content of conditioned medium was assessed at various time-points using the ABCAM (Cambridge, England) ADP colorimetric assay kit. The assay was performed according to the manufacturer protocol.

Forward transfection

As a means of investigating the cause of the biological endpoints of proliferation and apoptosis (described above) transfection of small-interfering RNA (siRNA) was performed. In the case of forward transfection, 6-well plates were inoculated with 50,000 cancer cells per well with a goal of transfection at 50% confluence within 12-24 hours of plating. Based on validation experiments, the appropriate amount of the siRNA of choice was incubated for 20 minutes at room temperature in serum-free culture medium (1 mL/well) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a 3.75:1 ratio of volume Lipofectamine 2000

to microgram siRNA. After incubation, cell culture media was removed from the 6-well plates and replaced with the siRNA/Lipofectamine-containing medium at 1 mL per well. Cells and transfection media were incubated at 37^{o}C for 4 hours, and then transfection medium was changed to serum-containing medium. Treatment experiments were started 48 hours after transfection. If forward transfection was performed as the second step of a "double" transfection (described below) then treatment experiments were started immediately in serum-free media.

Reverse transfection

As a means of investigating the cause of the biological endpoints of proliferation and apoptosis (described above) transfection of siRNA were performed. In the case of reverse transfection, and based on previously performed validation experiments, the appropriate amount of the siRNA of choice was incubated for 20 minutes at room temperature in serum-free culture medium (200 mcL/well) with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) at a 3:1 ratio of volume of Lipofectamine RNAiMAX to mcL siRNA. After incubation, 200 mcL of the solution was placed in each well, and then 50,000 cancer cells in 1 mL of serum-free media were added to each well. Cells were incubated with the transfection medium for 4-6 hours at 37^{o}C, and then medium was changed to serum-containing medium. Treatment experiments were started 48 hours after transfection.

Double transfection

If a longer duration of mRNA knockdown was needed than was offered by single forward or reverse transfection, then sequential double transfection was used. In this case, reverse transfection was initially performed, and 48 hours later, a forward transfection was performed, and treatment experiments were initiated within 1-2 hours of the completion of the forward transfection.

RNA Extraction

Total RNA was extracted from cells growing *in vitro* using RNeasy Kit (Quiagen, Venlo, Netherlands). Cells were lifted with trypsin/edta, washed with PBS, and centrifuged at 10,000 rpm for 5 minutes to form the pellet. The supernatant was discarded, and RNA was extracted according to the company protocol. RNA was quantified using a spectrophotometer and 1 mcg was transcribed into complementary DNA (cDNA) using the Verso cDNA kit (Thermo Fisher Scientific), closely following the manufacturer's protocol. Quantitative RT PCR was then used to assess levels of expression.

CD31 immunohistochemistry

In order to assess the vasculature in tumor tissue harvested from orthotopic models of ovarian cancer, immunohistochemistry was performed for CD31. The primary antibody was a rat anti-mouse antibody to CD31 manufactured by BD Pharmingen (Franklin Lakes, NJ), catalogue number 53370. The secondary antibody was goat anti-rat HRP manufactured by Jackson

ImmunoResearch (West Grove, PA), catalogue number 112-035-167. Frozen tumor sections were fixed in acetone, followed by acetone/chloroform, followed by acetone. Endogenous peroxides were blocked with 3% hydrogen peroxide in methanol, and non-specific proteins were blocked with 5% normal horse serum and 1% normal goat serum in PBS. Slides were incubated over-night in primary antibody at 1:800 dilutions in the same protein block. After washing with PBS and protein blocking, the sections were incubated in the secondary antibody at 1:200 in protein block. Slides were developed with DAB chromogen (Invitrogen, Carlsbad, CA) and counterstained with Gil No.3 hematoxylin (Sigma-Aldrich, St. Louis, MO).

Cleaved caspase 3 immunohistochemistry

In order to assess apoptosis rates in tumor tissue harvested from orthotopic models of ovarian cancer, immunohistochemistry was performed for cleaved caspase 3. The primary antibody was a rabbit polyclonal anti-human antibody to cleaved caspase 3 manufactured by BioCare Medical (Concord, CA), catalogue number CP229B. The secondary antibody was goat anti-rabbit biotinylated from BioCare Medical, catalogue number 111-036-047, and streptavidin HRP 4+ from BioCare Medical, catalogue number HP604H. Paraffin embedded tumor sections were heated to 60^{o}C for 45 minutes and were deparaffinized sequentially in xylenes and declining grades of ethanol prior to rehydration. Antigen retrieval was performed by steaming slides at 100ºC for 30 minutes in BioCare Medical borg-decloaker, Ready-to-Use. Endogenous

peroxides were blocked with 3% hydrogen peroxide in methanol, and non-specific proteins were blocked with 4% fish gelatin in PBS. Slides were incubated over-night in primary antibody (1:100) in the same protein block. After washing with PBS and protein blocking, the sections were incubated in the secondary antibody (ready-to-use) followed by streptavidin HRP (ready-to-use). Slides were developed with DAB chromogen (Invitrogen, Carlsbad, CA) and counterstained with Gil No.3 hematoxylin (Sigma-Aldrich, St. Louis, MO).

Ki67 immunohistochemistry

In order to assess the proliferation rates in tumor tissue harvested from orthotopic models of ovarian cancer, immunohistochemistry was performed for Ki67. The primary antibody was a rabbit polyclonal anti-human antibody to Ki67 manufactured by ThermoFisher Scientific (Houston, TX), catalogue number RB-90-43-P. The secondary antibody was goat anti-rabbit HRP (Jackson ImmunoResearch). Paraffin embedded tumor sections were heated to 60^{o}C for 45 minutes and were deparaffinized sequentially in xylenes and declining grades of ethanol prior to rehydration. Antigen retrieval was performed by steaming slides at 100ºC for 30 minutes in BioCare Medical Civa decloaking solution, diluted to 1x in water. Endogenous peroxides were blocked with 3% hydrogen peroxide in methanol, and non-specific proteins were blocked with 5% normal horse serum and 1% normal goat serum in PBS. Slides were incubated over-night in primary antibody (1:200) in the same protein block. After washing with PBS and protein blocking, the sections were incubated in the secondary antibody

(1:500 in protein block). Slides were developed with DAB chromogen (Invitrogen, Carlsbad, CA) and counterstained with Gil No.3 hematoxylin (Sigma-Aldrich, St. Louis, MO).

Platelet and CD31 co-immunofluorescence

Using the orthotopic model of ovarian cancer described above, mice were inoculated with tumor. At the time of sacrifice, the mouse was anesthetized with isofluorane, and intravital fixation was performed with 4% paraformaldehyde (described below) and tumor was frozen for future staining. The primary antibody was rat anti-mouse IgG to the GP1b-beta subunit of the GP1b-V-IX complex primarily labeled with DyLight488 (Emfret Analytics, Eibelstadt, Germany) at 1:100 dilutions. The second primary antibody was CD31 rat anti-mouse (BD Pharmingen, catalogue number 553370) and its secondary was goat anti-rat Alexa 594 (1:200) were applied. Frozen sections were fixed in cold acetone for 10 minutes. After protein block with 5% normal horse serum and 1% normal goat serum, the primary anti-GP1b-beta antibody was applied at 1:100 dilution over-night at 4^{o}C. After PBS wash, protein block was 5% normal rat serum (Jackson ImmunoResearch, catalogue number 012-000-120) for 1 hour followed by F(ab')$_2$ fragment Goat anti Rat IgG, F(ab')$_2$ fragment specific (Jackson ImmunoResearch, catalogue 112-007-003) diluted 1:50 in protein block, incubate for 2 hours. The slides were then incubated at 1:800 dilutions in protein block overnight at 4^{o}C. After washing and protein blocking, the secondary was added at 1:1000 dilutions and incubated at room temperature for 1 hour. Hoescht 33342

(Invitrogen) counterstain was applied at 1:10000 dilution in PBS for 10 minutes, and slides were mounted using propyl-gallate (Fisher Scientific, Hampton, NH) and glass cover slips. Slides were stored at 4^{o}C in the dark.

Intravital fixation

At the time of animal sacrifice, and after appropriate treatments, the relevant mice were anesthetized with isofluorane. An incision was made in the chest cavity allowing visualization of the left heart, and a 21-guage needle was used to deliver 4% paraformaldehyde in PBS through heart and into the ascending aorta. Slow, manual infusion was continued for approximately 5 minutes, infusing approximately 25 mL, and until all movement of the mouse had stopped. The absence of heartbeat or breathing was confirmed. Relevant dissection was then undertaken.

Platelet-depleting antibody

To deplete platelets in mice for *in vivo* experiments, use used a commercially available rat anti-mouse monoclonal antibody directed against mouse GP1b-alpha (CD42b, Emfret Analytics, Eibelstadt, Germany) that causes irreversible Fc-independent platelet depletion within 60 minutes of administration without inducing platelet activation. This antibody has been previously used and validated in our lab, including dose-kinetics.

TGF-beta receptor 1 siRNA validation and knockdown

Forward transfection was performed (as described above) using 5 mcg of siRNA per well, and quantitative RT-PCR was performed using the primer listed in Figure 5 compared to 18S control. Western blot was performed (as described above) to confirm effect. Double transfection was performed and validated using western blot. The validated sequence is listed in Figure 6 along with the validated control.

PDGFR-alpha siRNA validation and knockdown

Reverse transfection was performed (as described above) using 20 nM siRNA per well, and quantitative RT-PCR was performed using the primer listed in Table 4 compared to 18S control. Western blot was performed (as described above) to confirm effect. Double transfection was performed and validated using western blot. The validated sequences are listed in Table 5.

Sequence Name	Sequence
PDGFR-alpha	(F) 5'-TGAAAGCACACGGAGCTATG (R) 5'-TCTAGCATGGGGACATACTGTG
TGF-beta R1	(F) 5'-GATGCTGCTTCTCCAAAGTG (R) 5'-ACAAGTCAGGATTGCTGGTG
18S (Loading Control)	(F) 5'-CGCCGCTAGAGGTGAAATC (R) 5'-TTGGCAAATGCTTTCGCTC

Table 4: Primers used in the current work.

Sequence Name	Sequence
PDGFR-alpha (Sigma Aldrich)	5’-GAAGACAGTGGCCATTATA-3’
TGF-beta R1 (Sigma Aldrich)	5’-GAAGACAGUGGCCAUUAUAdTdT-3’
Control (Sigma Aldrich)	5'-AAUUCUCCGAACGUGUCACGU-3'

Table 5: siRNA sequences used in the current work.

Aspirinization of platelets

Pharmacy grade aspirin was acquired, and a single 325 mg table was dissolved in 500 mcM sodium acetate (pH 5.6). This was added 1:10 v/v to platelet rich plasma and the combination was incubated at 37^{o}C for 15 minutes. Incubation with an equivalent sodium acetate solution without aspirin was performed as a control.

Small molecule inhibitor of Focal Adhesion Kinase

GSK2256098, a novel small molecule inhibitor of Focal Adhesion Kinase (FAK) phosphorylation at the tyrosine-397 site, was obtained from GlaxoSmithKline (London, United Kingdom) under material transfer agreement. Pharmacokinetics and pharmacodynamics have suggested activity in ovarian cancer and at the level of the platelet in nude mice at 75 mg/kg given orally by

gavage in the carrier 10% 1-methyl-2-pyrrolidinone in polyethylene glycol 300. *In vitro*, kinase inhibition is seen at doses of 0.1 - 1 mcM. (Unpublished data, personal communication from Dr. Angela Sanguino, MD Anderson Cancer Center.)

Docetaxel

Docetaxel (Sanofi-Aventis, Paris, France) was obtained from leftover clinical samples through a material transfer agreement with the clinical pharmacy associated with the University of Texas M.D. Anderson Cancer Center. Stock concentration was 20 mg/mL and was maintained at $4^{o}C$.

Platelet protection against induced apoptosis *in vitro*

In order to assess the effect of platelets on induced and spontaneous apoptosis, cancer cell lines were plated in 6-well plates as described above. After exposure to serum-free conditions for 24 hours, treatment was initiated. Plasma-free platelets were isolated (as described above) and counted. Cells were co-cultured, either by direct or indirect exposure, with platelets at 1 x 10^{8} million cells/mL, docetaxel dosed to previously documented IC_{50}, or both. Appropriate buffer in an equivalent volume was added for control. In order to investigate the contribution of TGF-beta receptor 1 and PDGF receptor alpha, double-transfection was performed, and treatment was initiated after the second (forward) transfection. Flow cytometric analysis as described above was performed 72 hours after initiation of treatment.

Platelet acceleration of proliferation *in vitro*

In order to assess the effect of platelets on cancer cell proliferation, cancer cell lines were plated in 6-well plates as described above. After exposure to serum-free conditions for 24 hours, treatment was initiated. Plasma-free platelets were isolated (as described above) and counted. Cells were co-cultured with platelets at 10 million cells/mL with an appropriate volume of buffer added to the control wells. In order to investigate the contribution of TGF-beta receptor 1 and PDGF receptor alpha, reverse transfection was performed, and treatment was initiated 48 hours after transfection. Flow cytometric analysis as described above was performed 24 hours after initiation of treatment.

Blockade of platelet effect by platelet fixation

In order to assess whether platelet activation was necessary for the observed effects, the apoptosis and proliferation assays described previously were repeated including platelets that had been fixed with paraformaldehyde. Specifically, after plasma-free platelets were isolated, half were incubated in 1% paraformaldehyde in dPBS at room temperature for 15 minutes. Untreated platelets were incubated in an equivalent volume of PBS. Platelets were then centrifuged at 1800 rpm for 10 minutes and re-suspended in Buffer 1. A parallel solution of Buffer 1, paraformaldehyde, and PBS was processed in parallel to use as a control for the residual paraformaldehyde left after paraformaldehyde-fixed platelets were washed and re-suspended. After 24 hours (proliferation) and 72

hours (apoptosis) flow cytometric analysis was performed as previously described.

Platelet acceleration of tumor cell migration *in vitro*

In order to assess the effect of platelets on cancer cell migration, cancer cells were added to a Boyden chamber system as described above. Plasma free platelets were isolated and counted and added to the serum free media at 1 x 10^8 cells/mL. An equivalent volume of buffer was used as the control. After 6 hours of incubation at 37^{o}C, cells were removed and scored as described above.

Biodistribution

In order to determine relative docetaxel drug concentrations in human plasma and human platelet samples, uptake of drug into platelets with respect to other blood fractions was considered using High-Performance Liquid Chromatography (HPLC) with UV-spectrum capability (HPLC-UV). Fresh human blood from a healthy, adult female volunteer was collected in sodium citrate vacuum tubes (BD Pharmaceuticals, Franklin Lakes, NJ) and divided into 50 mL aliquots to be used to assess “high” and “low” concentrations. For the “high” concentration, docetaxel was added to a final concentration of 5 mcg/mL. For the “low” concentration, docetaxel was added to a final concentration of 1 mcg/mL.

At multiple time-points, fractions were taken to examine concentration of docetaxel in the whole plasma (plasma + platelets) compared to platelets separated from platelet-free plasma (PFP). Specifically, the whole blood was

centrifuged at 1200 rpm for 4 minutes, and 1 mL of plasma was removed. The plasma was centrifuged at 7500 rpm for 5 minutes, and the supernatant was analyzed as the PFP portion. Pelleted platelets were washed with 1 mL dPBS and were centrifuged again at 7500 rpm for 5 minutes. This supernatant was discarded and the remaining pellet was suspended in 1 mL of mobile phase for immediate HPLC-UV analysis using the column Nova Pak C-18 4µm. 3.9x150mm with the Nova Pak C-18 4µm Guard Pak. Results were reported in ng/mL with a lower limit of quantification of 50 ng/mL.

Thrombocytosis, thrombocytopenia, and effect on chemotherapy *in vivo*

Based on *in vitro* data obtained, the decision was made to proceed to *in vivo* with the cell lines A2780 and SKOV3-IP1 using an orthotopic model of nude mice.

In the first experiment, animals were inoculated on Day 0 with A2780 cells (as described above). On Day 7, treatment was initiated. The first group was given twice weekly tail vein injections of Control IgG (0.5 mcg/gram). The second group was given twice weekly Control IgG via tail vein injection and weekly docetaxel 35 mcg IP. The third group was given the platelet-depleting antibody described above at a dose of 0.5 mcg/gram via tail vein injection twice weekly. The fourth group was given the platelet-depleting antibody plus docetaxel. Mice were treated until they became moribund and were sacrificed at that time. Data taken at necropsy included mouse weight, aggregate tumor weight, number of tumor nodules, and location of tumor nodules. Tumor was taken for frozen storage, frozen section, and paraffin block.

In the second experiment, animals were inoculated on Day 0 with SKOV3-IP1 (as described above). On Day 7, treatment was initiated. The first group was given Control IgG. The second group was given platelet-depleting antibody. The third group was given tail vein transfusion of platelet rich plasma isolated from nude mice as described above. The fourth group was given Control IgG and docetaxel. The fifth group was given platelet-depleting antibody and docetaxel. The sixth group was given platelet transfusion and docetaxel. Mice were treated until they became moribund and were sacrificed at that time. Data taken at necropsy included mouse weight, aggregate tumor weight, number of tumor nodules, and location of tumor nodules. Tumor was taken for frozen storage, frozen section, and paraffin block.

