Determining the role of PI3K-β and PI3K-δ in Canine Hemangiosarcoma and Human Angiosarcoma

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Abstract

Neoplastic disease arising from endothelial cells occurs spontaneously in humans (angiosarcoma, AS) and pet dogs (hemangiosarcoma, HSA) and in both species exhibits aggressive biologic behavior including early metastasis and chemotherapy resistance. While AS is a rare cancer, with 300-500 new diagnoses per year, HSA is far more common, affecting over 50,000 pet dogs yearly. Molecular and genetic studies have shown that both AS and HSA exhibit dysregulation of the PI3K/AKT/mTOR pathway driven by *PIK3CA* mutations and PTEN mutations/deletions. While the contribution of PI3K-α to pathway activation has been interrogated in AS/HSA, the influence of signaling mediated by the other PI3K class I catalytic isoforms (PI3K-β and δ) remains unknown. We determined that of the 4 canine and 2 human cell lines studied, all express PI3K-β and all but one of the human cell lines express PI3K-δ. This was unexpected as PI3K-δ is typically only expressed in cells of hematopoietic/lymphoid origin. The canine HSA cell lines exhibited constitutive pathway activation despite serum starvations and both the canine and human HSA/AS cell lines were sensitive to treatment with a pan-PI3K inhibitor, resulting in decreased cell proliferation. However, isoform specific inhibitors had little effect, suggesting that the tumor cells rely upon multiple PI3K isoforms for survival and proliferation. Knockdown of PI3K-β and δ expression in the Dal-4 and EFB canine HSA cell lines had no effect on cell proliferation or migration through trasnwells however, the EFB cell line exhibited a marked decrease in invasive capacity following *PIK3CB* knockdown. In conclusion, we determined that the PI3K/AKT/mTOR pathway has a role in driving sustained proliferation of HSA/AS cells, with multiple PI3K isoforms contributing to this effect. Additionally, the PI3K-β isoform may play a role in supporting cell invasion, a finding that warrants further exploration with respect to isoform specific biologic effects.

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Vanhaesebroeck, B., Stephens, L. & Hawkins, P. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol* **13**, 195-203, doi:10.1038/nrm3290 (2012)

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List of Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AS	Angiosarcoma
bp	Base pair
BSA	Bovine Serum Albumin
CLL	Chronic lymphocytic leukemia
CNAs	Copy number aberrations
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FL	Follicular lymphoma
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
HRP	Horseradish peroxidase
HSA	Hemangiosarcoma
KD	Knockdown
LDM-C	Low-dose metronomic cyclophosphamide
mL	Milliliter
mM	Millimolar
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex - 1
NEAA	Non-Essential Amino Acids
Nm	nanometers
NTC	Non-template control
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK-1	3-phosphoinositide-dependent protein kinase-1
PET	Polyethylene terephthalate
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline with Tween-20
µg	Microgram
µM	Micromolar

Chapter 1: Introduction

1.1: The PI3K-AKT-mTOR Pathway:

The phosphoinositide 3-kinase (PI3K) lipid kinase family is comprised of three classes, with Class I being the most important in cancer[1,2]. This class is further subdivided into Class IA including p110α, p110β, and p110δ, and Class IB which is the p110γ isoform; these are encoded by the *PIK3CA, PIK3CB, PIK3CD and PIK3CG* genes, respectively. Class IA PI3Ks are heterodimers of the catalytic p110 subunits and the regulatory p85 subunit, while p110γ is bound to the p101 or p87 regulatory subunits[1] (Figure 1.1). PI3K-α, β, and δ are typically activated by receptor tyrosine kinases (RTKs). In contrast, PI3Kγ and occasionally PI3K-β are activated by G-protein-coupled receptors (GPCRs)[3]. Additionally, PI3K-δ and PI3K-γ are typically only expressed in leukocytes, while the other two isoforms are expressed ubiqioutsly[1,3,4].

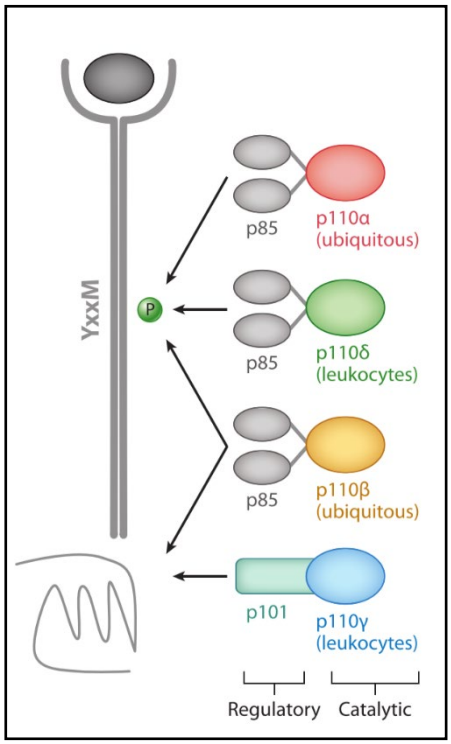


Figure 1.1: The Class I PI3K Isoforms. Illustration to demonstrate the differences between the Class IA and Class IB PI3Ks, both in regulation of the isoforms and their activation. Reprinted with permission from: Okkenhaug, K. Signaling by the phosphoinositide 3-kinase family in immune cells. *Annu Rev Immunol* **31**, 675-704, doi:10.1146/annurev-immunol-032712-095946 (2013).

The role of each PI3K isoform *in vivo* has been elucidated through mouse knockout experiments. Global knockout of the *PIK3CA* gene is embryonic lethal whereas mice with knockout of the *PIK3CB* gene die three days after birth[5] demonstrating the importance of these genes and respective isoforms on cellular homeostasis. In contrast,

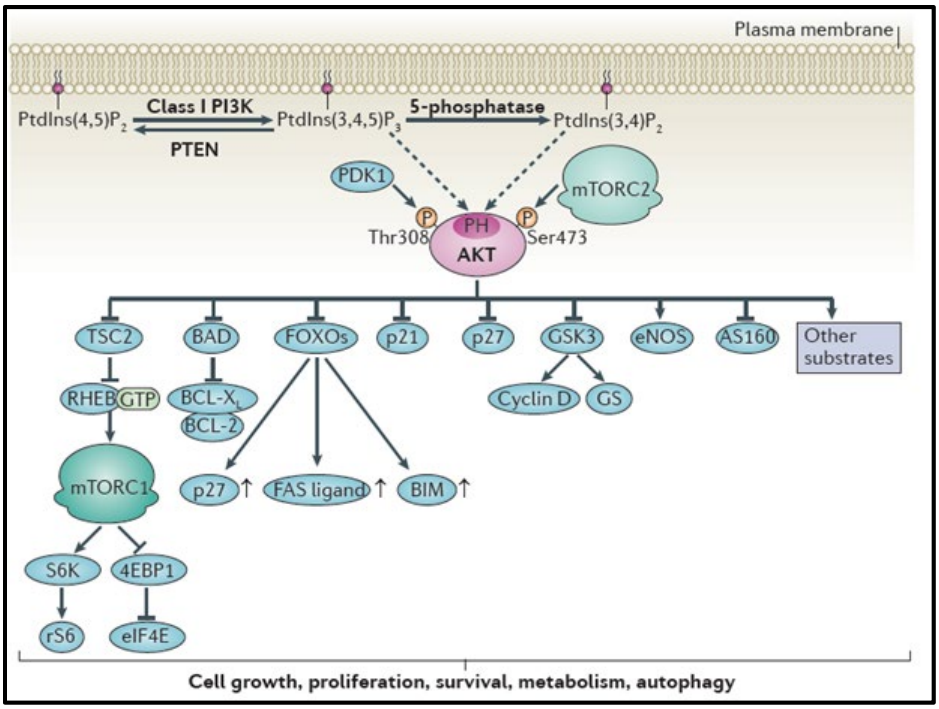


Figure 1.2: The PI3K/AKT/mTOR pathway. Schematic of the pathway describing downstream signaling of the PI3K pathway. Reprinted with permission from: Vanhaesebroeck, B., Stephens, L. & Hawkins, P. PI3K signalling: the path to discovery and understanding. Nat Rev Mol Cell Biol 13, 195-203, doi:10.1038/nrm3290 (2012).

knockout of *PIK3CD* leads to viable mice, although they have some functional deficits in lymphocytes such as a reduction in the number of mature B-cells and an absence of M1 macrophages[5]. Mice deficient in *PIK3CG* also have immunologic defects consisting primarily of fewer $CD8^+$ cytotoxic T-cells and apoptosis of thymus cells[5].

Tight regulation of the PI3K pathway in normal cells is essential to maintence of homeostasis (Figure 1.2). In the absence of an upstream signal, the regulatory subunits are bound to the p110 subunits, functionally inhibiting activation and downstream signaling[1]. Following RTK or GPCR stimulation, activated PI3K catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which in turn recruits several proteins to the cell surface, including PDK1 and AKT[1]. Phosphatase and tensin homolog (PTEN) negatively regulates this process by converting PIP3 back to PIP2, preventing downstream signal propogation[1].

PTEN is a tumor suppressor that is commonly mutated or deleted in many forms of cancer, resulting in loss of negative regulation and constitutive PI3K/AKT/mTOR signaling[1,6,7].

