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Ishani H. Rao April 2, 2022

Vasoactive Intestinal Peptide (VIP) Regulates ZEB1 in Cancer

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Abstract

Vasoactive Intestinal Peptide (VIP) Regulates ZEB1 in Cancer

By Ishani H. Rao

In cancer immunology, VIP (vasoactive intestinal peptide) is a neuropeptide with pleiotropic effects including inhibiting T-cell activation, increasing regulatory T-cell frequency, and augmenting the immunosuppressive activity of myeloid-derived suppressor cells. VIP expression by cancer may represent a paracrine immune-check-point pathways as well as having autocrine effects on cancer growth. We used an *in silico* model to identify genes whose over-expression in cancer was correlated with elevated VIP expression and confirmed the role of VIP-receptor signaling in vitro using VIP-R antagonists. The TCGA PanCan dataset (analysis per UCSC Xena) was used to compare mRNA expression data of VIP versus 760 genes involved in 48 cancer-related pathways. A positive association was defined as Pearson's Coefficient R-values >0.3. Expression of target genes and VIP were confirmed by Western blot analysis in cancer cell lines, and VIP-R signaling was inhibited using ANT008, a novel VIP-R inhibitor. A significant correlation (R>0.3) between VIP expression and 10 genes was observed (Table 1). Grouping cancers by germ-layer analysis demonstrated the strongest (R>0.4) associations in ectodermal and mesodermal-derived cancers. Histologically, the highest single association with VIP was with ZEB1 expression in stomach adenocarcinoma (R = 0.7643). *In vitro* experiments using Jurkat T-cells confirmed expression of VIP and ZEB1 protein. Treatment of Jurkat cells treated with the VIP antagonist ANT-08 (10 μM) demonstrated a reduction in ZEB1 total protein expression when VIP-R signaling was inhibited. The results demonstrate autocrine signaling between VIP expression and cancer-associated gene pathways. The ZEB1 transcription factor is a known EMT (epithelial-mesenchymal transition) regulator, and our *in vitro* studies demonstrate this pathway is therapeutically targetable using a VIP antagonist.

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Additionally, I would like to thank Dr. Sanjay Chandrasekaran, who mentored me remotely throughout the pandemic. His initial interest in an in-silico study using the TCGA database allowed me to start a novel project during a time when in-person research was difficult. His mentorship and support have been unwavering and led to my successful honors thesis defense.

Finally, I'd like to thank my committee members Dr. Arri Eisen and Dr. Simon Blakey for their guidance in completing this thesis.

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Introduction

Cancer: Cancer is characterized by the uncontrolled proliferation of abnormal cells. Abnormal function of a cancer cells ranges from preventing healthy body functioning, producing harmful impacts on a healthy body, and no longer serving its useful function in the human body. These cells divide rapidly to form masses and tumors that can further obstruct normal bodily functioning. Further progression of the cancer can lead to metastasis, the movement of cancer cells from the primary tumor site to a distant site previously uninhabited by cancer cells.

Tumor Germ Layer of Origin: A way to classify cancers is by embryonic origin. Cancers similar in morphology often respond differently to therapies, prompting a need for classifying cancers at the molecular level. Germinal layers are comprised of a primary group of cells that are involved in the initial formation of certain tissues and organs during embryonic development. These germinal layers are the endoderm (inner layer), ectoderm (outer layer), and mesoderm (middle layer). In brief, the endoderm gives rise to the digestive and respiratory tract, pancreas, and liver. Among other structures, the ectoderm gives rise to the epidermis of the skin, nervous system, and epithelial lining of the mouth and rectum. Finally, the mesoderm gives rise to the skeleton, muscular system, notochord, reproductive system, circulatory system, and excretory system. Studying cancers based on their tissue of origin is valuable because irregularities regarding gene regulation and signaling pathways during embryonic development show similar patterns in tumorigenesis (Gao, Cui, Zhang et al.). Interestingly, the processes of tumorigenesis and embryogenesis share multiple properties with respect to cell invasion and differential gene activation [DOI 10.1016/j.semcancer.2018.07.004], (Naxerova et al.).