Effect of aspirin on tumor cell proliferation *in vitro*

Based on the hypothesis that platelet effects on tumor may be due to platelet activation, and based on the knowledge that aspirin is a weak inhibitor of platelets, we dissolved a 325 mg tablet of aspirin in deionized, distilled water, filter sterilized it. Cancer cells were plated in 6-well plates as described above and exposed to serum-free conditions for 24 hours. Plasma-free platelets were isolated as above and half of the platelets were co-incubated with 30 mcM aspirin for 15 minutes. Platelets were counted. A control group was given an appropriate volume of buffer. An additional control group was given an equivalent concentration of aspirin. A group was treated with non-aspirin-exposed platelets at 1 x 10^8 cells/mL, and the final group was treated with the same number of

aspirinized platelets. After 24 hours of treatment, flow cytometric analyses for proliferation (as described above) was performed.

Effect of aspirin on thrombocytosis and malignancy *in vivo*

Based on accumulated *in vitro* and *in vivo* data, the hypothesis was formed that aspirinization of platelets would at least partially block platelet activation and therefore would at least partially abrogate the effects of platelet transfusion on progression of malignancy in our orthotopic mouse model of ovarian cancer. In this experiment, animals were inoculated on Day 0 with A2780 cells (as described above). On Day 7, treatment was initiated. The first group served as an untreated control. The second group was given intraperitoneal aspirin 20 mg/kg twice per week. The third group was given 500 mcL of platelet rich plasma isolated from nude mice and incubated with sodium acetate for 15 minutes (as described above) via tail vein injection weekly. The fourth group was given weekly tail vein transfusion of platelet rich plasma that had been incubated for 15 minutes with a 50 mcM solution of aspirin in sodium acetate (as described above). Mice were treated until they became moribund and were sacrificed at that time. Data taken at necropsy included mouse weight, aggregate tumor weight, number of tumor nodules, and location of tumor nodules. Tumor was taken for frozen storage, frozen section, and paraffin block.

Effect of the FAK inhibitor on proliferation *in vitro*

Based on the hypothesis that platelet effects on tumor may be due to platelet activation, and based on the knowledge that FAK participates in integrin signaling necessary for platelet activation, and based on reports that the absence of FAK leads to impaired migration and activity in platelets, we utilized the inhibitor described above to try to block any pro-proliferation effects of platelets. Cancer cells were plated in 6-well plates as described above and exposed to serum-free conditions for 24 hours. Plasma-free platelets were isolated as above and half of the platelets were co-incubated for 15 minutes with an adequate concentration of the inhibitor to yield 1 mcM inhibitor in the final incubation. Platelets were counted. A control group was given an appropriate volume of buffer. An additional control group was given an equivalent concentration of the inhibitor. A group was treated with non-inhibitor-exposed platelets at 1 x 10^8 cells/mL, and the final group was treated with the same number of inhibitor-exposed platelets. After 24 hours of treatment, flow cytometric analyses for proliferation (as described above) was performed.

Platelet infiltration of solid tumor

In order to elucidate whether platelets interact with tumor cells in a biological environment, we utilized our orthotopic model of ovarian cancer in nude mice. Mice were inoculated with 2774 (as described above). When tumor was palpable, the mice were sacrificed via intra-vital fixation. Tumor was harvested. Immunofluorescence was performed for the anti-GP42b antibody and

for anti-mouse-CD31 to assess for platelet infiltration into the tumor and for proximity of platelet infiltration to tumor blood vessels.

For comparison, non-tumor bearing mice were injected with 0.5 mL of 3% thioglycollate (PD Pharmingen, Franklin Lakes, NJ) by intraperitoneal injection or PBS by intraperitoneal injection. After 72 hours, these animals were sacrificed by intravital fixation, and peritoneal tissue was harvested and evaluated in the same fashion using the anti-GP42b antibody and anti-mouse-CD31 antibody.

Rebound tumor growth after short-term anti-angiogenic therapy

Based on pre-clinical and clinical evidence of tumor rebound after the withdrawal of anti-angiogenic therapy, we sought to develop a pre-clinical model of the phenomenon in our orthotopic model of ovarian cancer in nude mice in order to investigate the phenomenon. Animals were inoculated on Day 0 with SKOV3-IP1 cells (as described above). In the first group, animals were treated with pazopanib 100mg/kg daily for the week prior to inoculation with ovarian cancer. In the second group, mice were treated with pazopanib starting 7 days after inoculation, and treatment was stopped after 1 week. In the third group, mice were treated with pazopanib starting on Day 7 and were treated until the conclusion of the experiment. In the fourth group, mice were treated with the vehicle carrier for the pazopanib for the seven days prior to cell line inoculation. In the fifth group, mice were treated with the vehicle carrier for pazopanib starting on day 7 and continuing for the duration of the experiment. The experiment was ended when the mice became moribund, and they were sacrificed at that time.

Data taken at necropsy included mouse weight, aggregate tumor weight, number of tumor nodules, and location of tumor nodules. Tumor was taken for frozen storage, frozen section, and paraffin block.

Blockade of rebound tumor growth after short term anti-angiogenic therapy using FAK-inhibitor

Based on available *in vitro* data, it was hypothesized that bevacizumab would have similar effects to pazopanib when given for a brief interval of time. It was further hypothesized that the effects in question could be blocked by treating mice with an inhibitor of focal adhesion kinase, given its known effects on platelet activation and migration in response to fibronectin. Therefore, a series of *in vivo* experiments were undertaken utilizing our orthotopic model of ovarian cancer.

In the first experiment, nude mice were inoculated with SKOV3-1P1 on Day 0, and treatment was initiated on Day 7. The first group was an untreated control. The second group was treated daily with pazopanib 100 mg/kg by oral gavage from Day 7 until the end of the experiment. The third group was treated with pazopanib 100 mg/kg by oral gavage from Days 7 – 14 of the experiment. The fourth group was treated with pazopanib 100 mg/kg by oral gavage from Days 7 – 14 of the experiment, followed by the FAK-inhibitor GSK2256098 75 mg/kg daily by oral gavage until the end of the experiment. Groups 5 – 7 recapitulated Groups 2 – 4, except that bevacizumab 6.25 mg/kg IP twice weekly was substituted for pazopanib. The experiment was ended when the mice became moribund, and they were sacrificed at that time. Data taken at necropsy

included mouse weight, aggregate tumor weight, number of tumor nodules, and location of tumor nodules. Tumor was taken for frozen storage, frozen section, and paraffin block.

In the second experiment, nude mice were inoculated on Day 0 with HeyA8. The remainder of the experiment recapitulated the first in terms of drug dose and schedule. The experiment was ended when the mice became moribund, and they were sacrificed at that time. Data taken at necropsy included mouse weight, aggregate tumor weight, number of tumor nodules, and location of tumor nodules. Tumor was taken for frozen storage, frozen section, and paraffin block.

A third experiment was undertaken to address the interval of time between the short-term treatment with pazopanib and bevacizumab in groups 3 and 6 and the end of the experiment and to capture data regarding events in that interval. Nude mice were inoculated with SKOV3-IP1 on Day 0 and treatment was initiated group-by-group when tumor was palpable. The first group served as an untreated control. The second was given pazopanib 100 mg/kg daily by oral gavage for 7 days. The third was given bevacizumab 6.25 mg/kg IP every 4 days for two doses. The fourth was given the FAK-inhibitor GSK2256098 75 mg/kg by oral gavage daily for 7 days. The fifth was given aspirin 20 mg/kg every four doses for two doses. Mouse CBC was drawn immediately prior to treatment and after 1 week of treatment via retro-orbital collection. After 1 week of treatment, blood was also drawn for cytokine measurement, Alexa488-conjugated anti-

platelet antibody was given, and the mouse was sacrificed by intra-vital fixation. Tumor was taken for frozen storage, frozen section, and paraffin block.

Results

Platelets as a biomarker of disease recurrence

Patients were identified at the four participating institutions that received their diagnosis of epithelial ovarian cancer, their initial surgery and chemotherapy, and their post-therapy monitoring within that system. Patients were excluded if no recurrence was identified. In this population, enriched for recurrence, 341 patients were identified who met criteria for inclusion in this analysis. Demographic information is included in Table 4. Briefly, the majority of patients had high-stage, high-grade serious epithelial ovarian cancer. 32% met formal criteria for thrombocytosis at diagnosis (i.e. >450,000/mcL). Overall, the mean platelet level was 409,000/mcL (range 134,000-1,122,000/mcL). The mean platelet level for the cohort with thrombocytosis was 554,000/mcL and the mean for the cohort without thrombocytosis was 330,000/mcL.

Patient Clinical Characteristics	
Average	
- **Median**	**61 years**
- **Range**	**31-88 years**
- **Standard Deviation**	**11 years**
Stage	
- **Low (I, II)**	**29 (8.5%)**
- **High (III, IV)**	**311 (91.2%)**
- **Unknown**	**1 (0.29%)**
Grade	
- **Low**	**38 (11.1%)**
- **High**	**303 (88.9%)**
Histology	
- **Serous**	**269 (78.8%)**
- **Other**	**72 (21.1%)**

Table 6: Patient clinical characteristics.

Patients were typical of an ovarian cancer population with a median age 61 years. Disease was most commonly high-grade, high-stage serous cancer.

Survival analysis of this cohort (Figure 2) was performed to query whether elevated platelet levels at the time of diagnosis (greater than 450,000 cells per mcL) would be associated with adverse clinical outcomes. Our findings were consistent with previous finings that thrombocytosis at diagnosis was associated with significantly worse overall survival (median 480 days vs. 625 days, p = 0.007) and progression free interval (median 386 days vs. 440 days, p = 0.05).

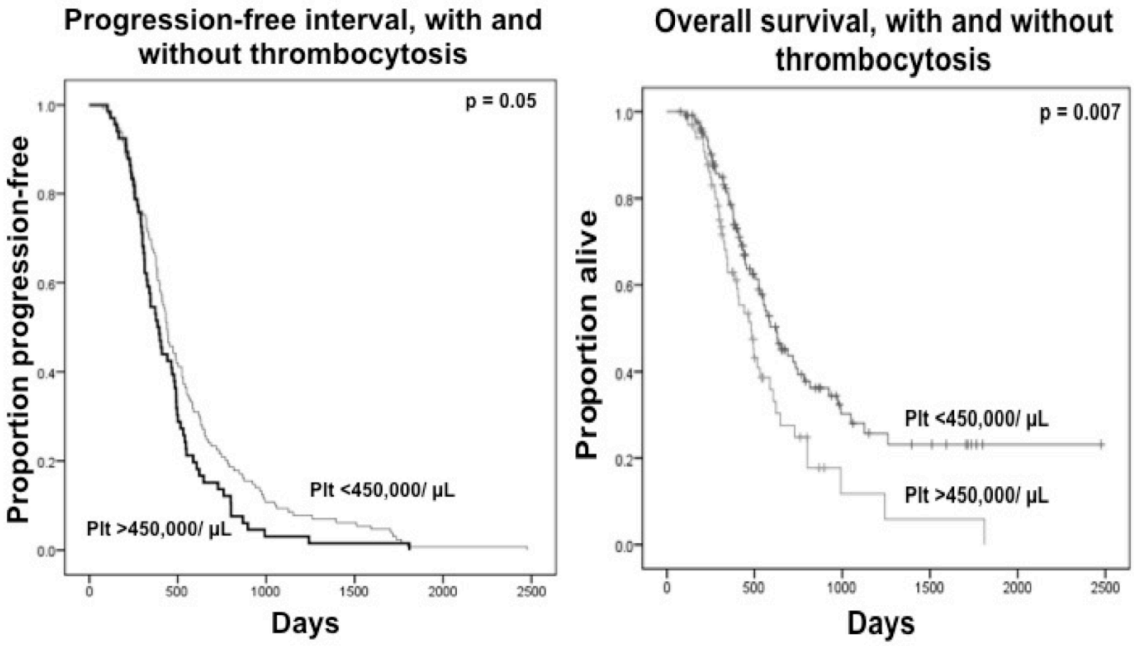
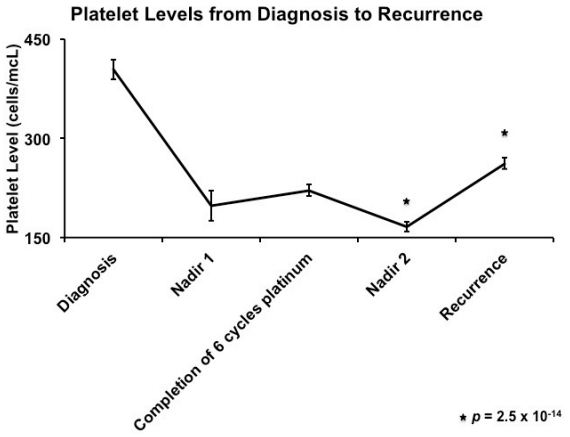


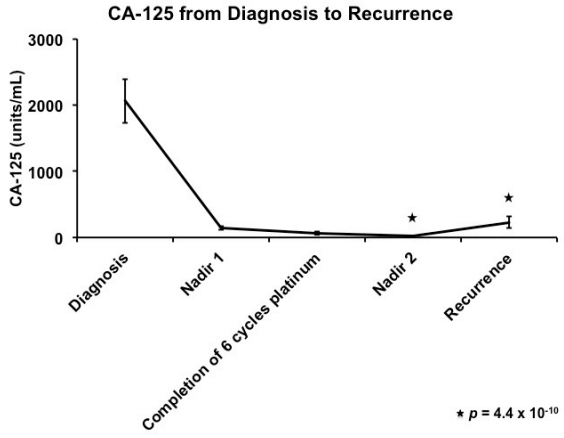
Figure 2: Elevated platelet levels adversely affect cancer prognosis.

Thrombocytosis at diagnosis (Platelets >450,000 cells/mcL) was associated with significantly worse overall survival (median 480 days vs. 625 days, $p = 0.007$) and progression free interval (median 386 days vs. 440 days, $p = 0.05$).

A total of 98 patients were identified who met criteria detailed in the methods section for full consideration of the question of whether platelet levels could be used as a biomarker of recurrence. (Figure 3) Following the completion of primary therapy, 86% of patients had a normal CA-125 level (< 35 U/mL) and 100% of patients had a platelet count < 450,000 cells/mcL with a mean of 206,000 cells/mcL. At the time of the clinical diagnosis of recurrence, CA-125 was elevated in only 75% of patients. The mean platelet count at recurrence was 262,000 cells/mcL, representing a 58% increase compared to the mean value at the conclusion of primary therapy ($p < 0.05$). Among patients with a normal CA-

125 at time of recurrence, platelets increased by an average of 108,400/μL at recurrence compared to the end of primary therapy (49% increase, $p < 0.05$).





	Platelet level (mean per microliter)	Absolute change from previous	Percent change from previous	Significance compared to previous
Diagnosis	404	NA	NA	NA
Nadir #1	198	-206	-51.0%	p < 0.001
Completion of 6 cycles platinum	221	23	11.6%	p = 0.36
Nadir #2	166	-55	-24.9%	p < 0.001
Recurrence	262	96	57.8	p < 0.001

Figure 3: Platelets as a biomarker of recurrence of disease.

Mean CA-125 and platelet levels are reflected at diagnosis, the lowest level during induction chemotherapy, at the completion of chemotherapy, the lowest level during post-therapy monitoring, and at the time of recurrence. Following the completion of primary therapy, 86% of patients had a normal CA-125 level (< 35 U/mL) and 100% of patients had a platelet count < 450,000 cells/mcL with a mean of 206,000 cells/mcL. At the time of the clinical diagnosis of recurrence, CA-125 was elevated in only 75% of patients. The mean platelet count at recurrence was 262,000 cells/mcL, representing a 58% increase compared to the mean value at the conclusion of primary therapy ($p < 0.05$). Among patients with a normal CA-125 at time of recurrence, platelets increased by an average of 108,400/μL at recurrence compared to the end of primary therapy (49% increase, $p < 0.05$).

Platelets mediate *in vitro* apoptosis protection in tumor cells

Direct contact co-culture experiments were performed in which various cancer cell lines were plated in 6-well plates, serum-starved for 24 hours, and then variously treated with buffer control, docetaxel dosed according to previously published IC_{50}, platelets dosed at 1 x 10^8 cells/mL, and platelets followed by docetaxel. After 72 hours of treatment, cells were processed for flow cytometry using Annexin and 7-aad staining to assess for total apoptosis. In the this first set of apoptosis experiments, platelets were included along with cancer cells in the analysis, and therefore it is understood that the additional

phosphatidylserine contribution of the platelets would increase the apoptosis score of groups containing platelets. (See Figure 4.)

In A2780, platelets decreased apoptosis by 46.7% ($p < 0.05$) compared to buffer control, and platelets decreased apoptosis by 20.4% ($p < 0.05$) in the context of docetaxel. In HeyA8, platelets decreased apoptosis by 66.0% ($p < 0.05$) compared to buffer control, and platelets decreased apoptosis by 53.6% ($p < 0.05$) in the context of docetaxel. In HeyA8-MDR, platelets decreased apoptosis by 70.0% ($p < 0.05$) compared to buffer control, and platelets decreased apoptosis by 66.8% ($p < 0.05$) in the context of docetaxel. In MDA.MB231, the most profound results were seen, as platelets decreased apoptosis by 90.3% ($p < 0.05$) compared to buffer control, and platelets decreased apoptosis by 62.1% ($p < 0.05$) in the context of docetaxel. In RUMG-S, a slightly different pattern emerged, as platelets did not change apoptosis (10% increase, $p = 0.33$) compared to buffer control, however platelets continued to decrease apoptosis in the context of docetaxel, decreasing it by 33.9% ($p < 0.05$).