Downstream effects of AKT membrane recruitment include the activation of the serine/threonine kinase mTOR that in turn phosphorylates ribosomal protein S6 kinases (S6K) and eukaryotic translation initiation factor 4E (eIF4E), ultimately promoting protein translation and cell cycle progression[1,4]. A positive feedback loop between mTOR and AKT also forms, supporting further activation of this pathway[1,4]. AKT acts to phosphorylate another serine/threonine kinase, glycogen synthase kinase 3β (GSK3β) resulting in increased expression of cyclin D1 and Myc, the latter leading to upregulation of cyclin-dependent kinase 4 (CDK4)[1,4]. Two inhibitors of CDK, p27 and p21 are downregulated by AKT, resulting in enhanced cell cycling[1,4]. Downstream effectors of AKT can mediate suppression of apoptosis, for example, reducing the levels on p53 in the face of DNA damage or oncogenic stress[1,4].

Mutations and dysregulation in the PI3K/AKT/mTOR pathway have been implicated in several types of cancer, making it an important potential target for therapeutic intervention[1,3,7]. Signaling through this pathway regulates key processes in normal cells, including proliferation, and apoptosis[1,3,7]. Dysregulation can occur at several stages of the pathway. For example, deletion or mutation of the tumor suppressor gene *PTEN* is one of the most commonly documented alterations of this pathway[4]. Additionally, activating mutations in *PIK3CA* are found in several forms of cancer including sarcomas, colorectal, brain, gastric, and breast cancers[3,4]. There are multiple hotspot mutation sites in *PIK3CA* which are conserved across these tumor types[8]. Mutations of the AKT isoforms have also been documented in cancer with a relatively low incidence of 3-5%[9].

1.2: Angiosarcoma

Angiosarcoma (AS) is a malignant tumor that presumptively originates from endothelial cells lining lymphatics and blood vessels[10,11]. The primary form of AS has no known underlying cause and arises spontaneously anywhere in the body, with more common locations including skin of the head and neck, the liver and the heart[10]. Secondary As typically arises in breast cancer patients following radiation therapy, and particularly in those patients who develop chronic lymphoedema[10-12]. Indeed, radiotherapy for any tumor type increases the risk of developing AS.

The incidence of AS is extremely low (approximately 300-500 new diagnoses per year), representing only 2% of all soft tissue sarcomas, making it extremely challenging. to recruit enough patients for prospective clinical trials[10,11]. For example, in a retrospective study at the University of Wisconsin only 81 patients were seen over a 25-year period[11]. Another currently enrolling study in patients with non-resectable AS tumors is studying the combination of paclitaxel and anti-PDL1, and is recruiting 32 patients, and is anticipated to take five years to complete[13]. While there are no established standards of care for treating AS, most patients will undergo surgical excision when possible followed by chemotherapy, typically with a doxorubicin (DOX) based protocol and/or local radiation therapy. The prognosis for most patients with AS is relatively poor; in the retrospective cohort mentioned above the overall five-year survival rate was only 40% with negative prognostic indicators including metastasis at diagnosis, visceral/deep soft tissue location, tumor size, and necrosis as poor prognostic factors[11]. For those patients that achieve remission, at least 50% will develop a metastatic relapse, which is typically refractory to therapy resulting in death[14].

Little data exist regarding the molecular and genetic drivers of AS. In the setting of secondary AS, one study found *MYC* amplification in 55% of tumors while another group found that all tumors analyzed had increased expression levels of *Myc*[15,16].

However, dysregulation of Myc is far less common in primary AS samples[15,16]. Another study of 62 AS tumors (30 primary, 32 secondary) specifically focused on alterations of the PI3K/AKT/mTOR pathway and p53 mutation[14]. Only two of the tumors, both secondary AS, carried *TP53* mutation and this occurred alongside *Myc* amplification[14]. While *PTEN* loss was not identified, 39% of the tumors had a truncating frame-shift mutation in exon 10 of *PIK3CA* and 42% of the tumors were positive for pS6K and/or p-4eBP1, downstream targets of mTORC1[14]. A larger study of 120 AS patients found similar alterations in secondary AS cases as published in other studies such as *Myc* amplification and *PLCG1/PTPRB* mutations[17]. Using paired-end RNA sequencing they were able to identify a small subset of young AS patients with *CIC* gene rearrangements[17]. They also showed a few cases (7%) of primary AS exhibiting *Myc* amplification[17]. This study, however did not investigate tumors for the presence of mutations in *TP53, RAS,* or *PIK3CA.*

Other research has focused on how the inhibition of key molecular pathways influences the progression of AS. A mouse model of AS has been developed by selectively deleting *Trp53, PTEN*, and *Ptpn12* in endothelial cells, resulting in multi-focal development of AS[18]. When these mice were treated with the combination of a MEK inhibitor, trametinib, and an mTOR inhibitor, rapamycin, the tumors grew at a significantly slower rate, with individual agents having a less pronounced effect[18]. Most of the mice treated with both inhibitors survived to 200 days, while individual inhibitor treatment resulted in early relapse and death[18]. This suggests that the combined inhibition of these pathways may lead to improved outcomes in AS patients. Another study investigated the potential role of 3-phosphoinositide-dependent protein kinase-1 (PDK-1), a kinase that phosphorylates AKT, as a target in AS tumors. This study was conducted *in vitro* only but found that inhibition of PDK-1 led to a decrease in the phosphorylation of downstream proteins such as S6 kinase and eukaryotic translation

initiation factor 4E-binding protein 1 (4E-BP1), as well as a decrease in colony formation in soft agar[19]. More research in use of inhibitors of this pathway *in vivo* is needed to determine if it is a viable candidate for AS patients, and to determine potential toxicities of these treatment options.

Due to the rarity of AS, there are only a handful of cell lines available for use *in vitro* and in mouse xenograft studies. The mouse model described above is one of the first described, and while it does lead to AS development, the mice also form other types of tumors[18]. Another recent development has been the production of a zebrafish model of AS by deleting PTEN in embryos, leading to spontaneous AS formation[20]. This model may be useful for large scale drug screening to determine what targets are viable in the setting of PTEN loss.

1.3: Canine Hemangiosarcoma

Hemangiosarcoma (HSA) is a tumor with several clinical, molecular and genetic features similar to AS that arises spontaneously in dogs. As with AS, it is thought to be derived from endothelial cell precursors; however, it occurs far more frequently in the dog population, with an estimated 50,000 new cases diagnosed annually[21]. HSA can develop in the spleen, liver, heart, muscle, retroperitoneal space, or subcutaneous space with some dogs exhibiting multi-organ involvement at diagnosis[22,23]. In nearly all cases microscopic and/or macroscopic metastasis is present at the time of diagnosis necessitating the use of adjuvant therapy (radiation, chemotherapy) post tumor resection[21]. Despite aggressive treatment with surgery and DOX chemotherapy, median survival times for most affected dogs range from 6-8 months with only 10-15% of dogs living one-year post diagnosis[21,23].

Several clinical trials of novel drug combinations and treatment options in dogs with HSA have been completed in the past five years. Two independent retrospective studies of dogs with splenic HSA analyzed the potential benefit of low-dose metronomic

cyclophosphamide (LDM-C) in addition to splenectomy and DOX based therapy[24,25]. Unfortunately, neither study found an increase in progression-free survival over DOX alone[24,25]. A prospective clinical trial of dogs with splenic HSA studied the use of the multitargeted kinase inhibitor toceranib (Palladia) following splenectomy and adjuvant DOX treatment, but again there was no difference in median disease free interval and median survival time for all dogs in the study[26]. More recently, a bispecific therapy that targets EGFR and uPAR (eBAT) and was shown to lead to death of AS and HSA cells *in vitro*[27]. Treatment of affected dogs with eBAT post surgery increased the median survival time to 10 months time compared to standard treatment alone[27].

Over the past decade several studies have been undertaken to define the molecular and genetic drivers of HSA. In one study, 75 tumors were analyzed using a microarray to identify DNA copy number aberrations (CNAs)[28]. The cohort exhibited limited global genomic instability and CNAs were predominantly of low amplitude with no distinct hallmark aberrations. However, CNAs involving CDKN2A, VEGFA, and the SKI oncogene were identified as potential driver aberrations of HSA development[28]. Whole exome sequencing on 20 HSA samples derive from the spleen identified somatic mutations in PIK3CA (45%), TP53 (35%), PTEN (10%), and PLCG1 (5%) (Figure 1.3). The mutation in PLCG1 was identical to that observed in human visceral AS[21].