Cancer Hallmarks: Cancerous cell behavior can largely be placed into one or many categories defined by the 10 hallmarks of cancer. Neoplastic transformation is engaged upon the

acquisition of key hallmark characteristics (D. Hanahan and Robert A. Weinberg; D. Hanahan and R. A. Weinberg). These 10 Hallmarks include evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, deregulating cellular energetics, and sustaining proliferative signaling (D. Hanahan and Robert A. Weinberg) **(Figure 1)**.

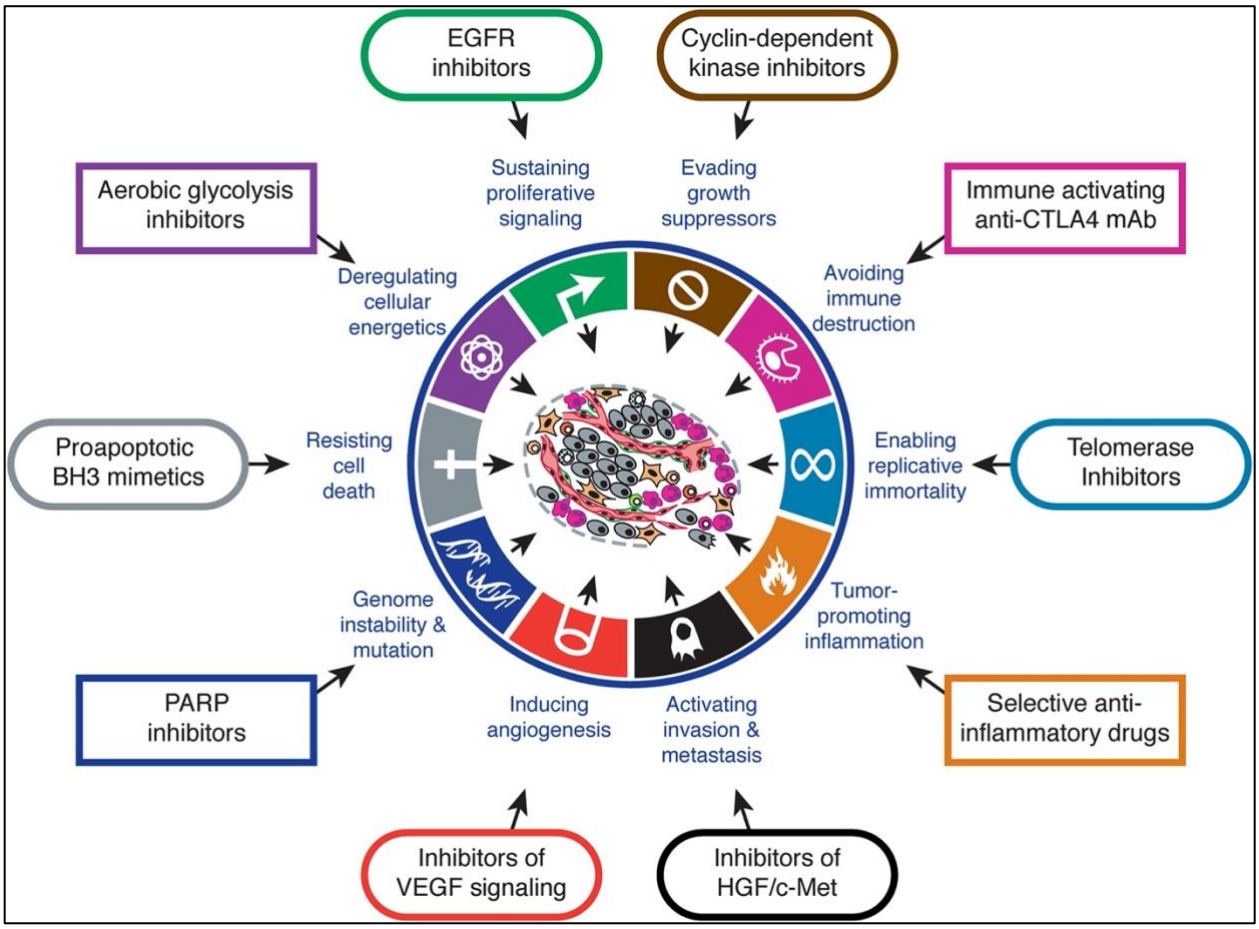
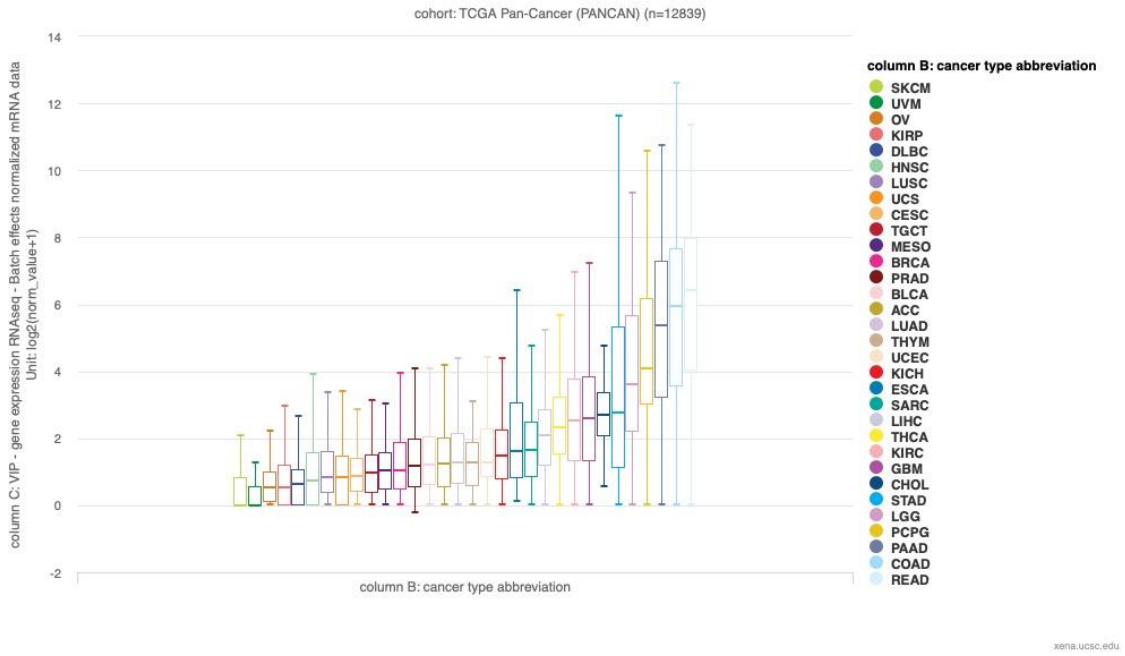