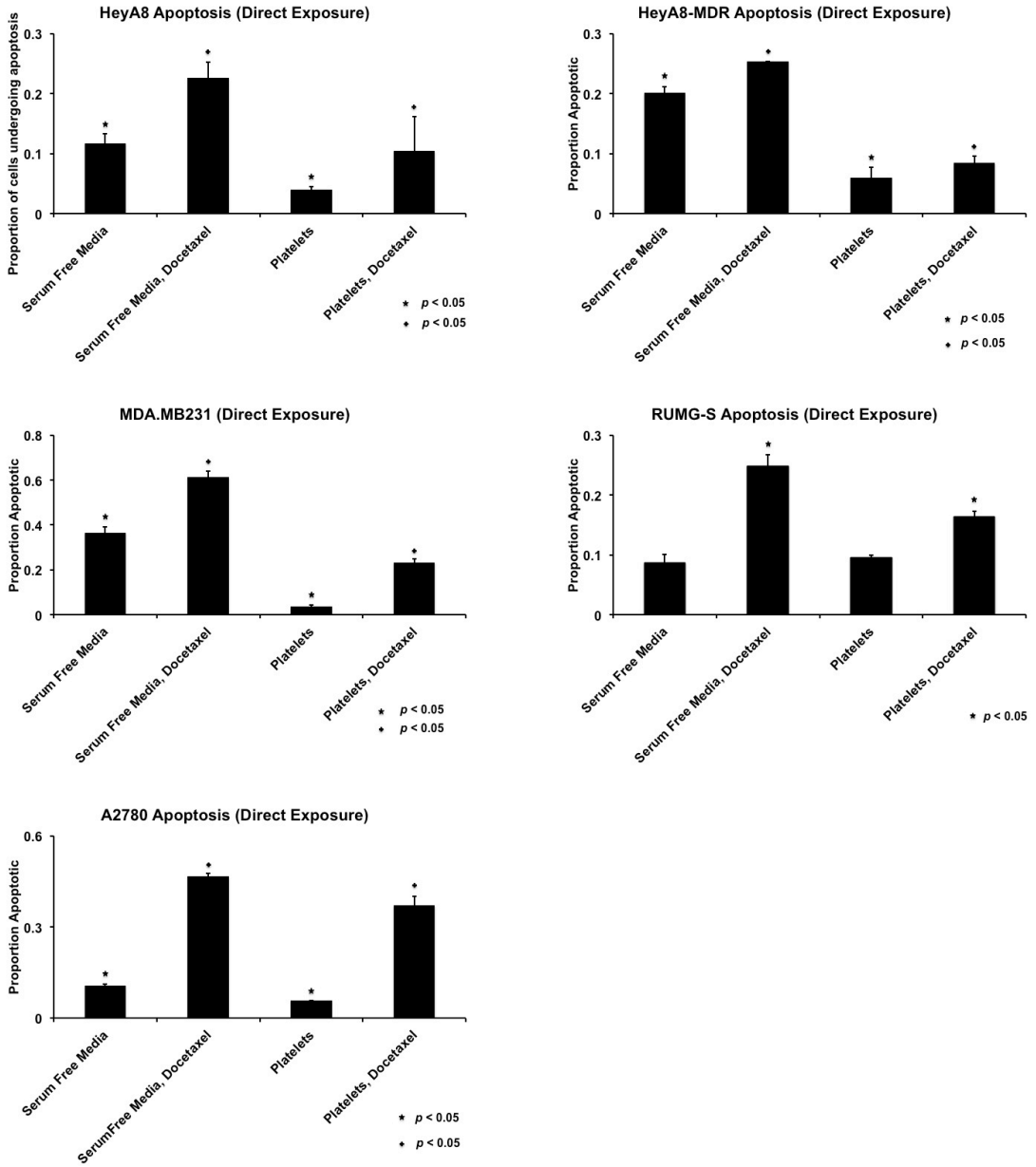


Figure 4: Platelet co-culture with direct exposure of platelets to tumor cells offers protection against apoptosis.

Indirect contact co-culture experiments were performed in which various cancer cell lines were plated in 6-well plates, serum-starved for 24 hours, and then variously treated with buffer control, docetaxel dosed according to previously published IC_{50}, platelets dosed at 1 x 10^8 cells/mL, and platelets followed by docetaxel. In this case, the tumor cells were separated from all components of the treatment by an insert with 0.4 mcm pores. After 72 hours of treatment, cells were processed for flow cytometry using Annexin and 7-aad staining to assess for total apoptosis. It is noted that platelets express phosphatidylserine that will also be marked by the Annexin. In the first set of apoptosis experiments, platelets were included along with cancer cells in the analysis, and therefore it is understood that the additional phosphatidylserine contribution of the platelets would increase the apoptosis score of groups containing platelets. In the present case, this effect should not be seen in these data, as platelets were kept separated from the cancer cells by the barrier. (See Figure 5.)

In A2780, platelets decreased apoptosis 60.8% ($p < 0.05$) compared to buffer control, and platelets decreased apoptosis 17.4% ($p < 0.05$) in the context of docetaxel. In MDA.MB231, platelets decreased apoptosis 52.3% ($p < 0.05$) compared to buffer control, and platelets decreased apoptosis 28.0% ($p < 0.05$) in the context of docetaxel. In 2774, platelets decreased apoptosis 25.3% ($p <$

0.05) compared to buffer control, and platelets decreased apoptosis 27.5% ($p <$ 0.05) in the context of docetaxel.

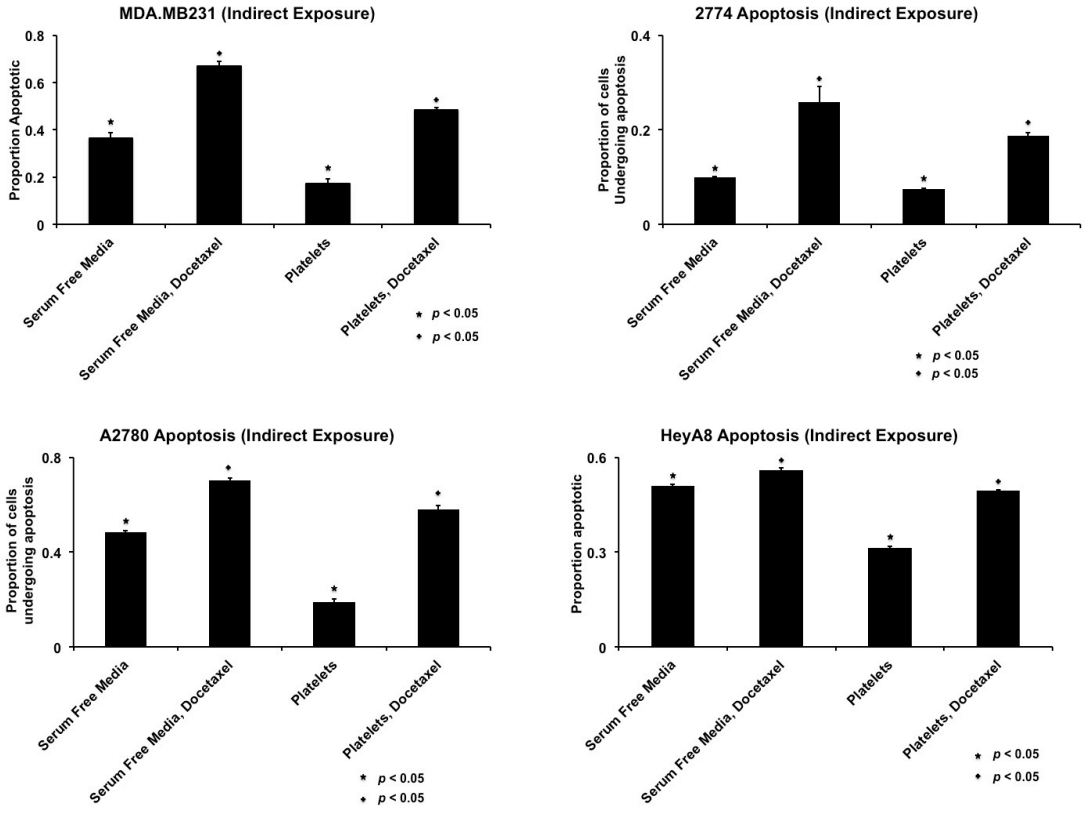


Figure 5: Platelet co-culture with indirect exposure of platelets to tumor cells offers protection against apoptosis.

Platelets mediate *in vitro* increases in proliferation in tumor cells

Direct contact co-culture experiments were conducted in which various cancer cell lines were plated in 6-well plates, serum-starved for 24 hours, and then treated with either control buffer or platelets at a density of 1 x 10^8 cells/mL for 24 hours. After treatment, they were incubated with EdU and processed as described above for flow cytometric evaluation of EdU incorporation as a proxy for degree of proliferation. (See Figure 6.)

Using HeyA8, co-culture with platelets resulted in mean increase in proliferation of 257.5% +/- 8.13% ($p < 0.05$) compared to control. Using SKOV3-Ip1, co-culture with platelets resulted in mean increase in proliferation of 78.9% +/- 1.47% ($p < 0.05$) compared to control. Using OVCAR5, co-culture with platelets resulted in mean increase in proliferation of 84.9% +/- 9.58% ($p < 0.05$) compared to control. Using ID8, co-culture with platelets resulted in mean increase in proliferation of 221.6% +/- 21.9% ($p < 0.05$) compared to control.

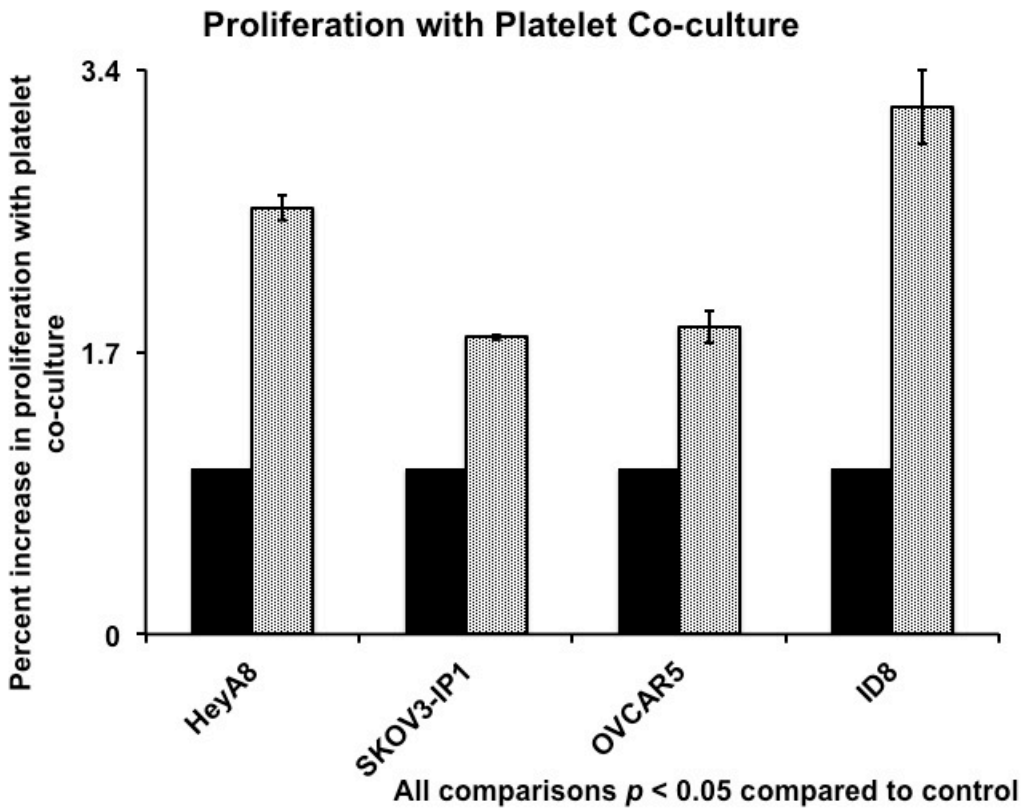


Figure 6: Platelet co-culture mediates increased tumor cell proliferation.

Platelets mediate *in vitro* increases in migration of tumor cells

Co-culture experiments were conducted in which various cancer cell lines were co-cultured with either control buffer or platelets at a density of 1×10^8 cells/mL using a modified Boyden chamber system for 6 hours. After incubation, fields were scored for migration (Figure 7).

Compared to buffer control, platelet co-culture with A2780 caused a 2.6-fold increase in the rate of migration ($p < 0.05$), platelet co-culture with HeyA8 caused a 2.8-fold increase in the rate of migration ($p < 0.05$), and platelet co-culture with 2774 caused a 2.1-fold increase in the rate of migration ($p < 0.05$).(37)

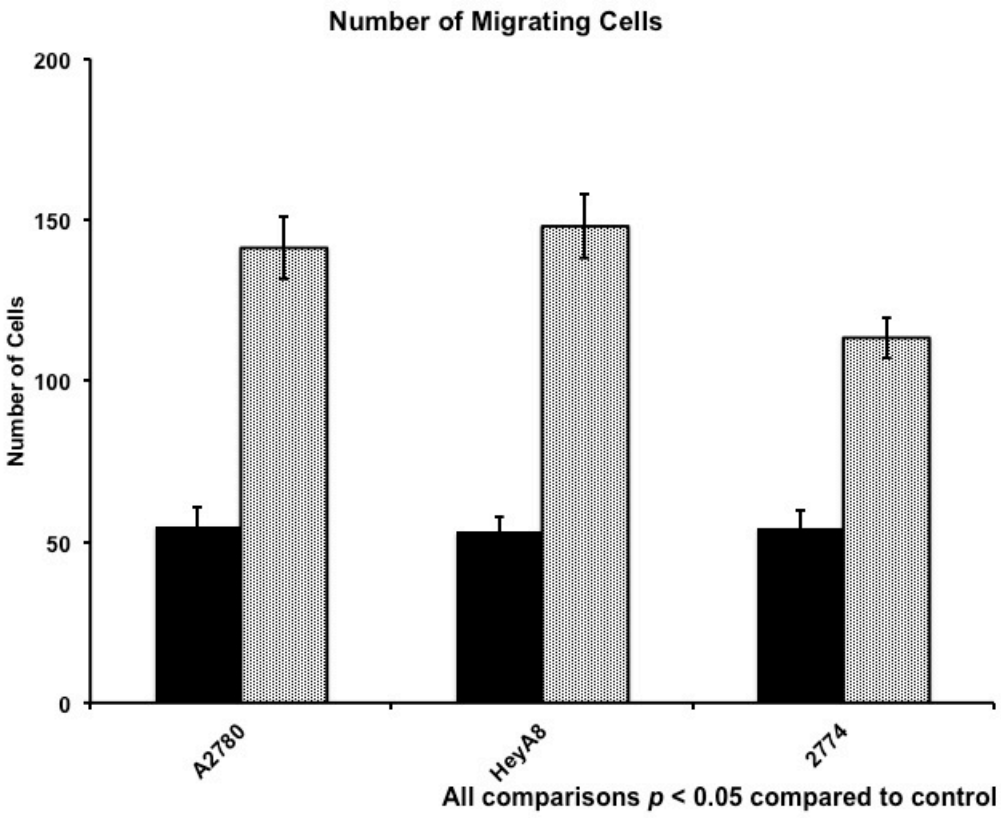


Figure 7: Platelet co-culture mediates increased tumor cell migration.

Hypoxia induces platelet-activating conditions *in vitro*, and effects of platelet exposure are maintained in hypoxic conditions *in vitro*.

Hypoxia is recognized as a mediator of angiogenic signaling that may contribute to changes in the tumor environment, both on a cellular and on an ecological level that may contribute to resistance to anti-angiogenic therapy. Given the apparent regulatory effects of platelets on angiogenesis, given platelet activity in wound healing, and given the powerful effects of ADP on platelet activation, we determined to examine the effects of hypoxia on cells and conditioned media. Specifically, SKOV3-IP1 was cultured in 10 cm plates in serum-free media in hypoxic and normoxic environments, and conditioned media was assessed at 24, 48, and 72 hours for the platelet-activating compound adenosine di-phosphate (ADP). (See Figure 8.)

At 24 hours, media from normoxic-cultured cells had 2.91 +/- 0.02 mM ADP compared to media from hypoxic-cultured cells that had 5.80 +/- 0.01 mM ADP ($p < 0.05$). At 48 hours, media from normoxic-cultured cells had 2.98 +/- 0.06 mM ADP compared to media from hypoxic-cultured cells that had 12.68 +/- 0.14 mM ADP ($p < 0.05$). At 72 hours, media from normoxic-cultured cells had 2.10 +/- 0.02 mM ADP compared to media from hypoxic-cultured cells that had 11.76 +/- 0.08 mM ADP ($p < 0.05$).

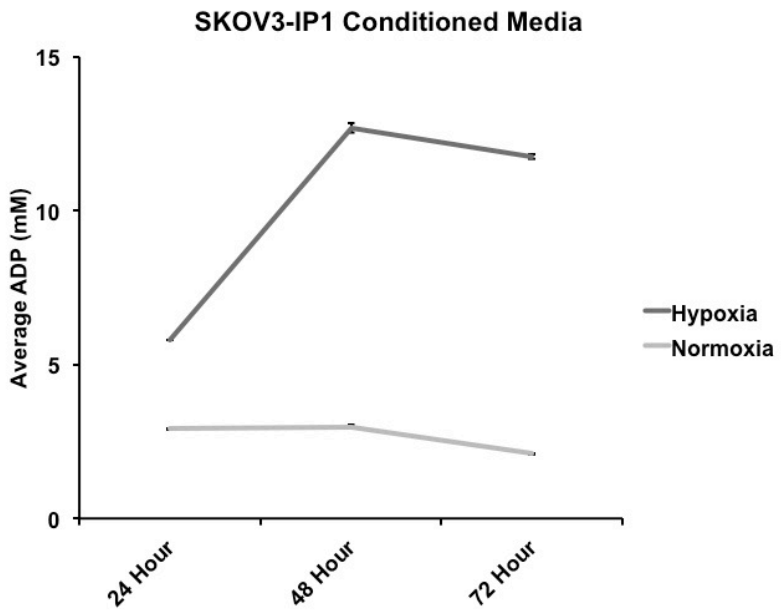


Figure 8: Conditioned media from SKOV-3 exposed to normoxic and hypoxic conditions.