Several studies such as the one described above have converged on the PI3K/AKT/mTOR pathway as a potential target for therapeutic intervention. An investigation of canine HSA cell lines showed constitutive activation of this pathway, even in the context of serum starvation, highlighting the crucial role of this pathway to the survival of these cells[29]. Another study has found the presence of a C-terminus truncating mutation of PTEN that leads to a dysfunctional protein in a subset of tumor samples[30]. An *in vitro* study of a PI3K/mTOR inhibitor VDC-597 used in HSA cell lines

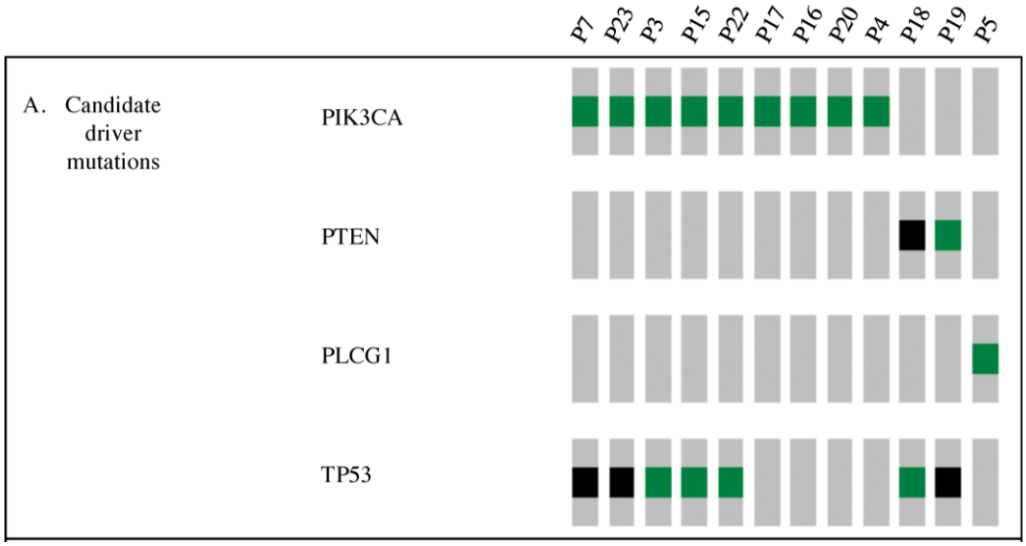


Figure 1.3: Candidate driver mutations of HSA Analysis of 20 splenic HSA cases identified *PIK3CA, PTEN, PLCG1*, and *TP53* mutations. Adapted with permission from Wang, G. *et al.* Actionable mutations in canine hemangiosarcoma. *PLoS One* **12**, e0188667, doi:10.1371/journal.pone.0188667 (2017). Changes include cropping of figure.

found that this small molecule inhibitor decreased cellular proliferation, migration, and wound healing, as well as inhibiting downstream components of the pathway[23]. A clinical trial to determine the efficacy of this drug *in vivo* is currently enrolling in dogs with splenic HSA at the Flint Animal Cancer Center at Colorado State University. Collectively, these studies show the importance of the PI3K/AKT/mTOR pathway in HSA and indicate the need for further study of how the dysregulation of this pathway can be leveraged to better patient outcomes.

1.4: PI3K/AKT/mTOR pathway inhibition:

Given the key role of PI3K/AKT dysregulation in cancer, there have been substantial efforts directed at modulating this pathway. One of the earliest examples is wortmannin, which was isolated from *Penicillium wortmannin* in 1957 and originally studied as an anti-inflammatory drug[31,32]. In1994 it was found that wortmannin actually functions as a noncompetitive, irreversible inhibitor of all three classes of PI3K at relatively low nanomolar concentrations[33]. While wortmannin has activity against several

different cancer cell lines,[34,35] it has poor oral bioavailability and induces liver toxicity, precluding its use in vivo[36]. Synthetic derivatives of wortmannin with broad activity against PI3K isoforms such as LY294002 and PX-866 have been developed to decrease toxicity and increase bioavailability[36]. However, their use *in vivo* is precluded by various toxicities, including hyperglycemia[36,37]. Despite promising activity *in vitro*, most pan-PI3K inhibitors have not shown tremendous efficacy *in vivo*, and toxicities have been problematic preventing long term use[38].

Effort has also been made to target other components of the pathway. The most well-known inhibitor of mTOR is rapamycin which was isolated from *Streptomyces hygroscopicus* in 1972, and was found to have antibacterial, antifungal, and immunosuppressive effects, and its main use was to prevent rejection in kidney transplant patients[39]. It was initially tested for its anti-cancer activity and its target, mTOR was discovered and named for the compound, and its role in cell cycle regulation was elucidated[39]. The poor bioavailability of rapamycin has sparked the development of several rapalogs like temsirolimus and everolimus both of which have been approved for use in renal cell carcinoma, as well as other forms of cancer[40]. AKT has also been a target of inhibitor development, most notably MK2206 is a pan-AKT inhibitor which has completed several phase I and II trials, as well as more recent focus on treatment options that involve combining MK2206 with other treatment options such as paclitaxel and other parts of the PI3K pathway[41].

Isoform selective inhibitors have been developed to reduce toxicity by through selective inhibition of PI3K isoforms. The PI3K-δ specific inhibitor Idelalisib was the first PI3K inhibitor approved by the FDA and is currently used to treat chronic lymphocytic leukemia (CLL)[42]. Two additional inhibitors have since been approved: Copanlisib which targets PI3K-α and δ and is used in follicular lymphoma (FL), and Duvelisib which targets PI3K-δ and γ and is used in CLL and FL patients[43,44].

As *PIK3CA* mutations are relatively common across several different types of tumors, the use of PI3K-α specific inhibitors has been of interest. There are several ongoing Phase I/II/III clinical trials of these agents across a variety of cancers[6,45]. Unfortunately, toxicity, particularly hyperglycemia, has continued to remain a significant obstacle[45,46]. Furthermore, the PI3K- α inhibitors have not been particularly effective in people, even in the setting of known driver mutations. More recently, PI3K-β selective inhibitors have become of interest, and a few are in Phase I/II clinical trials, and early results of some of these trials look promising[47-49]. An initial Phase I dose-finding study of GSK2636771 in 65 patients with solid tumors reported one partial response and 21 patients with stable disease, however, there were no complete responses observed in this cohort[49]. Currently enrolling studies include GSK2636771 as monotherapy in the MATCH study, as well as several combination studies in patients with melanoma, castration-resistant prostate cancer, and advanced gastric adenocarconima.

1.5: Summary:

Using dogs with spontaneous HSA to bridge the gap between drug development, *in vitro* testing, mouse models, and successful clinical trials in people with AS is appealing for several reasons. The large population of dogs diagnosed with HSA every year provides substantial numbers of patients to recruit for clinical trials, and owners are often highly motivated to enroll their pets in such trials, both for their benefit and to help develop better therapies for their human counterparts. Additionally, unlike traditional mouse models that are typically used to study novel therapies, canine patients have spontaneous tumors and possess an intact immune system, more closely recapitulating the molecular environment of the spontaneously occurring human counterparts.

Human AS and canine HSA tumors have many similarities including clinical presentation, histopathologic features, development of drug resistance metastatic disease, and PI3K/AKT/mTOR pathway activation. To most effectively leverage PI3K

pathway modulation, it is first necessary to interrogate the expression and function of the class I PI3K catalytic isoforms in HSA/AS across both species.

Chapter 2: Material and Methods

2.1: Cell Culture:

Three of the canine HSA cell lines, Dal-4, EFB, and DD-1 were obtained from Kerafast; these originated from the laboratory of Jaime F. Modiano, VMD, PhD, University Minnesota, Twin Cities. The fourth HSA cell line DEN-HSA was obtained from Dr. Thamm at Colorado State University. These cells were all cultured in Ham's F-12 Nutrient Mix supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamax, 1% NEAA, 1% HEPES, and 1% Sodium Pyruvate. The AS-M.5 cell line was obtained from the laboratory of C.J. Kirkpatrick, MD, PhD, DSc, Johannes Gutenberg-University, Mainz, Germany and maintained in Endothelial Cell Growth Medium MV supplemented with 1% Penicillin/Streptomycin (PromoCell). HAEND cell line was obtained from the Coriell Institute for Medical Research and maintained in RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamax, 1% NEAA, 1% HEPES, and 1% Sodium Pyruvate. The 293-T cells were maintained in DMEM containing 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamax, 1% NEAA, 1%HEPES, and 1% Sodium Pyruvate. The Jurkat human T-cell Leukemia line was obtained from ATCC and cultured in DMEM medium supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamax, 1% NEAA, 1% HEPES. All cells were maintained at 37°C in 5% CO_2.

2.2: Viral Vectors and Transduction:

Plasmids containing hairpins targeting the canine PIK3CB or PIK3CD genes, as well as a scramble control that does not target gene in the canine genome, were ligated into the plKO.1 TRC Cloning Vector purchased from Addgene (Plasmid #10878) and then transformed into *E. coli*. Hairpin sequences are shown below in 2.1. Colonies were then screened for the insert using sanger sequencing and those containing the hairpin transcript were purified and the plasmid was then used to produce virus using 293-T

cells and the TransIT®-LT1 Transfection Reagent (Mirus #2300). Media from these cells was collected, the virus was concentrated and used to transduce Dal-4 and EFB cells. Cells that incorporated the plasmid were then selected by puromycin until a control plate of cells died (typically 3-5 days after puromycin was added). Monoclonal cell lines were produced using selective dilution of cells at a concentration of 5 cells/well of a 96 well plate. Knockdown was then confirmed using qRT-PCR and western blotting.