Figure 1 shows the 10 hallmarks of cancers. Of these 10 hallmarks, activating invasion and metastasis is of most interest in the context of this study.

Signaling Pathways: Genetically altering signaling pathways is a common hallmark of cancer that occurs in many malignancies. Specifically, signaling pathways regarding resisting cell death, cell-cycle regulation, and activating invasion and metastasis are some of the most altered in cancer (Sanchez-Vega, Francisco et al.). There are a variety of genes that play roles in

proper regulation or dysregulation of these pathways and studying them provides insight for drug discovery along with which therapies should be employed for treatment. When pathways are dysregulated, it can lead to differential expression of specific genes, which can result in differential mRNA and protein expression. This can be used as yet another way to classify and study cancers. For instance, cancers that overexpress vasoactive intestinal peptide (VIP) can be studied together to find other genes that may be implicated in its pathways or serve as targets for anti-cancer therapy **(Figure 1A and Figure 1B).**

A



B

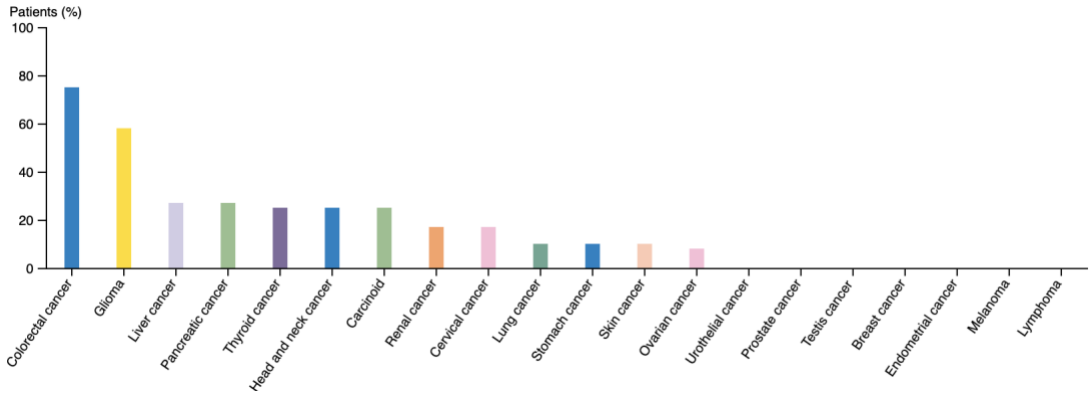


Figure 1 shows protein expression of VIP by tumor histology (A) (Human Protein Atlas, 2020) and MRNA expression of VIP by tumor histology (B) (Goldman et al, 2020). These graphs show that although varied by tumor histology, VIP is overexpressed by many cancers - namely pancreatic cancer and GI cancers.

Autocrine vs Paracrine Functions: VIP is a 28 amino acid peptide that is secreted by gastrointestinal tissue, nerve cells, and multiple immune cell populations including lymphocytes, mast cells, and granulocytes. VIP belongs to the glucagon/secretin family of peptides that signal through three class II G-protein coupled protein receptors (GPCRs), namely VPAC1, VPAC2 and PAC1 (Ref). Upon binding to the receptors, signaling occurs through effector enzyme adenylate cyclase (AC), which catalyzes cAMP synthesis and activates protein kinase A (PKA). PKA is known to activate cAMP-response element binding (CREB) transcription factor **(Figure 2)**. VIP has immunosuppressive properties in cancer immunology. VPAC1, VPAC2, and PAC1 are expressed on T and B lymphocytes, dendritic cells (DC), macrophages and many cancers, and In cancer immunology, VIP signaling disrupts immune homeostasis by inhibiting T-cell activation and proliferation, increasing the frequency of regulatory T cells and activity of myeloid-derived suppressor cells, and promoting immune tolerance (Forghani, Petersen et al. 2017). VIP receptVor antagonists downregulate inhibitory marker, PD-1, on CD8 cells and enhances cellular anti-viramunity as shown by the significantly improved survival in cytomegalovirus-challenged mice (Li et al.). In addition, inhibiting VIP signaling pathway

augments T cell dependent anti-leukemia response (Petersen, Li and Waller). VIP receptor antagonists increase T cell ex vivo expansion and persistence in murine lymphoma models (Petersen et al.).