The gross phenotypic effects of hypoxia and platelet-exposure were assessed in HeyA8 cells plated in 10 cm plates, placed in a serum-free media, and grown in normoxic versus hypoxic environments, with and without platelets dosed at 1 x 10^8 cells/mL. (See Figure 9.)

Comparing cells not exposed to platelets in the normoxic versus hypoxic environments, cell counts per 100x field at 72 hours were very similar, however cells in the hypoxic environment were less spiculated and had fewer cell-to-cell contacts compared to the normoxic environment. In both the normoxic and hypoxic contexts, co-culture with platelets resulted in a significant increase in cell density and eliminated the apparent differences in visual phenotype.

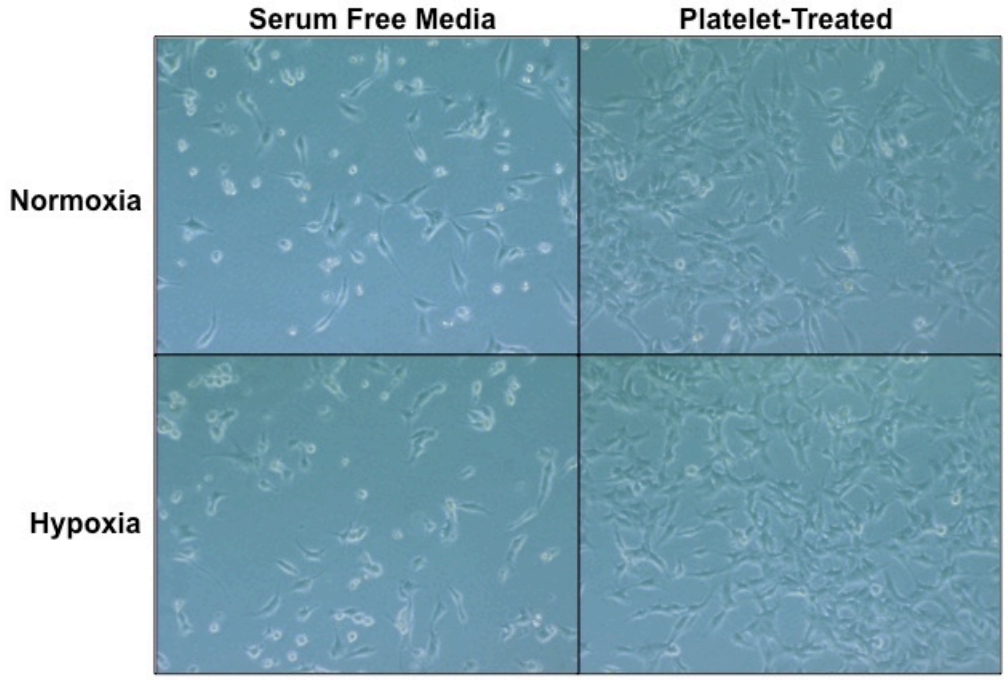


Figure 9: Gross phenotypic effects of hypoxia at 72 hours on HeyA8 exposed to hypoxic versus normoxic environments +/- platelets.

Taxane biodistribution in whole blood.

In order to consider whether platelets may be sequestering docetaxel to a degree that might explain the findings of the *in vitro* apoptosis experiments, human blood was inoculated *ex vivo* with docetaxel at two different concentrations ("high" dose = 5 mcg/mL and "low" dose = 1 mcg/mL) and incubated for 0.5 hr, 1.0 hr, 1.5 hr, 2.0 hr, 3.0 hr, 4.0 hr, 6.0 hr, 8.0 hr, and 24 hr. Biodistribution was determined by HPLC as described in the methods. (See Figure 10.)

Comparing docetaxel levels across time-points within groups, there were no significant differences between time-points, however there were remarkable differences between groups. Across all time-points, in the platelet fraction exposed to low-dose docetaxel, no docetaxel was detected in any of these samples at any time-point. In contrast, the low-dose exposed platelet-free plasma fractions and whole plasma fractions were not significantly different (1261 +/- 7.3 ng/mL versus 1211 +/- 7.0 ng/mL, $p = 0.12$) across all time-points. The platelet fraction exposed to high-dose docetaxel had detectable levels of 48.56 +/- 3.06 ng/mL, which actually falls below the lower limit of quantifiability. In contrast, the high-dose exposed platelet-free plasma fractions and whole plasma fractions were not significantly different (5123 +/- 37.9 ng/mL versus 5191 +/- 23 ng/mL, $p = 0.62$) across all time-points.

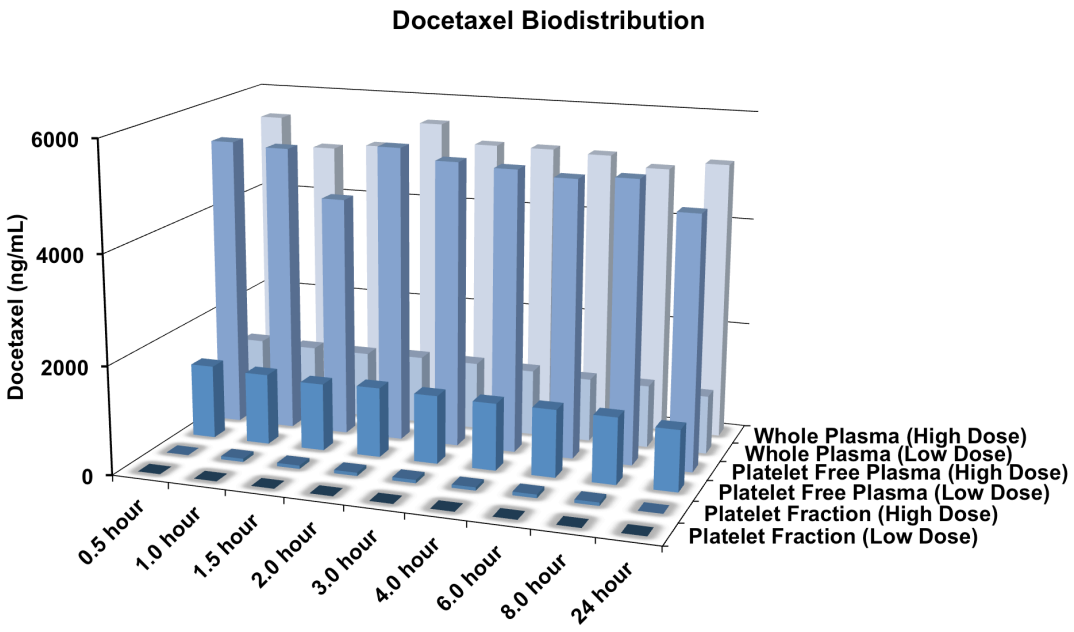


Figure 10: Docetaxel biodistribution in whole plasma compared to platelet-free plasma compared to platelets.

Platelet infiltration into tumor

In order to query whether platelets could plausibly be exposed to cancer cells in a biological system, e.g. in an organism with a growing tumor, nude mice were inoculated with the ovarian cancer cell line 2774. When tumor was palpable by physical examination of the mouse abdomen, the mouse was anesthetized and sacrificed by intravital fixation. Frozen tumor sections were taken for immune-fluorescence. Co-staining was performed for mouse-CD31 and for the mouse GP1b-beta subunit of the GP1b-V-IX complex. (See Figure 11.)

The numbers of infiltrating platelets were counted in representative 200x fields. It was found that representative tumor sections had a mean 69.3 +/- 14.5 cells positive for GP1b-beta staining suggesting platelet infiltration into the tumor.

This compared to almost no platelets observed in normal peritoneum and only a slightly increased number seen in a model of induced peritonitis (37).

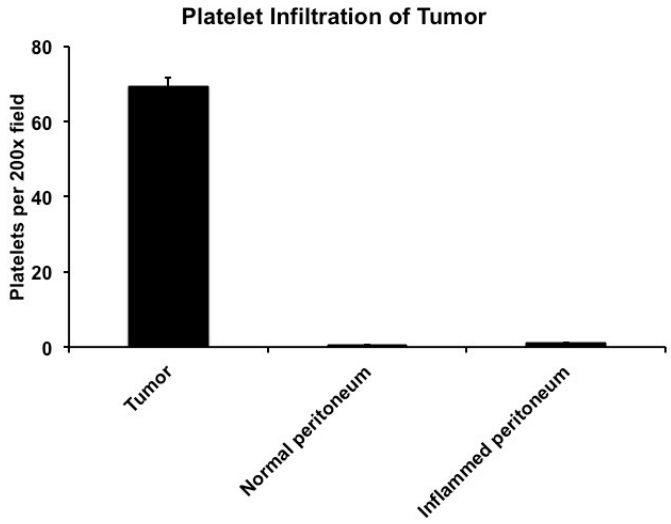


Figure 11: Platelets infiltrate tumor.

Modulation of platelet levels correlates with tumor growth, tumor spread, and chemotherapeutic efficacy.

Two experiments were performed to query the effects of platelet levels on tumor growth and chemotherapeutic efficacy using our orthotopic model of ovarian cancer in nude mice.

In the first experiment, mice were inoculated with A2780 on Day 0 and treatment was initiated on Day 7. Control mice were given a control IgG. The second groups of mice were given a platelet-depleting IgG previously validated in our lab. The third group was given control IgG and docetaxel. The fourth group was given platelet-depleting IgG in addition to docetaxel. Mice were sacrificed

when a significant number appeared moribund from disease. At necropsy, data was taken regarding tumor nodule number and aggregate tumor weight. (See Figure 12.)

Mice treated with control IgG had a mean aggregate tumor weight of 2.01 +/- 0.14 g per mouse and a mean number of tumor nodules of 25.7 +/- 2.1 nodules per mouse. Mice treated with platelet-depleting IgG had a mean aggregate tumor weight of 0.71 +/- 0.07 g per mouse and a mean number of tumor nodules of 11.1 +/- 1.2 nodules per mouse. Compared to control, mice treated with the platelet-depleting IgG had a significantly lower aggregate tumor weight ($p < 0.05$) and number of tumor nodules ($p < 0.05$). Mice treated with control IgG and docetaxel had a mean aggregate tumor weight of 0.60 +/- 0.07 g per mouse and a mean number of nodules of 4.9 +/- 0.7 nodules per mouse. The mean aggregate tumor weight for the mice treated with the platelet-depleting IgG and the control IgG plus docetaxel were not significantly different ($p = 0.70$). The mean number of nodules showed a non-significant trend toward a decrease when the platelet-depleting IgG mice were compared to those treated with control IgG and docetaxel ($p = 0.13$). Mice treated with platelet-depleting IgG and docetaxel had a mean aggregate tumor weight of 0.23 +/- 0.02 g per mouse and a mean number of tumor nodules of 2.0 +/- 0.1 nodules per mouse. Compared to mice treated with control IgG and docetaxel, these decreases in mean aggregate tumor weight ($p = 0.09$) and nodule number per mouse ($p = 0.17$) represented trends toward significance.

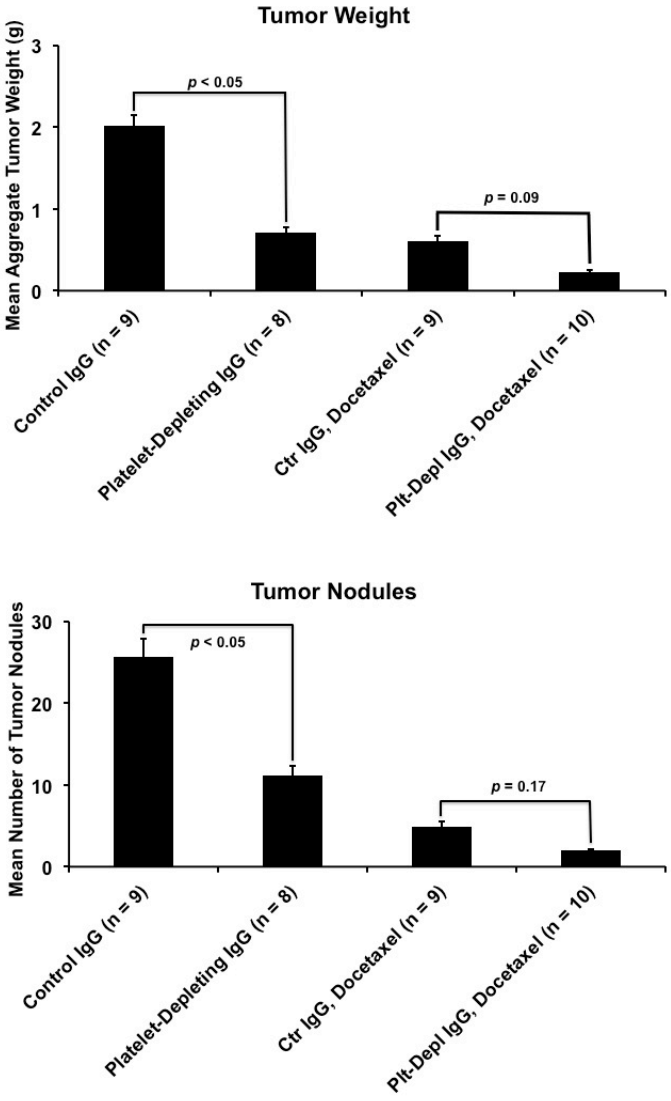


Figure 12: Effect of platelet-depletion on docetaxel chemotherapy in mice with orthotopically-inoculated A2780.

In the second experiment, mice were inoculated with SKOV3-IP1 on Day 0 and treatment was initiated on Day 7. Control mice were given a control IgG. The second groups of mice were given a platelet-depleting IgG previously validated in our lab. The third group was given platelet transfusion as described in the methods section. The fourth group was given control IgG in addition to docetaxel. The fifth group was given platelet-depleting IgG and docetaxel. The sixth group was given platelet transfusion and docetaxel. Mice were sacrificed when a significant number appeared moribund from disease. At necropsy, data was taken regarding tumor nodule number and aggregate tumor weight. It is noted that this experiment was ended because of the condition of the platelet-transfused mice. (See Figure 13.)

Mice treated with control IgG had a mean aggregate tumor weight of 0.37 +/- 0.037 g per mouse and a mean number of tumor nodules of 4.86 +/- 0.29 nodules per mouse. Mice treated with platelet-depleting IgG had a mean aggregate tumor weight of 0.21 +/- 0.017 g per mouse and a mean number of tumor nodules of 4.50 +/- 0.41 nodules per mouse. Compared to control, mice treated with the platelet-depleting IgG had a non-significant trend toward lower aggregate tumor weight (p = 0.15), while the number of tumor nodules was similar (p = 0.81). Mice treated with platelet transfusion had a mean aggregate tumor weight of 0.87 +/- 0.054 g and mean number of nodules of 13.25 +/- 1.13 nodules per mouse. Compared to control, platelet transfused mice had a significant increase in mean tumor weight ($p < 0.05$) and a significant increase in mean number of tumor nodules per mouse ($p < 0.05$). Mice treated with control

IgG and docetaxel had a mean aggregate tumor weight of 0.17 +/- 0.007 g per mouse and a mean number of tumor nodules of 2.22 +/- 0.11 nodules per mouse. Compared to control, mice treated with control IgG and docetaxel had a significant decrease in mean tumor weight ($p < 0.05$) and a significant decrease in mean number of tumor nodules per mouse ($p < 0.05$). By contrast, compared to mice treated with platelet-depleting antibody, mice treated with control IgG and docetaxel there was only a statistical trend toward decreased mean tumor weight ($p = 0.07$) and a statistical trend toward decreased mean number of tumor nodules per mouse ($p = 0.07$). Mice treated with platelet-depleting antibody in addition to docetaxel had a mean tumor weight of 0.057 +/- 0.005 g per mouse and a mean number of tumor nodules of 2.57 +/- 0.22 nodules per mouse. Compared to the mice treated with control IgG and docetaxel, there was a significant decrease in mean aggregate tumor weight ($p < 0.05$). Mice treated with platelet transfusion and docetaxel had a mean aggregate tumor weight of 0.47 +/- 0.043 g per mouse and a mean number of tumor nodules of 4.0 +/- 0.53 nodules per mouse. Compared to mice treated with control IgG and docetaxel, there was a significant increase in mean tumor weight ($p < 0.05$) and a non-significant trend toward an increase in mean number of tumor nodules ($p = 0.21$). Compared to mice treated with control IgG only, the mice treated with both platelet transfusion and docetaxel had statistically similar mean aggregate tumor weights ($p = 0.55$) and mean nodule number ($p = 0.61$).