Table 2.1: shRNA Hairpin Sequences

Gene Target	**Forward (5'>3')**
SCR	CCGGTTGCGCATCGATGGCCAACATCTCGAGATGTTGGCCATCGATGCGCAATTTTTG
PIK3CB	CCGGAAGACTCTGTGATGTCAGACCCTCGAGGGTCTGACATCACAGAGTCTTTTTTTG
PIK3CB	CCGGAATGATTATGTGTTGCAAGTCCTCGAGGACTTGCAACACATAATCATTTTTTTG
PIK3CD	CCGGAAGCTTGTCAACTCACAGATCCTCGAGGATCTGTGAGTTGACAAGCTTTTTTTG
PIK3CD	CCGGAATGAGACGCTGTGCAAGACGCTCGAGCGTCTTGCACAGCGTCTCATTTTTTTG
Gene Target	**Reverse (5'>3')**
SCR	AATTCAAAAATTGCGCATCGATGGCCAACATCTCGAGATGTTGGCCATCGATGCGCAA
PIK3CB	AATTCAAAAAAAGACTCTGTGATGTCAGACCCTCGAGGGTCTGACATCACAGAGTCTT
PIK3CB	AATTCAAAAAAATGATTATGTGTTGCAAGTCCTCGAGGACTTGCAACACATAATCATT
PIK3CD	AATTCAAAAAAAGCTTGTCAACTCACAGATCCTCGAGGATCTGTGAGTTGACAAGCTT
PIK3CD	AATTCAAAAAAATGAGACGCTGTGCAAGACGCTCGAGCGTCTTGCACAGCGTCTCATT

2.3: PCR and qRT-PCR:

Cells were collected and pelleted when they reached 75%-80% confluency. Pellets were then washed once in PBS before being lysed in 1mL of Trizol reagent (Invitrogen). RNA was extracted following manufacturers specifications[50]. The RNA concentration of each sample was analyzed with a Nanodrop spectrophotometer (Themo Scientific) and 2µg of RNA was used to produce cDNA by reverse transcription

using SuperScript IV Reverse Transcriptase (Thermo Scientific). PCR of canine and human samples was conducted with unique primers for each species and for the individual PI3K isoforms. Primer sequences used are listed in table 2.2 below, and PCR reactions were conducted using DreamTaq DNA Polymerase (Thermo Scientific). The PCR reactions were then run on a 2% agarose gel with ethidium bromide for approximately 30 minutes at 80V. The gels were then viewed and imaged using a BioRad ChemiDoc. qRT-PCR was carried out using the same primer sets listed in table 2.2 and was preformed using SyberGreen Master Mix (ABI) and the reactions were run on an ABI QuantStudio 3 Real-Time PCR System. Analysis was carried out using the double delta Ct method using GAPDH as a housekeeping gene[51].

Table 2.2: qRT-PCR and PCR Primers

Species/ Gene	**Forward (5'>3')**	**Reverse (5'>3')**
Canine/ PIK3CB	GTGACCTGCCACGAATGGCTCG	GTATTTACCCATGCCACAGG
Canine/ PIK3CD	CGCCGGGACCGACAGATAAG	GCAACAGGAAGTCAACAGCC
Human/ PIK3CB	TGGGAGGAGATCCATTACAGC	CACCTCGACTCTTATCGAAGGG
Human/ PIK3CD	TAACCCCAACACGGATAGCG	CTGCTCCTCCTCGGTGACAT
Hu/K9 GAPDH	GCAATGCCTCCTGCACCACCAACTG	GTTCAGCTCAGGGATGACCTTG

2.4: Western Blotting:

Cells were pelleted when they reached 75%-80% confluency and washed once in 1X PBS. Pellets were then lysed in lysis buffer containing 20mM Tris-HCl pH 8.0, 137mM NaCl, 10mM EDTA, 10% glycerol, 1% IGEPAL CA-630, 1µg/mL Aprotinin, 1µg/mL Leupeptin, 1µg/mL Pepstatin A, 1mM PMSF, 1mM Sodium Otrhanovandate, and 10mM Sodium Fluoride. Lysates were then rocked at 4°C for 1hr before being spun at 14,000rpm for 15min at 4°C. Finally, the protein concentration was quantified using a Bradford assay (Bio-Rad Protein Assay Dye). Lysates were resolved in denaturing

acrylamide gels with SDS-PAGE running buffer using the BioRad Mini-Protean System before being transferred to PVDF membranes. Membranes were then blocked in either 3% non-fat dry milk or 5% BSA in TBS-T (used for phospho-site and PI3K-δ blots). The primary antibodies used were: PI3K-β – CST#3011, PI3K-δ – Abcam#1678, p-AKT (Ser473) – CST#4060, AKT – BD Biosciences #610861, ribosomal p-S6 – CST#2211, ribosomal S6 – CST#2217, β-Actin – CST#3700, Vinculin – CST#4650 diluted 1:1000 in 3% non-fat dry milk or 5% BSA in TBS-T and incubated overnight at 4°C on a rocker. Blots were washed three times with TBS-T for 10 mins and then secondary antibodies were added for 1 hour, either Goat-anti-Mouse or Goat-anti-Rabbit conjugated with HRP (CST#7076 or #7074) diluted 1:20,000 in 3% non-fat dry milk or 5% BSA in TBS-T. Membranes were again washed three times with TBS-T and then placed in SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific) for 3 mins before using radiographic film to develop the blots for various time points.

2.5: Proliferation Assays:

Cell proliferation was analyzed using the CyQUANT Proliferation Assay (Molecular Probes). Cells were plated at a density of 1000 cells/well in 90μL of media in a 96 well plate, and each condition tested was plated in triplicate, as well as control wells and wells containing media only to control for background signal. Where indicated, 24 hours after plating, various drug treatments were added to the cells in triplicate wells. Small molecule inhibitors were purchased as lyophilized powders and reconstituted in DMSO at a concentration of 10mM and then diluted to various mM concentrations in DMSO. BKM-120 was purchased from AdipoGen Life Sciences and targets all class I PI3K isoforms, MLN-117 (PI3K-α inhibitor) and GSK2636771 (PI3K-β inhibitor) were purchased from Selleck Chemicals, and RV-1001 (PI3K-δ inhibitor) was obtained from Rhizen Pharmaceuticals where the inhibitor was developed. These stocks were then

diluted 1:100 into cell culture media corresponding to the cell line being treated, and 10μL of the dilution was added to each well, leading to a final dilution of 1:1000 in each well. The plates were then incubated for the indicated time points and then the media was removed by suction and the plates were stored at -80°C for at least 24 hours prior to analysis. The plates were then allowed to thaw at room temperature and CyQUANT reagent was added to the wells according to the manufacturer's specifications[52]. Fluorescence was read with an excitation of 480nm and emission of 520nm.

2.6: Transwell Migration and Invasion Assays:

Migration assays were conducted by placing transwell cell culture inserts (PET membrane with 8.0 μm pores) into 24 well plates. Cells were suspended in serum free media in the top well at a density of 10,000 cells/well, and each cell line was plated in duplicate. Media containing 10% FBS was placed in the lower well to act as a chemoattractant for the cells to migrate through the transwell membrane. Plates were then incubated for 24 hours before the media was removed from the plates and wells and the transwells were then stained with crystal violet solution for five minutes and rinsed 3X with PBS. To quantify migration, the number of cells in ten high power (20X) fields were counted and averaged. Invasion assays were completed in much the same way, but prior to adding cells to the upper chamber the transwells were coated with 100μL of Matrigel that was diluted 1:100 into serum free media. Cells were then plated in duplicate at a density of 100,000 cells/well in serum free media, and media containing 20% FBS was placed in the bottom chamber. The transwells were then incubated for 48 hours before they were stained and counted.

2.7: Statistical Analysis:

For CyQUANT proliferation, wells were plated in triplicate for each experiment, then averaged. The average results of three experiments were then averaged and a standard error of the mean was calculated and plotted as error bars. Statistical

significance was determined by a Kruskal-Wallis one-way ANOVA test. If the overall ANOVA showed significance, then Tukey's test was performed to determine significance between individual groups. For transwell migration and invasion assays, each cell line was plated in duplicate and the ten high powered fields counted were averaged. The experiment was then repeated a total of three times and the average number of cells/field of all six wells were then averaged and a standard error of the mean was calculated and plotted as an error bar. Statistical significance was determined by a Kruskal-Wallis one-way ANOVA test. If the overall ANOVA showed significance, then Tukey's test was performed to determine significance between individual groups. All analysis was performed using GraphPad Prism v.8.1.2.

Chapter 3: Results

3.1: Analysis of the PI3K isoform expression in HSA/AS Cell Lines:

To determine the presence of the PI3K isoforms in each of the HSA/AS cell lines used in this study we utilized standard PCR and western blotting. As expression of PI3K-α is ubiquitous and there is already a large body of literature evaluating the biology of this isoform in normal and neoplastic cells and PI3K-γ dysregulation appears to be restricted to hematopoietic cancers, we elected to focus on the contributions of PI3K-β and δ in HSA/AS[53]. PI3K-δ dysregulation was of interest to us because even though expression of this isoform is also typically restricted to hematopoietic cancers, there are several reports of its aberrant expression contributing to tumorigenesis in other forms of cancer such as breast cancer and colorectal cancer[54,55]. As shown in Figure 3.1, all four canine HSA cell lines (Dal-4, EFB, DD-1, and Den) and the two human AS lines (AS-M.5 and HAEND) expressed PI3K-β. Surprisingly, PI3K-δ was also expressed in all but one of the cell lines analyzed; the human HAEND cell line was positive by PCR analysis, but protein could not be detected by western blotting.