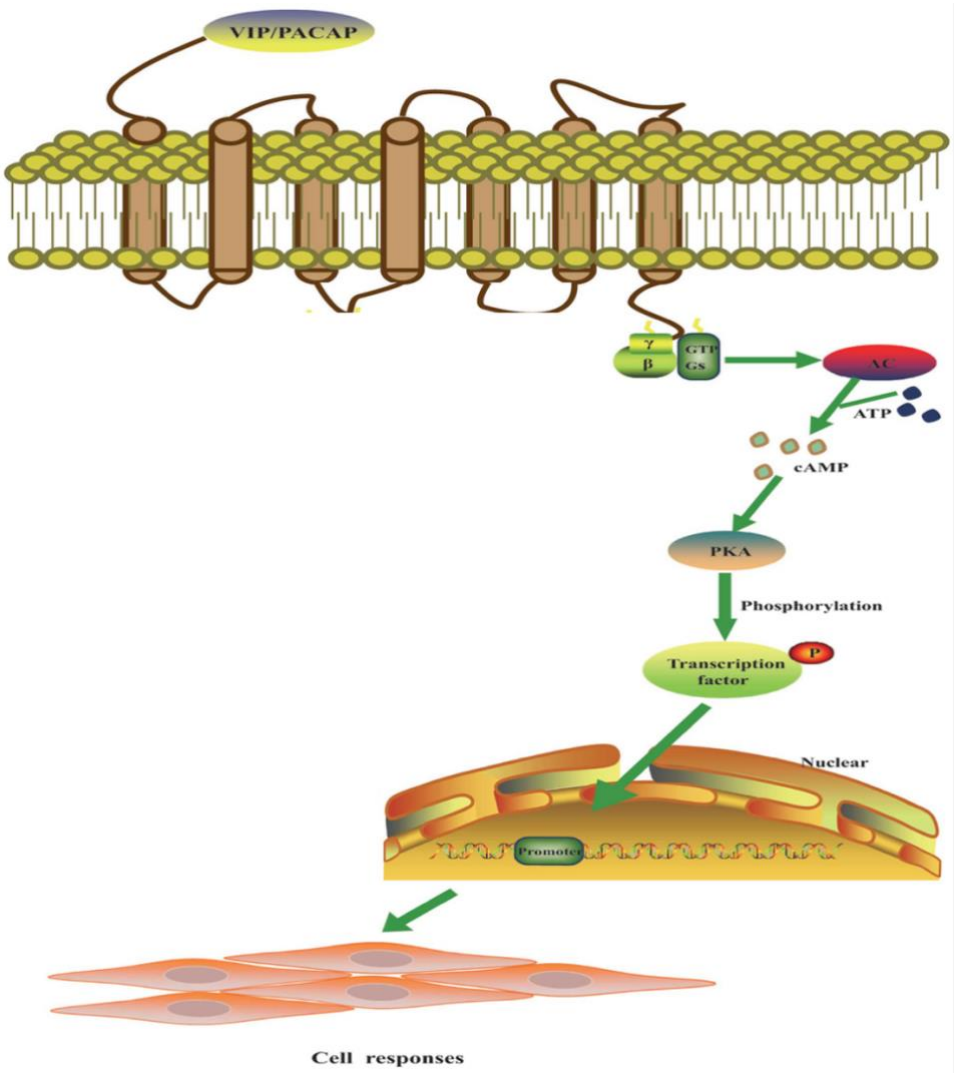


Figure 2 shows VIP's signaling pathway by which it produces its downstream effects via CREB signaling.

VIP is secreted in varying amounts by cancers of pancreas, gastrointestinal tract, kidneys, lungs, brain, and skin, evident by both total mRNA and protein levels **(Figure 1A and Figure 1B).** and the impact of autocrine VIP signaling remains elusive. Interestingly, VIP treated cytomegalovirus-challenged mice has shown increased serum VEGF, indicating VIP may play a

role in angiogenesis (Li et al.). PKA can also initiate mitogen-activated protein kinase (MAPK) pathway, further implicating the role of VIP in biological processes in addition to its established properties as an immunosuppressive peptide (Martínez et al.). Taken together, we hypothesize that VIP signaling may have broader autocrine mechanistic functions in cancer biology outside of immuno-oncology and serve as a target for anti-cancer therapy. Using our in-silico model, we have shown potential roles for VIP in cancer immunology, angiogenesis, and cellular energetics

Taken together, we sought to characterize the heterogeneous expression and activity of VIP and its receptors in different cancer histologies by their embryonic origin in the context of key cancer hallmarks by comparing VIP mRNA expression levels vs other cancer relevant genes. We employed an in-silico model using the TCGA Pan Can Database with a target-gene panel developed based on the Nanostring Tumor 360 panel.

The Pan Can TCGA dataset is comprised of 12,839 samples that span across 32 histologies. Nanostring's nCounter Tumor Signaling 360 Panel is a 760 gene panel across 44 core pathways of cancer, the tumor microenvironment, and the tumor immune response, 14 immune cell phenotypes, and 10 hallmarks of cancer.

2 Methods

TCGA Data Abstraction Analysis: We analyzed genomic expression data for VIP and genes within the Nanostring Tumor 360 profiling panel using the Pan Can dataset utilizing the University of California Santa Cruz (UCSC) Xena database, as previously described (Goldman et al, 2020). Of the 12,839 samples, 1912 were null value and were excluded from the data analysis. Data was imported into Microsoft Excel and analyzed using GraphPad Prism. (Expression of VIP, VPAC1, and VPAC2 by histology using median mRNA expression levels –

Rohan's figure) R-values between VIP and profile genes were evaluated. Positive and negative associations of interest were established if Pearson's Correlation Coefficient (*R*) with VIP mRNA levels vs profiled gene were > [.3] (primary genes) or > [.2] and <[.3] (secondary genes) (Shober et al, 2018). Associations between 0 and [.2] were not considered to be meaningful.