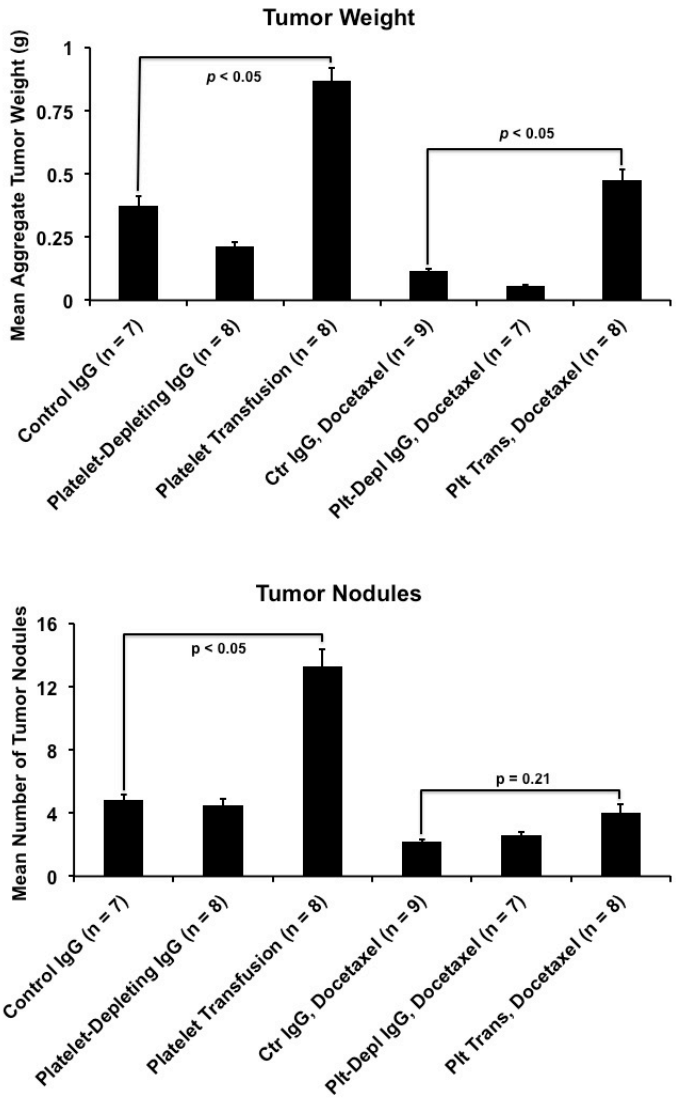


Figure 13: Effect of platelet-depletion and platelet-transfusion on docetaxel chemotherapy in mice with orthotopically-inoculated SKOV3-IP1.

Platelet activation is necessary for observed in vitro effects.

In order to determine whether platelet activation was necessary for the effects observed, the proliferation and apoptosis experiments described previously were repeated using the cell line HeyA8, in this cycle including a group treated with an equivalent number of platelets fixed with paraformaldehyde. (See Figure 14.)

The cell line HeyA8 was plated in 6-well plates and co-cultured with routine buffer control, plasma-free platelets (1 x 10^8 cells/mL), platelets fixed with 1% paraformaldehyde as described in the methods section, or a buffer processed including paraformaldehyde in parallel to the platelets prepared by paraformaldehyde fixation. Cells were analyzed by flow cytometry for apoptosis after 72 hours and for proliferation after 24 hours. Cells cultured with routine buffer control had a baseline apoptotic rate of 9.6 +/- 0.3%. Cells co-cultured with platelets had an apoptotic rate of 3.3 +/- 0.08%, which was significantly decreased compared to control ($p < 0.05$). The cells incubated with the paraformaldehyde prepared buffer and with the paraformaldehyde-fixed platelets had apoptosis rates of 10.6 +/- 0.2% and 10.4 +/- 0.2% respectively. In both cases, this was not significantly different than the control group ($p = 0.20$ and $p = 0.28$, respectively). Cells cultured with standard buffer had a baseline proliferation rate of 11.3 +/- 0.25%, which was significantly increased to 59.0 +/- 0.47% ($p < 0.05$) with co-culture with platelets. Paraformaldehyde fixation of platelets abrogated the increase in proliferation, decreasing the rate to 14.2 +/- 0.68%, which was not significantly different than control ($p = 0.084$)

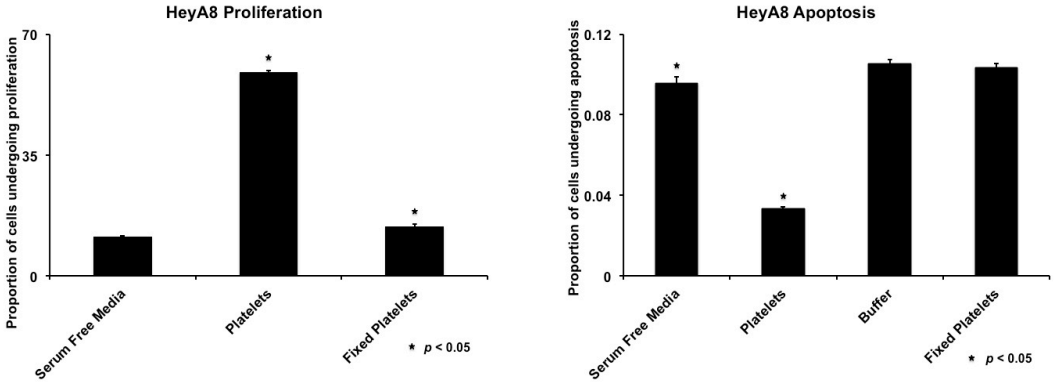


Figure 14: Apoptosis protection and proliferation augmentation effects of platelet co-culture are eliminated by platelet fixation.

In order to consider what options may be clinically available to block platelet activation, the proliferation end-point was re-examined, but instead of blocking platelet activation with highly-toxic paraformaldehyde, aspirin and a small molecule inhibitor of focal adhesion kinase were used to block platelet activation. (See Figures 15 and 16.)

SKOV3-IP1 cells were co-cultured with control buffer, platelets, aspirin, and aspirin—pretreated platelets. After 24 hours of treatment, flow cytometric analysis was performed to assess degree of proliferation. In the control group the proliferative rate was 21.7 +/- 0.72%. In the platelet-treated group, the proliferative rate was 34.1 +/- 0.94%. In the aspirin treated group, the proliferative rate was 23.1 +/- 1.73%. In the cells treated with both aspirin and platelets, the proliferation rate was 27.3 +/- 2.05%. The increase in proliferation between control and platelet-treatment was statistically significant ($p < 0.05$). Interestingly, because aspirin incompletely abrogated platelet-induced proliferation, and because variances were relatively large, the proliferation rate of the cells treated with aspirin and platelets was not significantly different from the primary control ($p = 0.22$) or from the platelet-treated tumor cells without aspirin ($p = 0.157$).

HeyA8 cells were co-cultured with control buffer, platelets, FAK-inhibitor, and FAK-inhibitor plus platelets. After 24 hours of treatment, flow cytometric analysis was performed to assess degree of proliferation. In the control group the proliferative rate was 14.5 +/- 1.94%. In the platelet-treated group, the proliferative rate was 50.2 +/- 2.66%. In the FAK-inhibitor treated group, the proliferative rate was 17.2 +/- 2.15%. In the cells treated with both FAK-inhibitor

and platelets, the proliferation rate was 16.9 +/- 1.33%. The increase in proliferation between control and platelet-treatment was statistically significant ($p < 0.05$). By way of comparison, there were no significant differences between the other three groups. Comparing the control to the FAK-inhibitor plus platelets, $p = 0.58$.

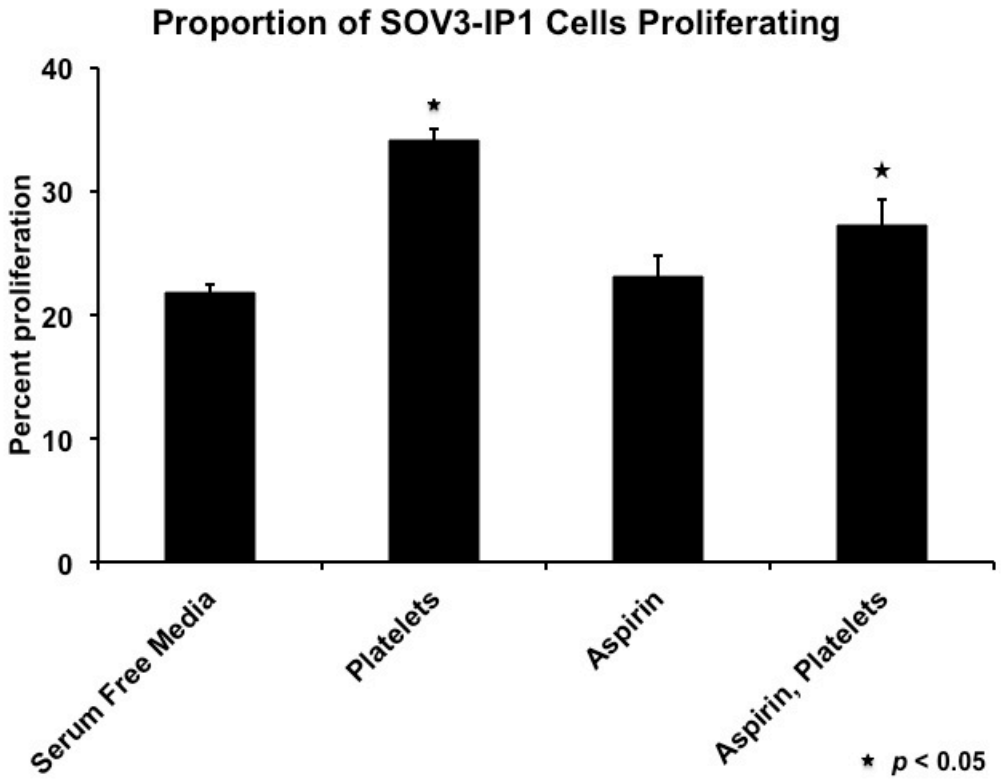


Figure 15: Blockade of the tumor cell proliferative response to platelets with aspirin.

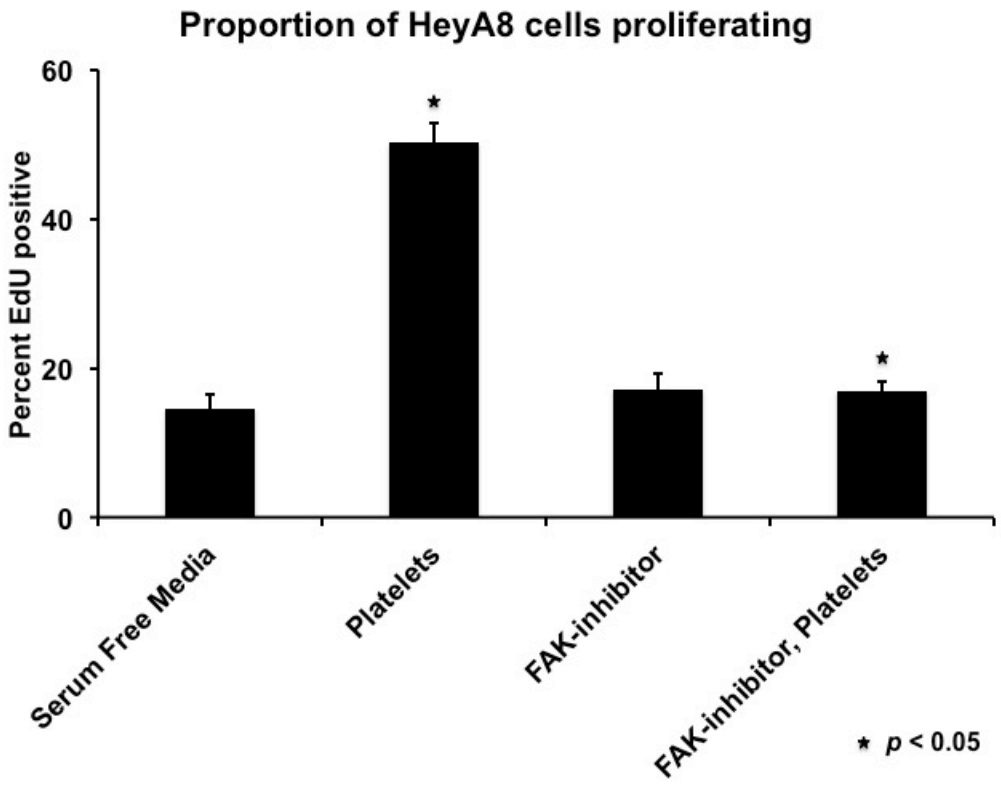


Figure 16: Blockade of the tumor cell proliferative response to platelets with the FAK-inhibitor.

Platelet-derived growth factor (PDGF) and transforming growth factor beta-1 (TGFB1) are involved in the observed mitogenic signaling.

In order to validate the causality of platelet-derived signaling factors in the proliferation and apoptosis-protection observed in our experiments, siRNA transfection methods were validated to knockdown expression of platelet-derived growth factor receptor alpha (PDGFRA) and transforming growth factor beta-1 receptor (TGFBR1), as these factors are known to be released from platelets on activation. Transfection was used to attempt to demonstrate that these pathways could be blockaded. (See Figures 17 and 18.)

After validation of siRNA knockdown, reverse transfection was performed on HeyA8 cells, and 48 hours later, treatment with control buffer or platelets was initiated. After 24 hours of treatment, flow cytometric analyses for proliferation was performed. As anticipated, in the cells transfected with control siRNA, platelet co-culture resulted in a significant increase in proliferation (5.41% versus 23.35%, $p < 0.05$). There was no difference in proliferation between the cells transfected with control siRNA compared to PDGFRA siRNA (5.41% versus 5.00%) that were not exposed to platelets. However, in the cells transfected with PDGFRA siRNA, cells exposed to platelets only demonstrated an increase from 5.00% proliferation to 17.13% proliferation ($p < 0.05$). While that increase was significant, it was not as large an increase in proliferation as that seen in the cells transfected with control siRNA and exposed to platelets (23.25% versus 17.13%, $p < 0.05$).

After validation of siRNA knockdown, double transfection as described in the methods section was performed on SKOV3-IP1 cells. Immediately following the second-phase of the transfection, treatment was initiated with control buffer, docetaxel 5 nM, platelets 1 x 10^8 cells/mL, or platelets followed immediately by docetaxel. In the cells transfected with control siRNA, baseline apoptosis was 34.3 +/- 1.04%, docetaxel-mediated apoptosis was 60.8 +/- 1.05%, apoptosis after platelet co-culture was markedly decreased at 6.07 +/- 0.32% ($p < 0.05$ compared to control), and platelets also decreased docetaxel-mediated apoptosis to 40.2 +/- 0.86% ($p < 0.05$ compared to docetaxel alone). In cells transfected with siRNA targeted to TGFBR1, baseline apoptosis was similar to control siRNA transfection at 36.4 +/- 0.65%. Docetaxel-mediated apoptosis was also similar to control siRNA transfected cells at 64.7 +/- 1.73%. However, platelet co-culture only decreased apoptosis to 23.3 +/- 0.39% compared to 6.07% in the control siRNA transfected cells ($p < 0.05$), representing a 60.9% blockade of platelet-mediated apoptosis protection. Likewise, platelet co-culture in the context of docetaxel only decreased apoptosis to 50.4 +/- 1.71% in TGFBR1 transfected cells compared to control siRNA transfected cells, representing a 69% return of apoptosis ($p < 0.05$).

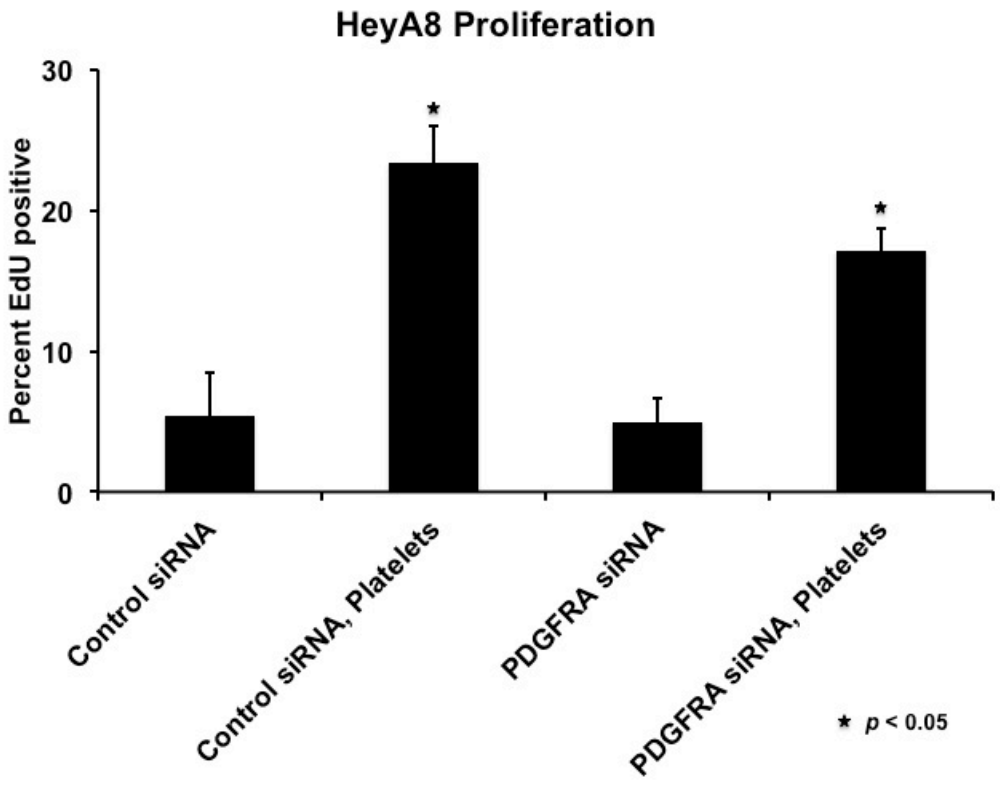


Figure 17: Proliferative response to platelets is partially mediated by PDGFRA signaling.