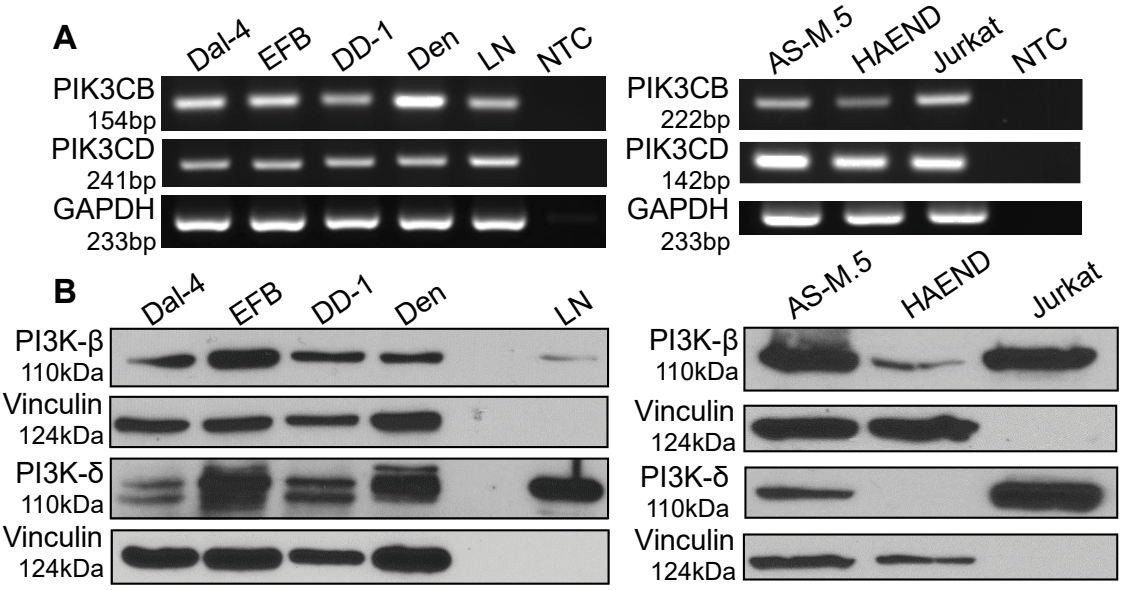


Figure3.1: Analysis of the PI3K Isoforms in HSA/AS Cell Lines. A) Primers specific to each species and isoform used in PCR reactions with cDNA produced from each of the six cell lines analyzed. B) Western blots of the cell lysates in all six cell lines with antibodies specific to each PI3K isoform. For both figures, canine lymph node, designated LN and the human T-cell lymphoma cell line, Jurkat were used as positive controls. NTC = non-template control and represents a negative control where water was used in place of cDNA. Note that is documented that lymph node tissue and Jurkat cells do not express vinculin at high levels.

3.2: PI3K/AKT/mTOR pathway activation in HSA/AS.

We next wanted to determine whether PI3K/AKT/mTOR pathway dysregulation was present in HSA/AS. Given that PTEN is a tumor suppressor and negative regulator of this pathway, we evaluated protein expression and found that all of the canine and human cell lines were positive by western blotting (Figure 3.2 A). Therefore, pathway activation through loss of PTEN expression does not appear to be a common event in HSA/AS. Next, we determined the phosphorylation status of AKT at the Ser473 site, which is indicative of a fully activated form of AKT and only occurs when AKT is recruited to the membrane by PIP3 formation following PI3K activation[56]. We also analyzed the samples for phosphorylation status of ribosomal S6, which is downstream of mTOR-1. To determine the influence of growth factors in the tissue culture medium, we serum starved cells for 0, 2, or 6 hours prior to collection and subsequent western blotting. As

shown in Figure 3.2 B, all cell lines evaluated demonstrated continued AKT phosphorylation despite serum starvation. Additionally, although ribosomal S6 demonstrated a slight decrease in phosphorylation over time, activation was still evident at the 6-hour time point. An additional control for this experiment to show that serum starvation is effective to reduce activation of the PI3K pathway, would be to include primary endothelial cells which have previously been shown to have reduced AKT and GSK-3β phosphorylation[57].

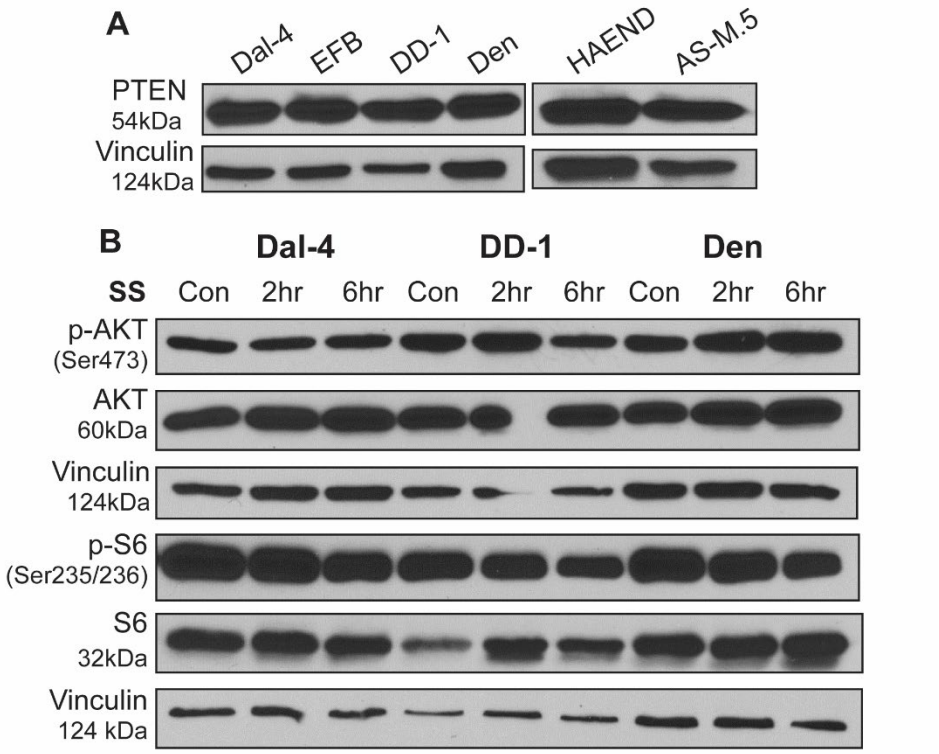
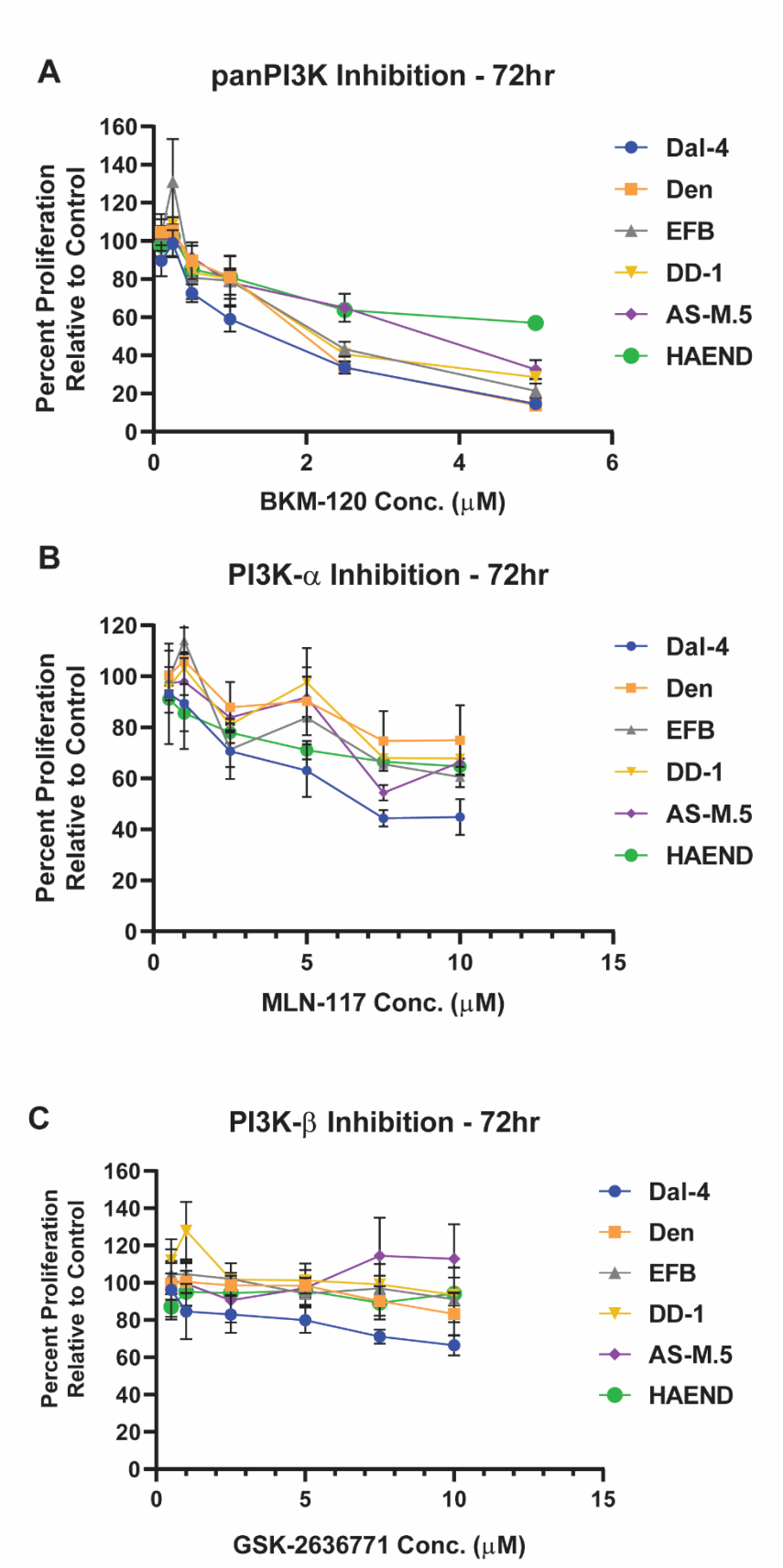


Figure 3.2: PI3K/AKT/mTOR pathway analysis: A) Western blotting to determine presence of PTEN in the six cell lines used throughout this study. B) Cell pellets were collected following 0, 2, or 6 hours of serum starvation (SS) and then phosphorylation status of AKT and ribosomal S6 was determined by western blotting.