Characterization by Tissue Histology and Germ Layer: The 32 histologies in the TCGA dataset were further stratified by germ-layer (Ectoderm, Endoderm, Mesoderm) per SEER categorization. Of 34 cancer histologies, PCPG was excluded in the analysis due to unclear histologic origin and LAML was excluded as it was comprised exclusively of null values. R-values between VIP and profiled genes were evaluated by germ-layer groups and individual tumor histologies. Particular attention was given to positive and negative associations if Pearson's Correlation Coefficient (*R*) with VIP mRNA levels vs profiled gene were > [.4] (Shober et al. 2018).

Statistical Analysis Methods: Primary genes were those that showed a Pearson's R value of > [.3] with VIP mRNA levels, and secondary genes were those that showed an R value of [.2] < x < [.3] with VIP mRNA levels. This system of labeling genes as primary genes and secondary genes based off their Pearson's R value was adapted from: Shober et al. 2018. Associations between 0 and [.2] between genes and VIP were not considered to be meaningful.

Cell culture methods: To further study the relationship between VIP and ZEB1, in vitro modeling was employed. Jurkat T cells, a CD4+ human acute T lymphocyte cell, were used as the model organism. Jurkat T cells have served as good model organisms to study T cell signal transduction pathways as they can simulate T lymphocyte function (Chen and Nong). In this experiment, Jurkats were seeded at a concentration of 1 million cells/ 1.5 mL of media. They

were treated with VIP antagonist (Ant 308) and VIP agonist at concentrations 0μM, 1μM, 3μM, 5μM, and 10μM for 24 hours. Cell lysates were promptly made after 24 hours incubation period.

Western blot methods: For western blotting, 30μg of sample was loaded and run at 200V. Blots were blocked with 1% BSA for 1.5 hours. Blots were probed with primary antibody ZEB1 - 1:500, primary antibody VIP - 1:500, primary antibody GAPDH - 1:1000, secondary antibody - 1:2000. Blots were imaged using SynGene GeneSnap imaging machine/software. Molecular weights of interest: ZEB1 190-210kDa, VIP 19kDa, GAPDH 37 kDa.

3 Results

Pathway Associations with VIP: Initially, 760 genes that were related to at least one of 43 Cancer pathways were analyzed for associations with VIP across all cancer types. After abstracting data for 760 genes, 10 genes – presented with R-values of [.3] or higher (MAPK3, ZEB1, NOS2, TEK, PTCH1, EIF4G1, GMPS, CDK2, RUVBL1, and TIMELESS) and were characterized as "primary" genes **(Figure 3)**. To follow the pathways of potential interest, Lead genes and "Follower" genes (> [.2]) were compared by cancer pathway implication. Of 44 cancer pathways identified in Nanostring's Tumor Signaling Panel, 14 target pathways, were identified in which at least one "Lead" gene and one "Follower" gene were involved in. The pathways include, MAPK Signaling, EMT, Epigenetic & Transcription Regulation, HIF1 Signaling, Myeloid Immune Evasion, Hedgehog, Inflammation, FGFR, Cell Cycle, DNA Damage Repair, mTOR Signaling, Immortality & Stemness, Interferon Response, and WNT Signaling **(Figure 4)**.