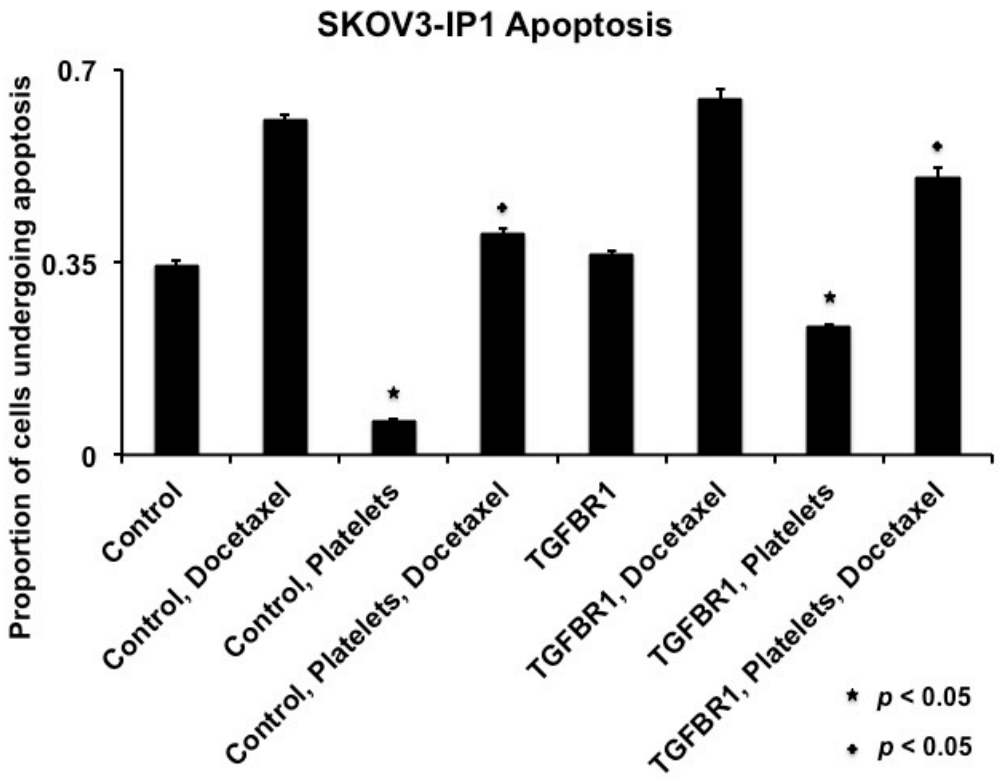


Figure 18: Apoptosis protection from platelets is partially mediated by TGFBR1 signaling.

Aspirin can block *in vivo* effects of platelet transfusion on tumor growth.

In order to consider the mechanism of platelet effect on tumor growth and spread, we used our orthotopic model of ovarian cancer in nude mice to query whether those effects could be blocked by aspirin therapy. Nude mice were inoculated with A2780 on Day 0 and treatment was started on Day 7. Control animals were untreated. The second group received aspirin therapy as described in the methods section. The third group received platelet transfusion as described in the methods section. The fourth group was given transfusions of aspirinized platelets per the protocol described in the methods section. When a

substantial number of mice became moribund, the animals were sacrificed. (See Figures 19-22.)

Control animals with no treatment had a mean aggregate tumor weight of 2.14 +/- 0.50 g per mouse and a mean number of tumor nodules of 20.57 +/- 6.91 nodules per mouse. Animals treated with aspirin were not statistically different, having a mean aggregate tumor weight of 1.93 +/- 0.26 g per mouse and a mean number of tumor nodules of 23.0 +/- 4.51 nodules per mouse. Mice receiving platelet transfusions had a significantly increased mean aggregate tumor weight of 4.11 +/- 0.59 g per mouse ($p < 0.05$ compared to control and aspirin-treated groups) and mean number of tumor nodules of 62.3 +/- 9.86 nodules per mouse ($p < 0.05$ compared to control and aspirin-treated groups). Transfusion of aspirinized platelets did not have the same effect as non-aspirinized platelets and was statistically similar to the control and aspirin treated groups; mean tumor weight was 2.06 +/- 0.34 g per mouse and mean number of nodules was 24.4 +/- 6.79 nodules per mouse (both $p < 0.05$ compared to platelet transfused mice).

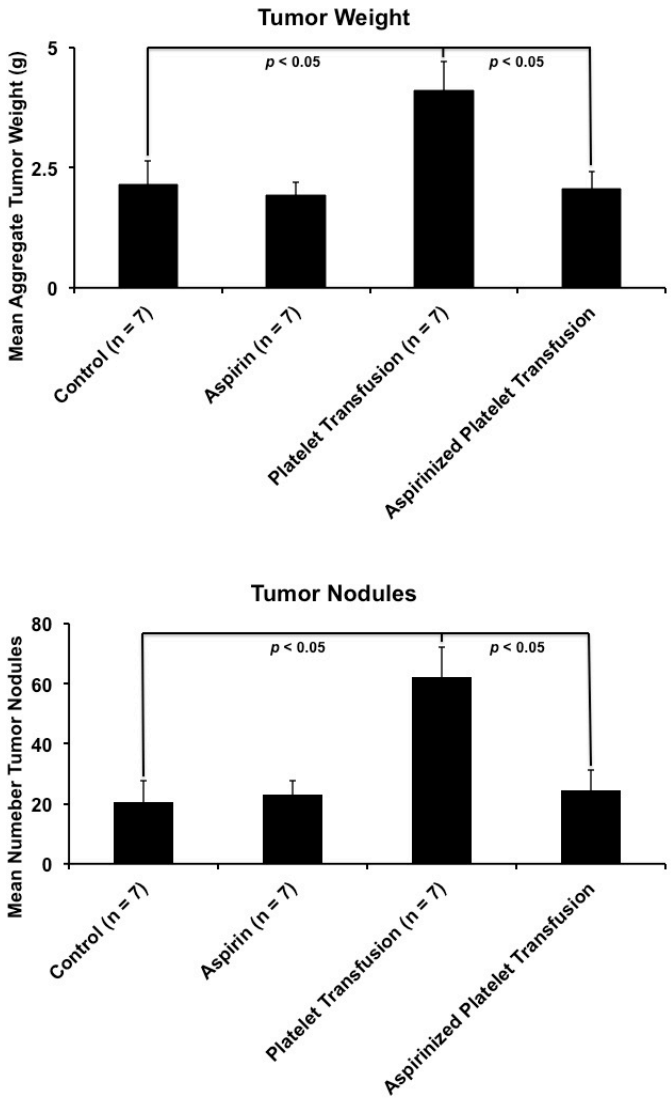


Figure 19: Aspirin treatment blocks *in vivo* effects of aspirin therapy.

The initial evaluation of tumor vasculature was performed based on conventional scoring of microvascular density as determined by identified clusters of 3 or more CD31-positive cells. Based on this system, tumor from platelet transfused mice demonstrated a significant decrease in microvascular density per 200x field, control 19.07 +/- 0.39 vessels per HPF versus 14.0 +/- 0.25 vessels per HPF, $p < 0.05$. By contrast, the aspirin treated mice had a microvascular density of 16 +/- 0.56 ($p = 0.28$) and the mice treated with aspirinized platelets had a microvascular density of 14.8 +/- 0.39, which approached statistical significance at $p = 0.059$. These differences were not overwhelming and obligated more careful consideration.

As a contrasting analysis, we re-scored the CD31 considering the concept of vascular stabilization by scoring for the ratio of lumen-containing CD31-positive clusters to non-lumen-containing clusters. In this case, platelet-transfused mice had tumors with a significantly higher ratio, scoring a mean 2.56 +/- 0.117 compared to the control value of 1.038 +/- 0.063, $p < 0.05$. By contrast, the aspirin-only treated mice had a mean ratio of 1.039 +/- 0.043, which was statistically indistinguishable from control, $p = 0.997$. The tumor from the mice treated with aspirinized platelets had a ratio of 1.91 +/- 0.147 that trended toward a significant increase compared to control with $p = 0.20$.

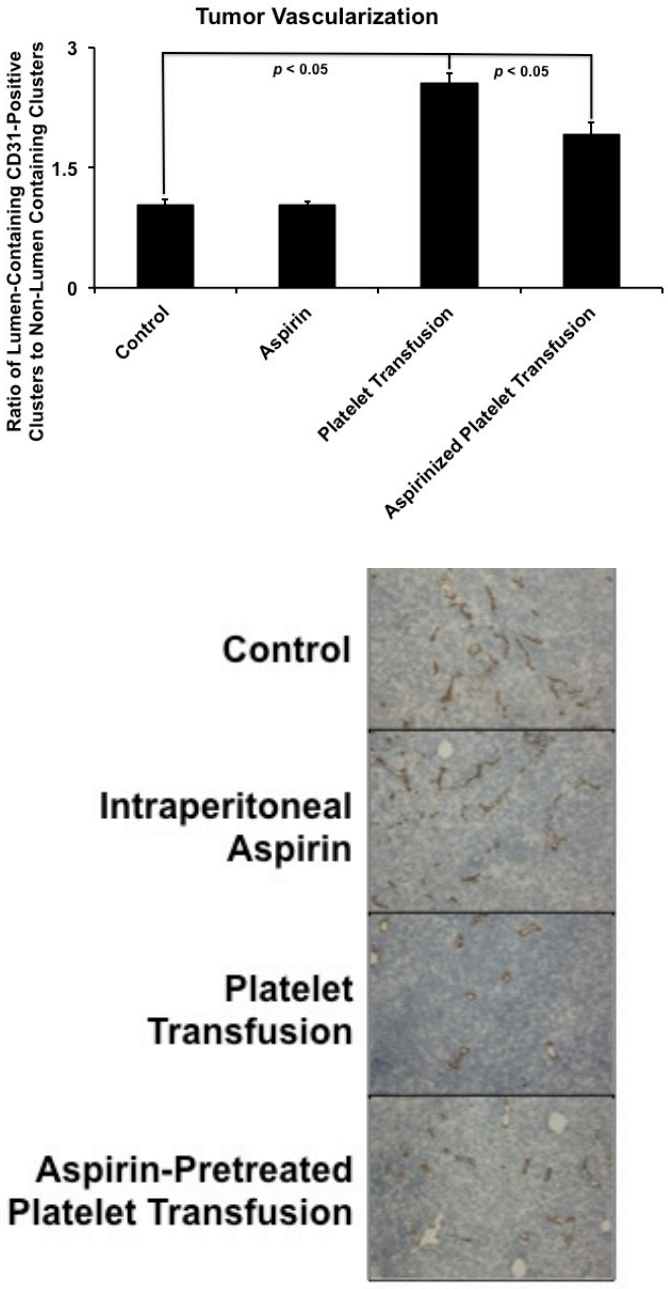


Figure 20: Platelet transfusion normalizes tumor vasculature.

Tumor harvested from the mice at the time of sacrificed was assessed using Ki67 immunohistochemistry for ratio of proliferating nuclei to total nuclei counted in a 200x field (proliferative index). The tumor from control animals was noted to have a proliferative index of 66.3 +/- 0.77%. Aspirin significantly decreased this rate of proliferation by itself, with the aspirin-only treated mice scoring 56.8 +/- 0.77% ($p < 0.05$). Platelet transfusion had the opposite effect, increasing the mean proliferative index to 82.9 +/- 0.43% ($p < 0.05$). Transfusion of aspirinized platelets resulted in a non-significant trend toward an increased proliferative index at 72.0 +/- 0.62% ($p = 0.14$), suggesting that aspirinized platelets may have had an incompletely blocked effect. Comparing the tumor of platelet-transfused mice to tumor from mice that had been transfused with aspirinized platelets, the difference in proliferative index (82.9% versus 72.0%, $p < 0.05$) was significant, again suggesting that aspirin at least partially blocked he effects attributable to platelet transfusion.

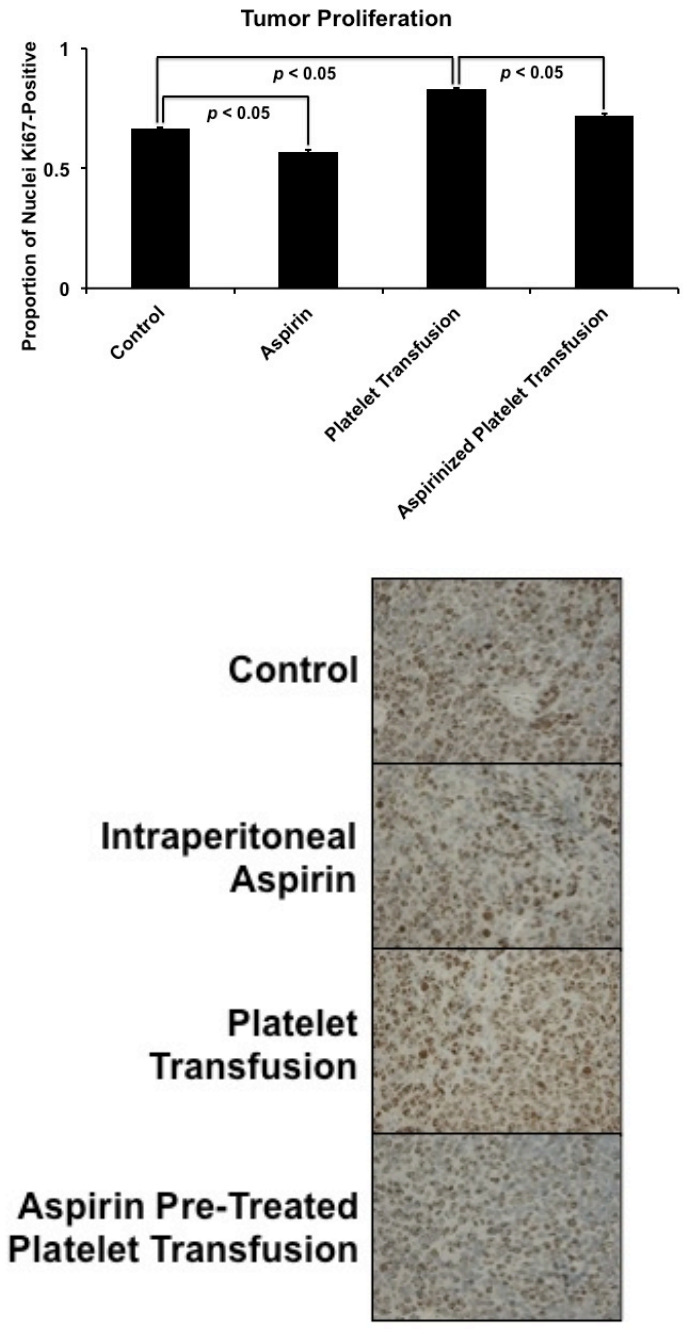


Figure 21: Platelet transfusion increases tumor cell proliferation that is partially blocked by aspirin.

In order to query whether platelet transfusion would impact tumor cell apoptosis, ex vivo tumor from treated animals was immunostained for activated caspase-3 that was scored based on the number of positive cells per 200x field. Tumor from control animals demonstrated a mean of 28.1 +/- 3.43 positive cells per 200x field. Tumor from aspirin-treated animals had a nearly identical mean of 28.9 +/- 2.32 positive cells per 200x field. In sharp contrast, tumor from platelet-transfused animals had a statistically significantly lower rate of positive (apoptotic) cells at 17.6 +/- 2.10 positive cells per 200x field ($p < 0.05$). Interestingly, the tumor from the mice given transfusions with aspirinized platelets had a rate of positivity (22.5 +/- 1.62 positive per 200x field) greater than that of the platelet-transfusion mice and lower than that of the control and aspirin-treated mice ($p = 0.113$ compared to control and $p = 0.098$ compared to platelet-transfused mice). However, the data again suggest that aspirin was only able to partially block the effects of platelet transfusion on this orthotopic model of ovarian cancer.

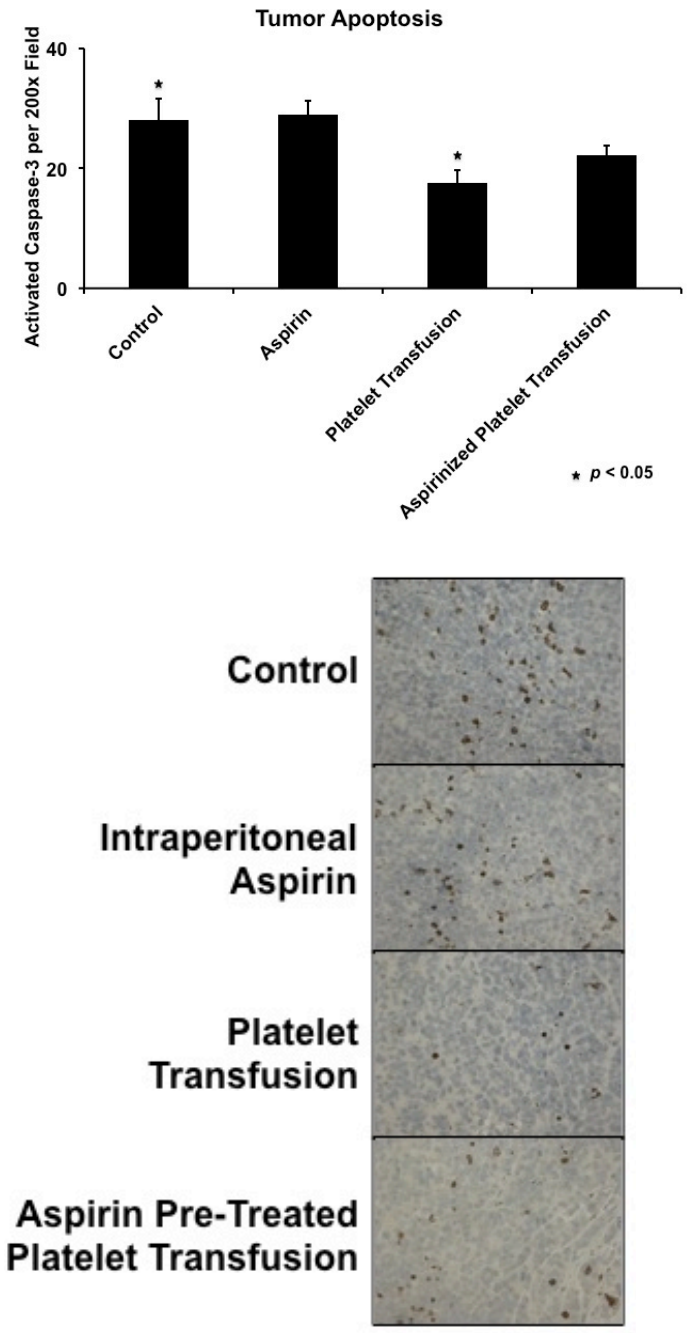


Figure 22: Platelet transfusion results in decreased tumor cell apoptosis.