3.3: PI3K inhibition in AS/HSA Cells:

To begin study the role of the PI3K-α, β, and δ isoforms in HSA/AS, we utilized commercially available small molecule inhibitors including a pan-PI3K inhibitor, BKM-

120, which inhibits all four class I PI3K isoforms. This was developed by Novartis Pharmaceuticals which when assessed in a kinase panel was shown to have activity to the PI3K class I isoforms at 50-300nM and activity against class III and IV PI3Ks at 2->5μM and more than 25μM to inhibit other kinases in the panel[58]. MLN-117 was used to specifically inhibit PI3K-α, and was initially developed by Intellikine and is now owned by Takeda[59]. It has been shown to have a 100-fold greater activity on PI3K-α than the other PI3K isoforms and a kinase panel[59]. The PI3K-β inhibitor GSK2636771 was developed by Glaxo-Smith-Kline and has a >900 fold selectivity over PI3K-α and γ and >10 fold selectivity over PI3K-δ[49]. A PI3K-δ specific inhibitor, RV-1001, was developed by Rhizen Pharmaceuticals and has exhibited activity against PI3K-δ at 39nM and a 20-250 fold selectivity over the other class I PI3K isoforms[60]. These inhibitors were obtained from various sources which are listed above in the materials and methods section. BKM-120 is a pan-PI3K inhibitor that was developed by Novartis and is fo We found that all lines were sensitive to pan-PI3K inhibition, with all but one of the cell lines reaching an IC50 below 5μM (Figure 3.3 A). Interestingly, with the exception of the DAL-4 line treated with MLN-117, none of the isoform specific inhibitors had substantial effects on cell proliferation even at concentrations as high as 10μM (Figure 3.3 B-D).


A
panPI3K Inhibition - 72hr
Percent Proliferation Relative to Control
BKM-120 Conc. (μM)
Dal-4
Den
EFB
DD-1
AS-M.5
HAEND
B
PI3K-α Inhibition - 72hr
Percent Proliferation Relative to Control
MLN-117 Conc. (μM)
Dal-4
Den
EFB
DD-1
AS-M.5
HAEND
C
PI3K-β Inhibition - 72hr
Percent Proliferation Relative to Control
GSK-2636771 Conc. (μM)
Dal-4
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EFB
DD-1
AS-M.5
HAEND

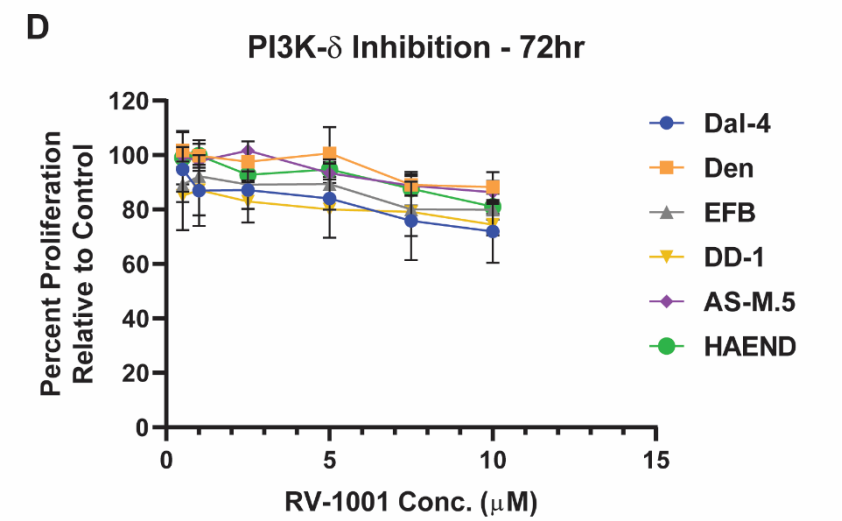


Figure 3.3: Inhibition of PI3K isoforms with small molecule inhibitors: Inhibitors were added to the culture media at indicated doses and the plates were incubated for 72 hours before collection and analysis with the CyQUANT cell proliferation assay. The inhibitors used and their targets were A) BKM-120 – pan-PI3K, B) MLN-117 – PI3K-α, C) GSK-2636771 – PI3K-β, and D) RV-1001 – PI3K-δ.

3.4: Combination of PI3K isoform inhibitors *in vitro*

To determine if there was an additional effect on proliferation if multiple PI3K inhibitors were used, we combined two of the inhibitors used in Figure 3.3 above. First, we treated the canine and human tumor cell lines with both PI3K-α (MLN-117) and PI3K-δ (RV-1001) inhibitors at a concentration of 1.0μM each for 72 hours. We observed no enhanced effect on cell proliferation over that observed with the individual inhibitors alone (Figure 3.4A). We repeated this experiment using the PI3K-β (GSK-2636771) and PI3K-δ (RV-1001) inhibitors in combination at concentrations of 1.0 and 2.5μM, this time only in 3 of the canine lines. Again, there was no appreciable impact on cell proliferation (Figure 3.4B). Note that both of these experiments were only repeated once, with each condition plated in triplicate.

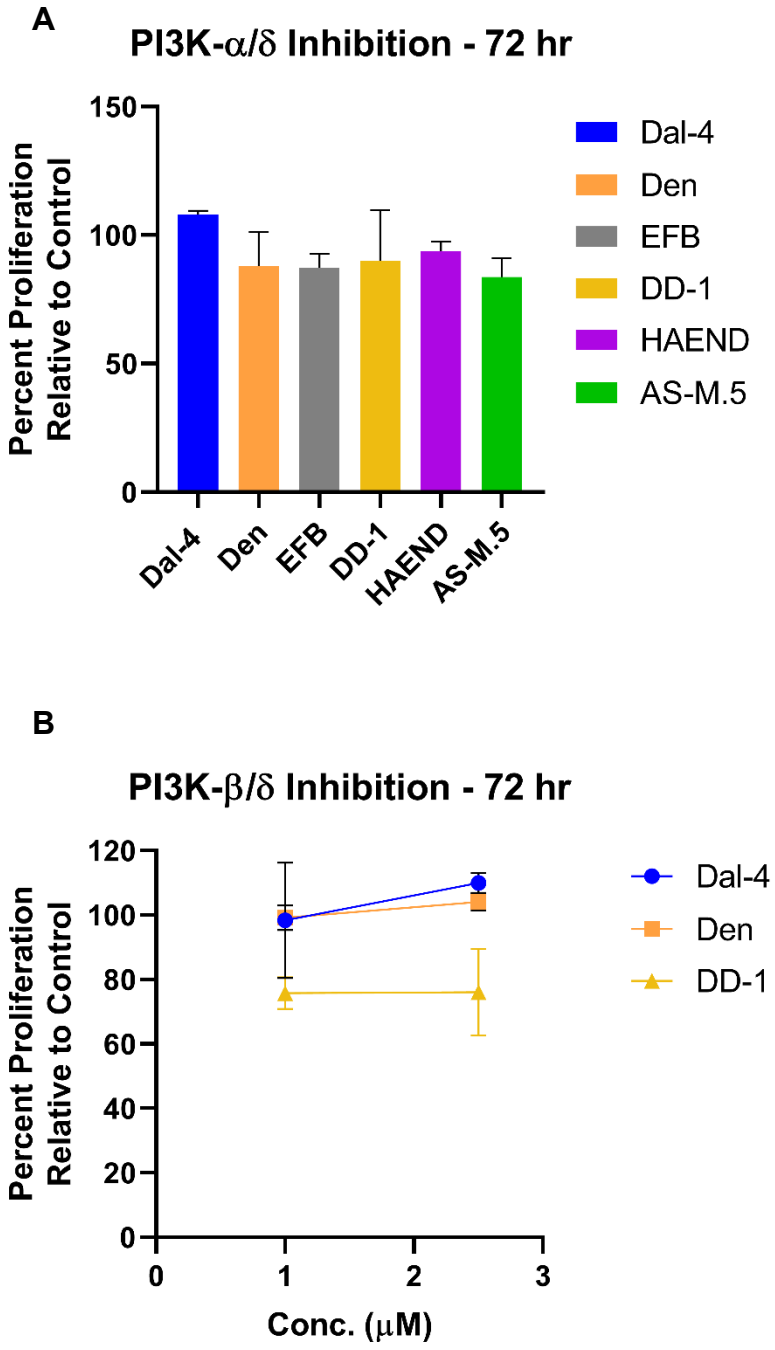
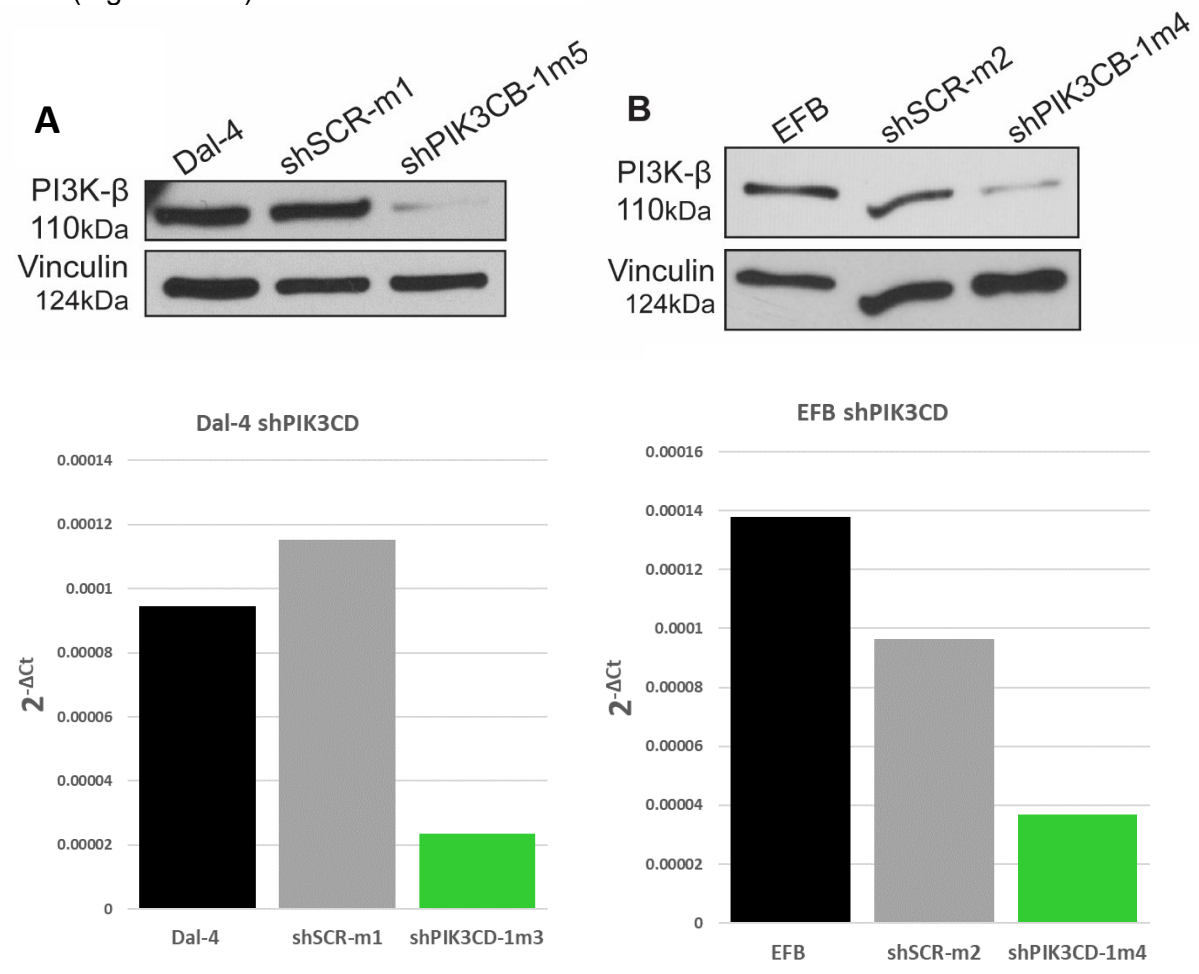