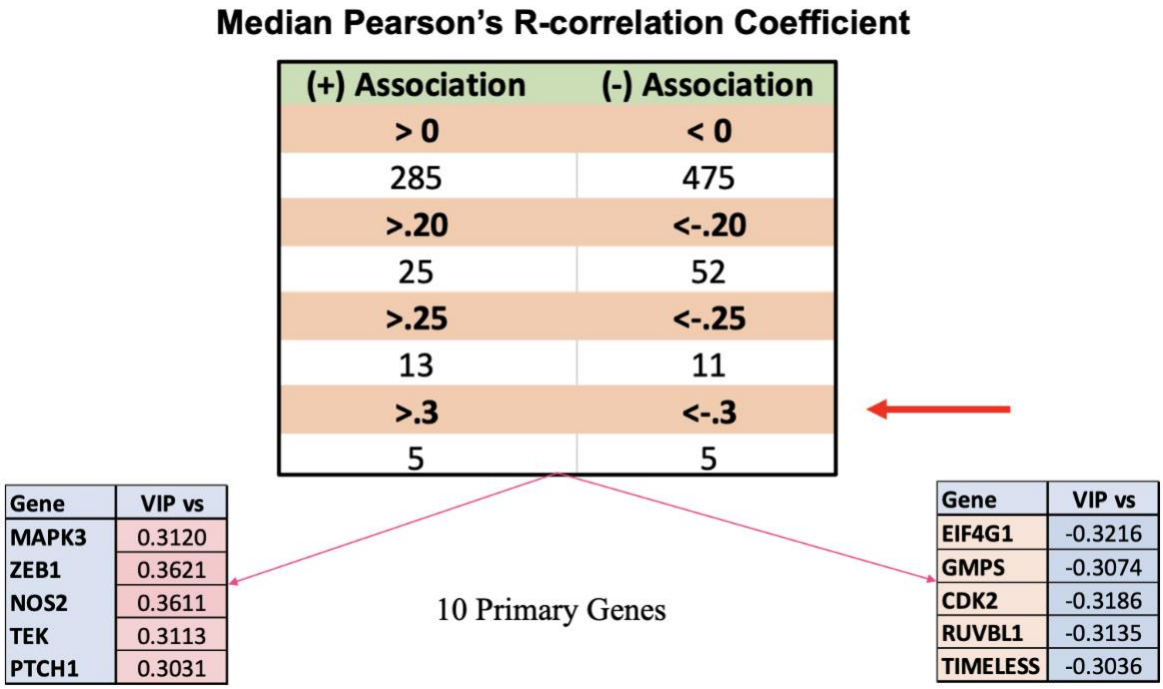


Figure 3 shows how many genes from the TCGA database fall into each category of association by Pearson's R-correlation coefficient. 10 genes denoted "primary genes", have R values > [.3].

EMT	MAPK Signaling	Epigenetic & Transcrip. Regulation	HIF1 Signaling	Myeloid Immune Evasion	Hedgehog	Inflammation	FGFR
MAPK3	MAPK3	ZEB1	TEK	NOS2	PTCH1	NOS2	MAPK3
ZEB1	TEK	ARID4A	NOS2	NT5E	SHH	VCP	BRAF
SHH	IL3RA	KDM1A	FLT1		PTCH2	GNAI3	
TLR4	HSPB1		NOS3		P4HB		
MTOR	BRAF		EDN1		VCP		
KDM1A							
ESR1							

Cell Cycle	DNA Damage Repair	mTOR Signaling	Immortality & Stemness	Interferon Response	WNT Signaling
RUVBL1	RUVBL1	EIF4G1	RUVBL1	EIF4G1	RUVBL1
CDK2	TIMELESS	GMPS	RFC2	KPNA2	RAC3
MCM2	PARP1	MCM2	RFC4	MAPK3	PTCH1
TUBG1	FANCI	TUBG1	FEN1		
RFC2	FANCD2	STIP1	LIG1		
FEN1	CHEK2	MCM4	POLE		
LIG1	DTL	BUB1B	ABCG2		
POLE	EME1	MTOR	EPAS1		
EXO1	FEN1	PRDX1	CD34		
RAD51	LIG1				
MCM4	POLE				
BUB1B	EXO1				
RFC4	RAD51				
CDC20	KPNA2				
KIF20A	VCP				
KIF23					
KIF2C					
CCNB1					
CCNB2					
SKA1					
CCND2					

Figure 4 shows 14 mechanisms with which at least one "primary" gene and one "secondary" gene are associated with. These are mechanisms in which VIP may be implicated.

Gene Associations by Germ Layer: To obtain further insight into histologies where this may be more prevalent the "Lead" genes were analyzed for association with VIP by germinal layer **(Figure 5 and Figure 6)**. Strong shifts in associations were seen between the germ layers. Lead genes appeared to have greater associations (R values of .4 and greater or -.4 or lower) in the ectodermal and mesodermal germ layers, for genes ZEB1, PTCH1, GMPS, CDK2, and TIMELESS (ectodermal) and genes ZEB1, NOS2, TEK, and RUVBL1 (mesodermal).