Acceleration of tumor growth after withdrawal of anti-angiogenic agents

As discussed in the introduction, response to anti-angiogenic therapy has been modest in the clinical setting, and recent pre-clinical models have even suggested a rebound in tumor growth following the withdrawal of certain anti-angiogenic agents.

In consideration of this concern, and given the probability that micro-environmental factors may play a central role in these phenomena, we initially sought to establish a pre-clinical model in which we could study the effects of anti-angiogenic therapy on tumor. In order to query the phenomenon of resistance to anti-angiogenic therapy and rebound tumor growth that has recently been described in the literature, we sought to establish a working *in vivo* model. Ultimately, the goal was to develop working *in vivo* models of both antibody-based treatment resistance (e.g. bevacizumab) and small molecule inhibitor-based treatment resistance (e.g. pazopanib). For our first experiment, we started with pazopanib because it is a well-tolerated and studied drug in pre-clinical models and is of current interest in early-phase ovarian cancer trials. We inoculated nude mice with SKOV3-IP1 on Day 0. In the first group of mice, the animals were treated with pazopanib for 7 days prior to Day 0 inoculation with tumor. Two other groups were given drug vehicle as controls from Day 7-14 and starting on day 7 for the duration of the experiment. A fourth group was given pazopanib daily from Day 7-14. The fifth group was given pazopanib daily starting on day 7 for the duration of the experiment. Animals were sacrificed when a significant number appeared to be suffering morbidity related to tumor

burden. Data were taken with respect to aggregate tumor weight and the number of identified tumor nodules. (See Figure 23.)

Mice were sacrificed primarily related to morbidity seen in the mice treated with pazopanib for only seven days. There were no statistical differences seen between mean aggregate tumor weight or number of tumor nodules for the mice pre-treated with pazopanib (0.30 +/- 0.053 g/mouse; 10 +/- 2.22 nodules/mouse), the mice treated with vehicle for 7 days (0.35 +/- 0.06 g/mouse; 12.9 +/- 3.34 nodules/mouse), and the mice treated with vehicle continuously starting at Day 7 (0.32 +/- 0.06 g/mouse; 12.2 +/- 2.75 nodules/mouse). Choosing a representative vehicle control for comparison, treatment with continuous pazopanib resulted in a decreased mean aggregate tumor weight (0.11 +/- 0.01 g/mouse versus 0.35 +/- 0.06 g/mouse, $p < 0.05$) and decreased number of tumor nodules that approached statistical significance (6 +/- 0.97 nodules/mouse versus 12.9 +/- 3.34 nodules/mouse, $p = 0.06$). In sharp contrast, treatment of the mice with pazopanib from days 7-14 resulted in increased aggregate tumor weight compared to control that approached statistical significance (0.54 +/- 0.05 g/mouse versus 0.35 +/- 0.06 g/mouse, $p = 0.08$) and an increased average number of tumor nodules that was non-significant but of interest (17.0 +/- 1.87 versus 12.9 +/- 3.34 nodules/mouse, $p = 0.29$). The differences in mean tumor weight and mean number of nodules between animals treated continuously with pazopanib and for 7 days with pazopanib were both statistically significant, $p < 0.05$.

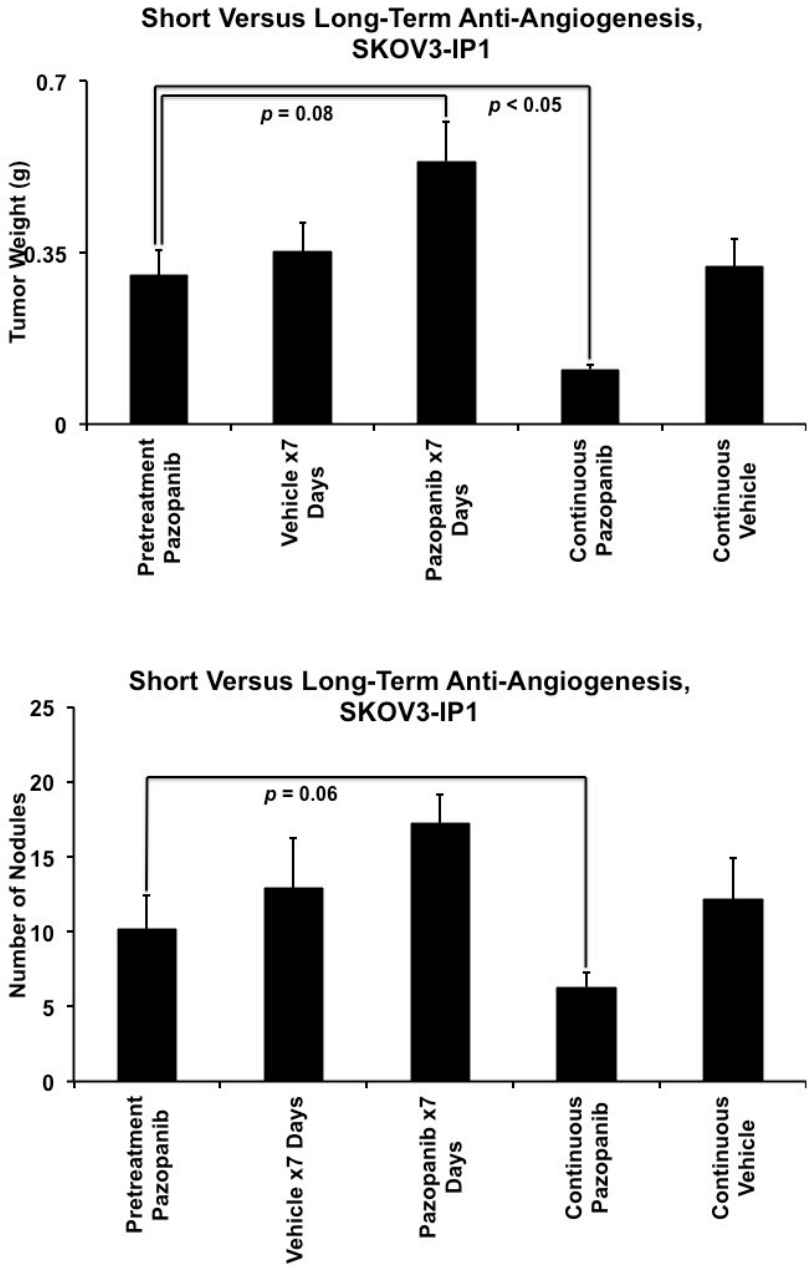


Figure 23: Short-term anti-angiogenic therapy results in acceleration of tumor growth.

Acceleration of tumor growth after withdrawal of anti-angiogenic agents is abrogated by FAK blockade.

Based on the preliminary finding that short-term treatment with pazopanib in our orthotopic model of ovarian cancer resulted in an increase in the aggregate tumor mass and number of tumor nodules per mouse compared to control and to continuously treated animals, based on the knowledge that FAK-inhibition is effective in platelets, based on demonstrated platelet infiltration into tumor, and based on literature suggesting that the absence of focal adhesion kinase signaling in platelets leads to impaired migration, we formed a two-fold hypothesis: (1) short term anti-angiogenic therapy leads to local hypoxia and increased local activation of platelets through ADP-driven mechanisms, and (2) that impairment of FAK-mediated signaling would lead to impaired platelet infiltration into tumor, correlating with a blockade of the observed “rebound” effect.

Based on this hypothesis, a series of *in vivo* studies were designed utilizing the orthotopic model of ovarian cancer in nude mice. In the first experiment, nude mice were inoculated with SKOV3-IP1 on Day 0 and treatment was initiated on Day 7. Control mice were untreated. In groups 2-4, pazopanib was initiated and given daily. In groups 5-7, bevacizumab was administered on a twice-weekly schedule. In groups 2 and 5, treatment was discontinued after 1 week. In groups 3 and 6, treatment was continued for the duration of the experiment. In groups 4 and 7, treatment of the anti-angiogenic medication was discontinued after 1 week, and the FAK-inhibitor was administered on a daily

basis thereafter. The experiment was ended and animals sacrificed when a significant degree of cancer-related morbidity was observed. (See Figure 24.)

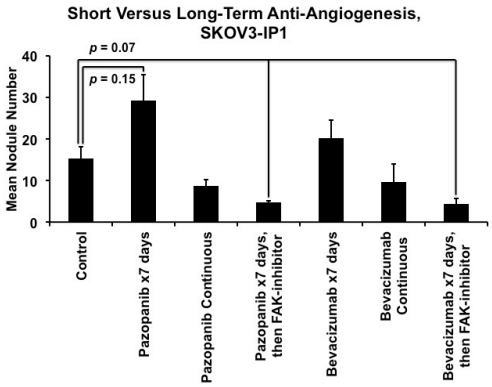
In the second experiment, the design detailed above was replicated using the cell line HeyA8. (See Figure 25.)

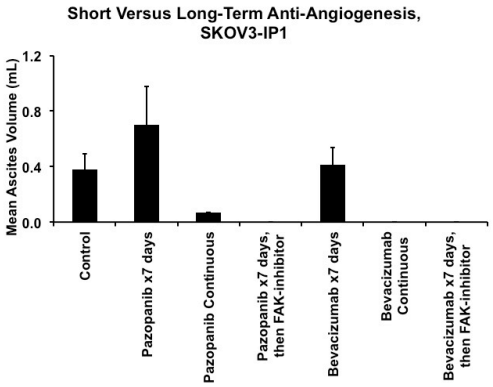
In the third experiment, nude mice were inoculated with SKOV3-IP1 and tumor was allowed to develop until palpable. One group was an untreated control, one group was given twice-weekly bevacizumab, one group was given daily pazopanib, and one group was given FAK-inhibitor. After one week of therapy, animals were sacrificed and tumor was harvested for analysis. Blood was also taken at that time for CBC. Tumor was analyzed by immunofluorescence to determine whether the platelet infiltration was altered either by the use of anti-angiogenic agents or the use of the FAK-inhibitor. (See Figure 26.)

In this first experiment, mice were inoculated with SKOV3-IP1 prior to the initiation of therapy, and therapy was as described above. The experiment was stopped because of the condition of the mice treated with only 7 days of pazopanib. Compared to the untreated control mice, animals treated with continuous pazopanib were noted to have a significant decrease in the aggregate mean tumor weight (0.95 +/- 0.22 g/mouse versus 0.26 +/- 0.06 g/mouse, $p < 0.05$) and a trend toward a significantly decreased number of tumor nodules (15.33 +/- 2.8 nodules/mouse versus 8.7 +/- 1.58 nodules/mouse, $p = 0.152$). By contrast, mice treated with only 7 days of pazopanib had increased aggregate mean tumor weight (0.95 +/- 0.22 g/mouse versus 1.39 +/- 0.27 g/mouse, $p =$

0.22) and number of nodules (15.33 +/- 2.8 nodules/mouse versus 29.11 +/- 6.36 nodules/mouse, $p = 0.065$) that trend toward statistical significance. Mice treated with short-term pazopanib followed by the FAK-inhibitor also had significantly decreased tumor weight compared to control (0.95 +/- 0.22 g/mouse versus 0.26 +/- 0.05 g/mouse, $p < 0.05$) and decreased number of nodules compared to control (15.33 +/- 2.8 nodules/mouse versus 4.87 +/- 0.50 nodules/mouse, $p = 0.065$).

In a similar fashion, compared to untreated control animals, mice treated with only 7 days of bevacizumab experienced non-significant increases in mean aggregate tumor weight and number of nodules. In contrast, mice treated with continuous bevacizumab were noted to have decreased aggregate mean tumor weight compared to control (0.95 +/- 0.22 g/mouse versus 0.29 +/- 0.06 g/mouse, $p < 0.05$). Similarly, animals treated with 7 days of bevacizumab followed by the FAK-inhibitor were noted to have decreased aggregate mean tumor weight compared to control (0.95 +/- 0.22 g/mouse versus 0.14 +/- 0.06 g/mouse, $p < 0.05$).



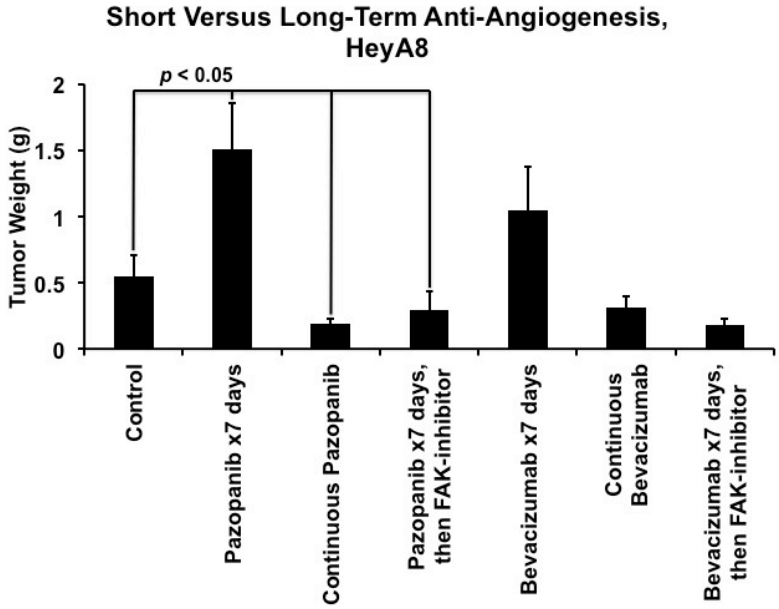


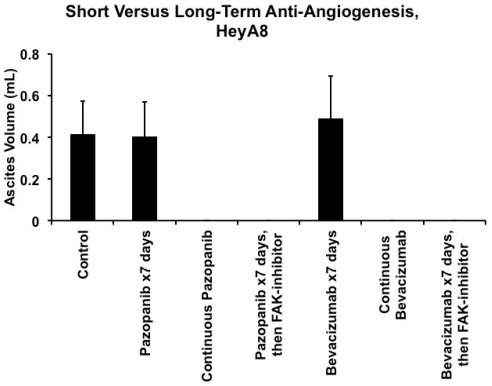
Group (SKOV3-IP1)	Mean Tumor Mass (g)	Standard Error (MTM)	Mean Nodule Number	Standard Error (MNM)	Mean Ascites Volume (mL)	Standard Error (MAV)
Control	0.95	0.22	15.33	2.80	0.38	0.11
Pazopanib x7 days	1.39	0.27	29.11	6.36	0.70	0.28
Pazopanib Continuous	0.26	0.06	8.70	1.58	0.07	0.00
Pazopanib x7 days, then FAK-inhibitor	0.26	0.05	4.67	0.50	0.00	0.00
Bevacizumab x7 days	1.05	0.23	20.25	4.19	0.41	0.13
Bevacizumab Continuous	0.29	0.06	9.63	4.26	0.00	0.00
Bevacizumab x7 days, then FAK-inhibitor	0.14	0.03	4.43	1.27	0.00	0.00

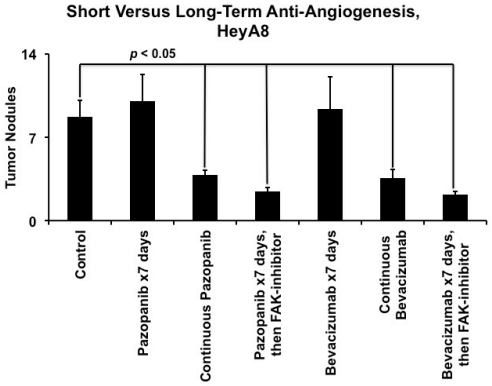
Figure 24: Short-term anti-angiogenic therapy accelerates tumor growth, and the effect is blocked by the FAK-inhibitor.

In the second experiment, mice were inoculated with HeyA8 prior to the initiation of therapy, and therapy was as described above. The experiment was stopped because of the condition of the mice treated with only 7 days of pazopanib. Compared to the untreated control mice, animals treated with continuous pazopanib were noted to have a significant decrease in the aggregate mean tumor weight (0.55 +/- 0.16 g/mouse versus 0.19 +/- 0.03 g/mouse, $p < 0.05$). By contrast, mice treated with only 7 days of pazopanib had increased aggregate mean tumor weight (0.55 +/- 0.16 g/mouse versus 1.51 +/- 0.35 g/mouse, $p < 0.05$). Mice treated with short-term pazopanib followed by the FAK-inhibitor also had decreased tumor weight compared to control (0.55 +/- 0.16 g/mouse versus 0.29 +/- 0.14 g/mouse, $p = 0.24$) that trended toward significance and was not statistically distinct from mice treated with continuous pazopanib ($p = 0.50$).