Figure 3.4: Combination of PI3K Inhibitors: A) Combination of PI3K-α (MLN-117) and PI3K-δ (RV-1001) inhibitors in four canine HSA and two human AS cell lines. Both drugs were added at a dose of 1.0μM. B) Combination of PI3K-β (GSK-2636771) and PI3K-δ (RV-1001) in three canine HSA cell lines. In both cases, the drugs were added at the indicated doses and then the plates were incubated for 72 hours.

3.5: Effects of PIK3CB and PIK3CD knockdown on proliferation in HSA cells

To study the contribution of PI3K-β and δ to HSA/AS without the potential off target effects of small molecule inhibitors, we used shRNA to knockdown (KD) the expression of these isoforms in two HSA cell lines, Dal-4 and EFB. The specific hairpin sequences used are shown above in Table 1. Monoclonal lines were produced and analyzed for efficiency of KD with western blotting and qRT-PCR (Figure 3.5 A/B). As assessed by the CyQUANT assay, *PIK3CB* KD in the Dal-4 cell line resulted in a slight reduction in cell proliferation when compared to the parental cell line, scrambled control, or PIK3CD KD, although this difference was not significant (Figure 3.5 C). No change in proliferation was observed when *PIK3CB* or *PIK3CD* was knocked down in the EFB cell line (Figure 3.5 D).



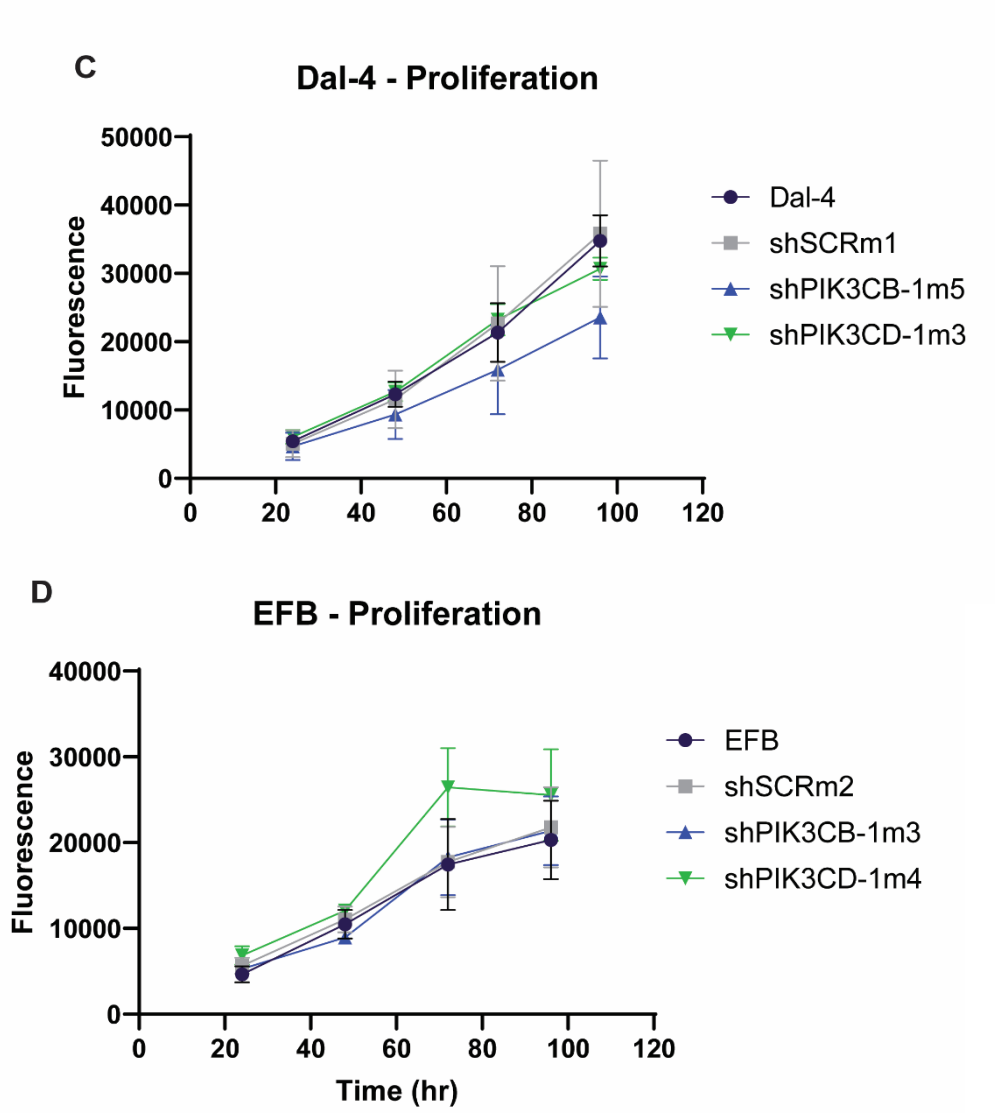


Figure 3.5: PIK3CB and PIK3CD KD in Canine HSA Cell Lines: A/B) Efficiency of KD of PIK3CB and PIK3CD in Dal-4 and EFB cells was determined using western blotting for PI3K-β and qRT-PCR for *PIK3CD*. C/D) CyQUANT Cell Proliferation Assay of Dal-4 and EFB cells with KD of *PIK3CB* and *PIK3CD*. Cells were plated at a density of 1000 cells/well and incubated for 24, 48, 72, and 96 hours before collection and analysis.

3.6 Migratory and Invasive Capacity of HSA Cells with *PIK3CB/PIK3CD* KD.

The ability of cells to migrate to and invade through new tissues are both essential processes for metastatic tumors to form. To determine the role of the PI3K-β and δ isoforms in the ability of HSA cells to migrate and invade, we used two variations of the transwell assay. Migratory capacity was investigated by plating control and

PIK3CB/D KD cells suspended in serum free media in the upper chamber of the transwell chamber and placing media with FBS in the lower chamber to act as a chemoattractant. In both the Dal-4 and EFB cell lines, neither *PIK3CB* or *PIK3CD* KD led to any change in the ability of the cells to migrate (Figure 3.6 A/B). To study the effects of gene KD on invasive capacity, we used a variation of this assay where the transwell surface is coated with Matrigel prior to placing cells in the upper chamber. This experiment is a representation of the ability of cells to move through extracellular matrix proteins, a necessary step of metastasis. Interestingly, KD of *PIK3CB* markedly reduced the ability of EFB cells to invade through Matrigel, although there was no significant change the Dal-4 cell line (Figure 3.6 C/D). Conversely, *PIK3CD* KD did not appear to have a significant effect on either cell line (Figure 3.6 C/D).