In a similar fashion, compared to untreated control animals, mice treated with only 7 days of bevacizumab experienced increases in mean aggregate tumor weight that trended toward significance (0.55 +/- 0.16 g/mouse versus 1.05 +/- 0.33 g/mouse, $p = 0.182$). In contrast, mice treated with continuous bevacizumab were noted to have decreased aggregate mean tumor weight compared to control (0.55 +/- 0.16 g/mouse versus 0.31 +/- 0.08 g/mouse, $p = 0.21$). Similarly, animals treated with 7 days of bevacizumab followed by the FAK-inhibitor were noted to have decreased aggregate mean tumor weight compared to control (0.55 +/- 0.16 g/mouse versus 0.18 +/- 0.05 g/mouse, $p = 0.060$).







Group (HeyA8)	Mean Tumor Mass (g)	Standard Error (MTM)	Mean Nodule Number	Standard Error (MNM)	Mean Ascites Volume (mL)	Standard Error (MAV)
Control	0.55	0.16	8.71	1.41	0.42	0.16
Pazopanib x7 days	1.51	0.35	10.00	2.24	0.40	0.16
Pazopanib Continuous	0.19	0.03	3.86	0.34	0.00	0.00
Pazopanib x7 days, then FAK-inhibitor	0.29	0.14	2.43	0.37	0.00	0.00
Bevacizumab x7 days	1.05	0.33	9.33	2.70	0.49	0.20
Bevacizumab Continuous	0.31	0.08	3.57	0.75	0.00	0.00
Bevacizumab x7 days, then FAK-inhibitor	0.18	0.05	2.17	0.31	0.00	0.00

Figure 25: Short-term anti-angiogenic therapy accelerates tumor growth, and the effect is blocked by the FAK-inhibitor.

In animals inoculated with SKOV3-IP1, after tumor was palpable, animals were treated for one week with nothing, bevacizumab, pazopanib, or the available FAK-inhibitor. At the end of the week, animals were sacrificed. Blood was taken for CBC. All groups had similar platelet levels except for the animals treated with bevacizumab. In this case, compared to control, bevacizumab-treated animals had an elevated platelet level compared to control that approached statistical significance (1399 +/- 18 cells/mcL versus 1746 +/- 91 cells/mcL, p = 0.07). Tumor was harvested and flash frozen after paraformaldehyde intra-vital fixation. Double-immune fluorescence staining of tumor sections were significant for both intravascular and intra-tumor platelet accumulation that was scored according to the number of discrete aggregates per 200x field. Compared to the control mice, those treated with bevacizumab had an increased number of platelet aggregates that reached statistical significance (3.88 +/- 0.17 aggregates/200x versus 14 +/- 0.55 aggregates/200x, $p < 0.05$). Compared to the control mice, those treated with pazopanib had an increased number of platelet aggregates that approached statistical significance (3.88 +/- 0.17 aggregates/200x versus 5.83 +/- 0.17 aggregates/200x, p = 0.06). In contrast, animals treated with FAK-inhibitor had a decreased number of platelet aggregates that did not reach statistical significance (3.88 +/- 0.17 aggregates/200x versus 2.93 +/- 0.19 aggregates/200x, p = 0.45).

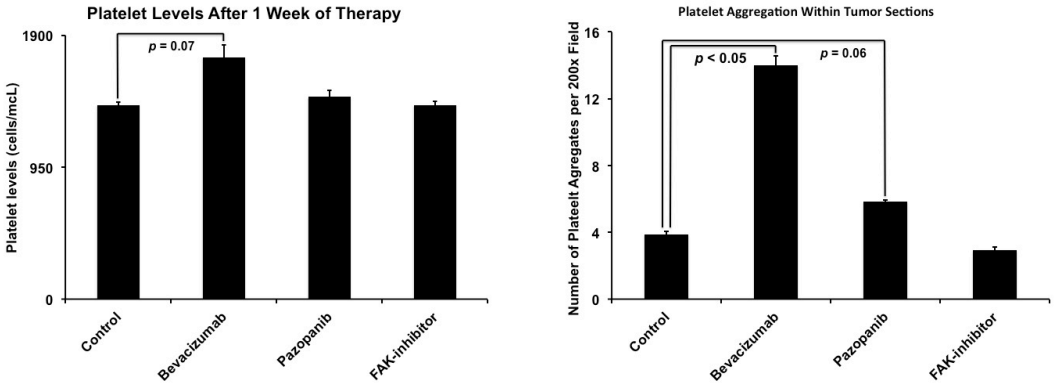


Figure 26: Short-term anti-angiogenic therapy causes increased platelet invasion into tumor that is blocked by the FAK-inhibitor.

Discussion

Over the past 30 years, significant time, effort, and money have been spent in an on-going fight to decrease mortality related to cancer. Aside from some notable victories, e.g. imatinib therapy for chronic myelogenous leukemia, progress has been incremental, and progress has been significantly less than that seen in other chronic diseases of aging. Of late, there has been significant focus placed on determining a more detailed understanding of the detailed molecular biology of cancer. This point of view may be reductionist, and it is at significant risk of failing to recognize both the dizzying heterogeneity of a cancer, but is also the myriad interactions of those heterogeneous tumor cells with otherwise normally functioning cells and systems of the human body. This work is not a rejection of pathway-level thinking, however the logic of the current work operates at the level of cellular systems and considers net effect at the level of population and ecology; we specifically consider the contribution of platelets to selection and promotion of clonal populations via mitogenicity and the augmentation of angiogenesis.

One of the primary reasons that cancer has proven difficult to treat, including in the case of ovarian cancer, is that we continue to lack adequate biomarkers to provide early identification of more readily treated disease. In the present work, we provide evidence to support the notion that platelet levels may be considered as part of a screening algorithm for early identification of malignancy. We correlate elevated platelet levels at the diagnosis of malignancy

with decreased interval to progression and decreased overall survival. We provide evidence that normalization of platelet levels reflects relative success of therapy, and we provide evidence that elevation of platelet levels during the post-therapy monitoring period may be useful as part of a monitoring program. Historic evidence would suggest that ovarian cancer patients who have normalized CA-125 and negative imaging at the conclusion of induction therapy continue to have up to a 50% rate of persistent disease. And, in the case of ovarian cancer, recent prospective data has raised concern that CA-125 monitoring after the conclusion of therapy may not improve cancer-related outcomes. Given, in the case of ovarian cancer, that CA-125 has proven to be of at best controversial utility in monitoring disease, and given that the present data is weakened by its retrospective collection, we would advocate for the inclusion of CBC's as part of a prospective monitoring program both during and after therapy in order to better identify patients with persistence of disease at the conclusion of therapy, but also in the hope of obtaining early identification of patients who may have a recurrence amenable to definitive therapy.

In the present work, we go beyond established literature on the effects of platelets on endothelial cells, and we show in multiple cell lines that platelets confer apoptosis resistance, increase rates of proliferation, and encourage the migration of tumor cells. Understanding that platelets are known to sequester and release multiple mitogens and regulators of angiogenesis, we were able to show via siRNA transfection and protein knock-down that at least a portion of the

observed effects on apoptosis were due to signaling through TGFBR1 and at least a portion of the proliferative effects were mediated through PDGFRA signaling. It is recognized that platelet activation results in the release of multiple mediators, and therefore we approached the question of therapy not from the perspective of attempting to block one or another pathway, but from the perspective of blocking platelet activation and release of the mediators in question.

A simplified schematic is offered in Figure 27. Briefly, the simplified model reflects a biology in which tumor is subjected to routine platelet trafficking as a result of characteristically haphazard and leaky vasculature. Platelets in the tumor are activated by various stimuli within the tumor, and this activation results in the release of mitogenic cytokines, including PDGFA and TGFB1, that lead to tumor proliferation, apoptosis resistance, and increased migration. We propose this process of co-opting the normal, wound-healing functions of platelets to be a routine part of tumor biology that contributes to the ability of tumor cells to survive in an adverse environment, to propagate in the body, and to metastasize.

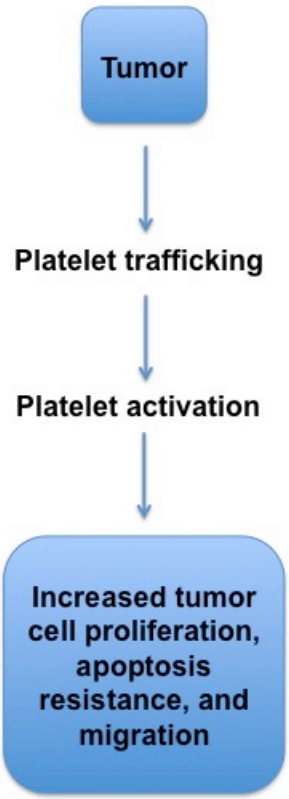


Figure 27: A simplified model of co-opted platelet function contributing to tumor cell proliferation, survival, and metastatic potential.

Recognizing that platelet activity is dependent on activation, we first demonstrated that platelet-mediated mitogenic functions, i.e. apoptosis resistance and proliferation, could be blocked simply by platelet fixation with paraformaldehyde. We were subsequently able to demonstrate that proliferative functions were able to be (at least partially) be blocked by co-culture in the context of aspirin and an inhibitor of FAK.

As part of the consideration of how platelets would interact with tumor as regulators of angiogenesis, we began or investigation *in vitro* with the use of an

induced hypoxic environment. We were able to demonstrate that hypoxia resulted in a 6-fold increase in ADP in the environment around tumor cells. It is important in consideration of this fact to recall that ADP is a powerful activator of platelets, and concentrations of ADP three orders of magnitude less than that seen in our experiments have been shown to activate platelets that have been treated with high doses of aspirin. Also, our work showed that platelets facilitate growth of tumor cells in the hypoxic environment in a manner visually identical to that seen in the normoxic environment, suggesting that platelet activity (that would be increased by ADP activation in a hypoxic micro-environment) may rescue cell populations from the stress imposed by hypoxia. It is important to recall that tumors have characteristically leaky vasculature, and our work demonstrated platelet transit into the bulk of tumor.

In order to test these hypotheses in a more typical biologic environment, we used orthotopic models of ovarian cancer in mice that are well known to our lab. In our first experiment, we showed that depletion of platelets using an anti-platelet antibody had anti-tumor effects by itself, and depletion of platelets increased the efficacy of cytotoxic chemotherapy. In the next experiment, we showed that platelet transfusion had the opposite effect, increasing tumor growth and decreasing the efficacy of cytotoxic chemotherapy. After that, we attempted to block the effects of platelet transfusion with aspirin. Two interesting observations emerged. The first was that aspirin by itself seemed to have minimal effect in this model; in fact, pre-treatment of transfused platelets with

aspirin only partially blocked their effect on the tumor. Within this context, it is important to recall that an adequate amount of ADP will activate platelets despite aspirin therapy, and so many of these platelets may well have been activated in the tumor despite aspirin therapy. The second observation was that platelet transfusion was associated with increased proliferation of tumor cells, decreased apoptosis of tumor cells, and an increased frequency of lumina identified in vessels suggesting vascular stabilization, normalization, and maturation. Again, these effects were incompletely blocked by aspirin pre-treatment of transfused platelets.

These observations, coupled with the sheer magnitude of angiogenic mediators housed by platelets, to consider whether platelets may be at least partially responsible for the poor performance of angiogenic drugs in clinical trials and for emerging evidence of resistance to anti-angiogenic agents that has been described in recent literature. In order to consider this possibility, we first established a model system *in vivo* using short versus continuous treatment with anti-angiogenic agents. This model consistently demonstrated an acceleration of growth after short-term treatment with anti-angiogenic drugs, however variances and inadequate mouse numbers to meet strict statistical significance in many cases limited the model. Despite this limitation, we were able to in two cell lines and using two anti-angiogenic agents demonstrate that the acceleration in tumor growth seen after short-term anti-angiogenic therapy could be blocked by the use of the FAK-inhibitor. Recalling that platelets deficient in FAK signaling have

diminished capacity to spread and activate, and recalling our *in vitro* findings with respect to the FAK-inhibitor blocking platelet-mediated proliferation, we sought to establish that the effects of the FAK-inhibitor on the observed tumor growth were at least partially attributable to platelet-specific effects. We were able to offer preliminary evidence that that platelet infiltration into tumor is significantly increased given seven days of hypoxia-inducing anti-angiogenic therapy, supporting the hypothesis that local platelet activation is increased.

In view of these findings, we propose the following model to account for the effects of hypoxia, both biological resulting from tumor growth and iatrogenic from the use of anti-angiogenic therapy, in Figure 28.

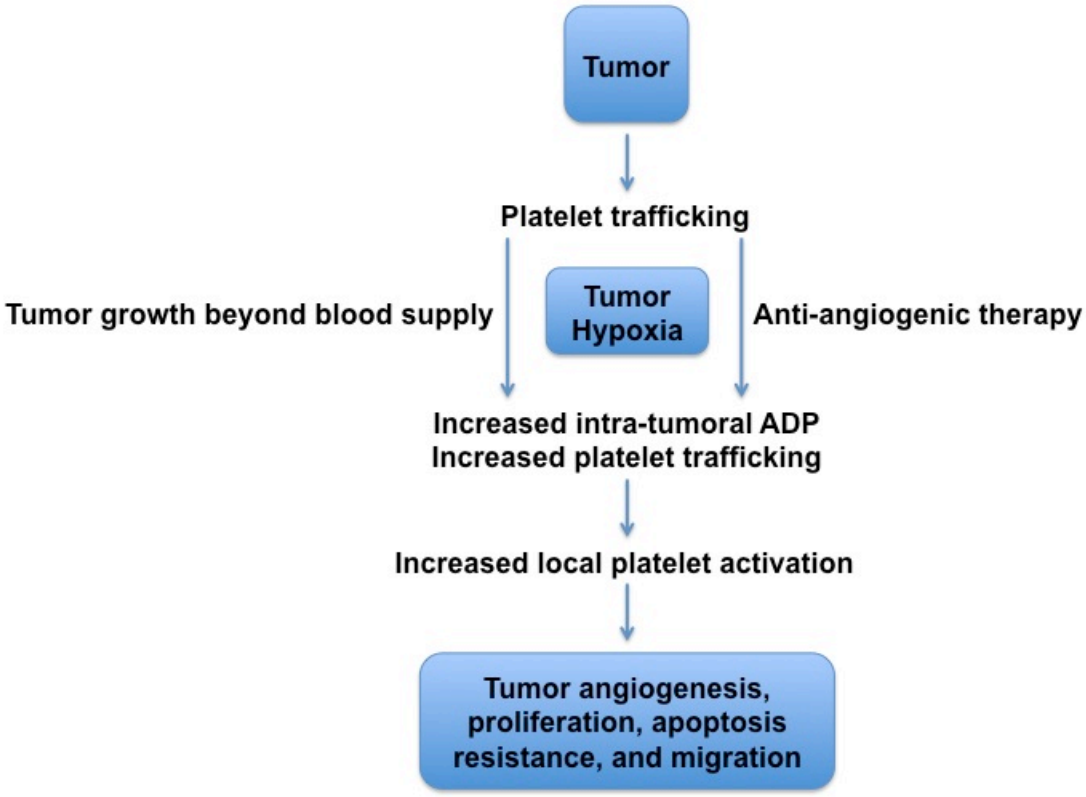


Figure 28. The effect of tumor hypoxia on platelet trafficking and activation.

In this model, we propose that hypoxia results in an increase in local ADP in the tumor microenvironment that increases the capacity of the tumor to activate platelets. At the same time, increased vascular irregularity permits increased incidental trafficking of platelets. In fact, our preliminary data suggests that platelet levels may systemically increase as a result of tumor hypoxia, a result supported by data that tumor IL-6, which is known to drive malignant thrombocytosis, is produced in response to hypoxia. Platelet activation results in improved tumor angiogenesis and neovascularization, along with the increased proliferation, decreased apoptosis, and increased migratory behavior of tumor

cells already considered. This entire process can be slowed, or even stopped, with the use of agents such as aspirin and FAK-inhibitors that block activation. It is noted that FAK-inhibitors have the added benefit of slowing platelet migration in response to fibrinogen.

Better understanding of the full spectra of factors, especially those normal and co-opted, regulating angiogenesis and mitogenic behavior will necessarily lead not only to more effective treatment of angiogenesis specifically, but it may also lead to a better understanding of the most optimal dosing of various, more common, chemotherapeutic regimens. And increased understanding of the behavior of platelets as a mediator of angiogenesis should cause a reconsideration of the degree of thrombocytopenia that constitutes a toxicity of chemotherapy and what degree of thrombocytopenia might be seen as a desirable treatment effect. Similarly, it is appropriate to consider the broad ramifications of treatment effects of more traditional agents, not just on tumor cells specifically, but on other cells in the micro-environment. In the same way that taxanes have been show to impact platelet function beyond direct tumor effect, we have shown that inhibition of FAK impacts platelet function and the interaction of platelets with tumor above and beyond well-known direct tumor effect. Future attempts to understand and treat cancer may demand a greater emphasis on a systemic, ecological understanding of cancer and its context within the body. In this work, we consider the effects of platelets directly on tumor and within the context of angiogenic regulation necessary for tumor growth. We

demonstrate previously unknown mitogenic effects of platelets on tumor cells, we show that platelets and tumor cells interact, and we show that platelet-driven modulation of angiogenesis contributes to therapeutic resistance.

The current data argue strongly for consideration of the routine use of combination therapy when an anti-angiogenic agent is considered. Specifically, when considering anti-angiogenic therapy, one should consider adding agents that have anti-activation effects on platelets. Many of these drugs are already available on the market, such as aspirin, clopidogrel, and lovenox. FAK-inhibitors, with the added benefit of limiting platelet migration, are entering clinical trials both as single agents and as combination therapy with anti-angiogenic agents. These data offer an additional biological rationale for this combination.

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Vita

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