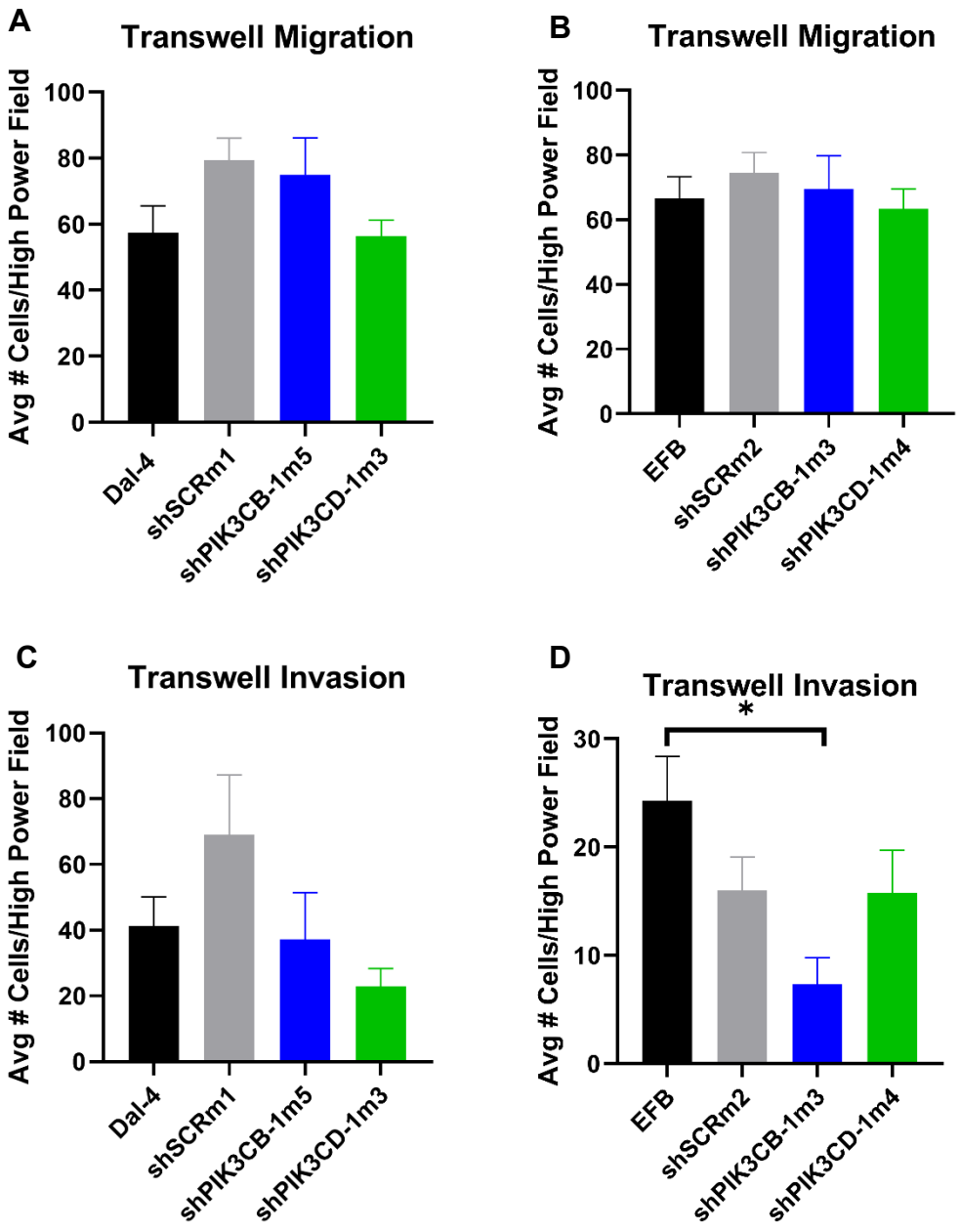


Figure 3.6: Migratory and Invasive Capacity of HSA cells with KD of *PIK3CB/PIK3CD* A/B) Transwell migration of Dal-4 and EFB cells with KD of *PIK3CB/D.* Cells were plated at a density of 10,000 cells/well in serum free media and placed in the upper chamber of a transwell with media containing 10% FBS in the bottom well. Wells were collected and stained with crystal violet after 24 hours of incubation. C/D) Transwell invasion of Dal-4 and EFB cells with KD of *PIK3CB/D.* Transwells were coated with Matrigel before 100,000 cells suspended in serum free media were added on top of the Matrigel and media containing 20% FBS was placed in the bottom well. Wells were collected and stained with crystal violet after 48 hours of incubation. * = $p<0.05$

Chapter 4: Discussion

In this study we aimed to establish the role of two of the class I catalytic PI3K isoforms, PI3K-β and PI3K-δ in canine HSA and human AS. We decided not to pursue study of the PI3K-α isoform since this has been the most researched isoform in previous studies. Not surprisingly, all six of the cell lines analyzed were found to express the ubiquitous PI3K-β isoform. A unique finding of this study was the expression of the PI3K-δ isoform in five of the six cell lines analyzed. The sixth cell line, HAEND, demonstrated expression of the *PIK3CD* at the mRNA level but not at the protein level. There are several possibilities to explain this, including the production of a truncated, nonfunctional form of the protein, which could be formed due to a mutation in the gene that has not been identified, leading to misfolding of or splicing of the protein. The protein could also be getting tagged through the ubiquitination pathway so that it is degraded prematurely. Aberrant expression of PI3K-δ has been documented other non-hematologic cancers such as breast cancer, neuroblastoma, glioblastoma, and colorectal cancer, although its functional properties in these settings are not entirely clear[54,55,61,62]. Since expression of PI3K-δ had not been previously demonstrated in AS or HSA, we were particularly interested in exploring the role of this isoform in the context of these cancers and hypothesized that signaling through this isoform may confer unique properties to the tumor cells.

Analysis of the downstream PI3K/AKT/mTOR pathway was also informative of how this pathway is contributing to the progression of these cancers. We analyzed phosphorylation of AKT and ribosomal S6 in three of the canine HSA cell lines and found that both proteins were still phosphorylated after six hours of serum starvation. This constitutive activation, even without the presence of growth factors, indicates that this pathway likely plays a role in tumor cell growth and survival.

We then evaluated how various PI3K small molecule inhibitors affected the growth of these cells *in vitro*. Pan-PI3K inhibition with BKM-120 affected the proliferation of both the human and canine tumor cell lines. In order to show that this inhibitor targeted PI3K, we could also determine the status of AKT and ribosomal S6 phosphorylation in cells treated with BKM-120. For the canine HSA cell lines, the IC50 for each line was between 2 and 3μM. The two human AS cell lines were slightly less responsive, but still had decreased growth. To determine which of the PI3K isoforms contributes this growth inhibition, we used isoform selective inhibitors to block PI3K-α, β, and δ independently. However, none of these inhibitors alone was sufficient to decrease cellular proliferation *in vitro.* We hypothesized that the combination of inhibition of two or more of the PI3K isoforms is necessary to inhibit the growth of these cells, however the combination of PI3K-α with PI3K-δ and PI3K-β with PI3K-δ inhibition did not lead to a reduction in the proliferation of the cells. Additionally, since inhibition of multiple PI3K isoforms often results in adverse events that are not tolerable in people, combination of a selective PI3K isoform inhibitor with blockade of a different pathway (i.e., synthetic lethality) may be more effective and more tolerable for long-term therapy.

To further interrogate the function of PI3K-β and PI3K-δ, we used shRNA to knockdown expression of both genes in two HSA cell lines. Analysis of several phenotypes related to cancer progression highlighted the potential relevance of PI3K-β as a potential avenue for targeted treatment of AS/HSA. While proliferation or migratory capacity was not affected by KD of either gene, the invasive capacity of EFB cells markedly reduced in the absence of PI3K-β, suggesting an important role in the process of metastasis.

More studies will be necessary to determine the role of these isoforms in AS/HSA. It would be informative to determine the presence of mutations within this pathway in the cell lines used, particularly in PTEN, the PI3K isoforms, and the AKT

isoforms. We have also yet to determine the role PI3K-δ in these cells and studies regarding the functional consequences of signaling through both PI3K-β and PI3K-δ in AS remain to be delineated. It may be revealing to study the combined inhibition of PI3K-β and PI3K-δ in the context of experiments such as transwell invasion to determine if there is an additive effect by the inhibition of both isoforms. *In vivo* studies would also help to inform the role of both of these proteins, including determining if KD of these isoforms impacts growth in the setting of xenograft mouse models and/or lung seeding (metastasis) models in mice.

Collectively, the findings of this study indicate the crucial role that the PI3K/AKT/mTOR pathway plays in AS/HSA. The pathway is constitutively active and inhibition of all the PI3K isoforms leads to decreased growth of all six cell lines used in this study. Further analysis of this pathway, particularly the ability to grow *in vivo* will help to inform the potential benefits that inhibition of these isoforms will have in patients.

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