We further characterized the associations of PACAP, an analog peptide thought to competitively bind to the VPAC1 and VPAC2 receptors to establish if these act and signal through similar pathways **(Figure 7)**. Lead genes showed weak associations across ectodermal and mesodermal germ layers. The only gene that displayed a greater association (R values of .4 and greater or -.4 or lower) with VIP was ZEB1 in the endodermal germ layer. These results in contrast with results obtained with VIP vs "Lead" genes by germ layer further support the notion that VIP's role in pathways involving the "Lead" genes may be of some significance.

VIP				
Gene	**All**	**Ectoderm**	**Endoderm**	**Mesoderm**
MAPK3	0.3120	0.3537	0.3380	0.2252
ZEB1	0.3621	**0.4341**	0.3348	**0.4901**
NOS2	0.3611	0.3186	0.3428	**0.4849**
TEK	0.3113	0.3336	0.2600	**0.5131**
PTCH1	0.3031	**0.4040**	0.2879	-0.0061
Gene	**All**	**Ectoderm**	**Endoderm**	**Mesoderm**
EIF4G1	-0.3216	-0.4268	-0.2064	-0.3779
GMPS	-0.3074	**-0.4698**	-0.0928	-0.3005
CDK2	-0.3186	**-0.4687**	-0.0563	-0.2345
RUVBL1	-0.3135	-0.3270	-0.1886	**-0.4716**
TIMELESS	-0.3036	**-0.4284**	-0.1505	-0.3717

Figure 5 shows associations of VIP with "primary" genes by germinal layer. VIP appears to have strongest associations with lead genes in the ectodermal and mesodermal layers.

Abbreviation	Gene	Germ Layer
ZEB1	Zinc Finger E-box-binding Homeobox 1	Mesoderm
NOS2	Nitric Oxide Synthase 2	Mesoderm
TEK	TEK Receptor Tyrosine Kinase	Mesoderm
RUVBL1	RuvB Like AAA ATPase 1	Mesoderm
PTCH1	Patched 1	Ectoderm
GMPS	Guanine Monophosphate Synthase	Ectoderm
CDK2	Cyclin Dependent Kinase 2	Ectoderm
TIMELESS	Timeless Circadian Regulator	Ectoderm
MAPK3	Mitogen-Activated Protein Kinase 3	Ectoderm
EIF4G1	Eukaryotic Translation Initiation Factor 4 Gamma 1	Ectoderm

Figure 6 shows which germ layer each primary gene was most associated with.

PACAP				
Gene	**All**	**Ectoderm**	**Endoderm**	**Mesoderm**
MAPK3	0.2005	0.0982	0.2167	0.2093
ZEB1	0.3542	0.3340	**0.4527**	0.2073
NOS2	0.1987	0.1741	0.1410	0.2823
TEK	0.3508	0.3963	0.3423	0.3360
PTCH1	0.1618	0.1713	0.1976	0.1865
Gene	**All**	**Ectoderm**	**Endoderm**	**Mesoderm**
EIF4G1	-0.1174	-0.1383	0.0026	-0.0989
GMPS	-0.1128	-0.1467	0.0295	-0.1224
CDK2	-0.2204	-0.3366	0.0167	-0.2019
RUVBL1	-0.1761	-0.1728	-0.1164	-0.1898
TIMELESS	-0.2003	-0.1882	-0.1139	-0.2722

Figure 7 shows associations of PACAP with "lead" genes by germinal layer. PACAP appears to have weak associations with lead genes in the ectodermal and mesodermal layers.

Gene Associations by Histology: Further analysis for associations of VIP with "primary" genes by the cancer types that fall into either ectoderm, endoderm, or mesoderm revealed tissue histologies in which VIP is suspected to have impact **(Figure 8)**. Within ectodermal tumors, associations > [.4] were noticed in breast invasive carcinoma (BRCA), brain lower grade glioma (LGG), lung squamous cell carcinoma (LUSC), and rectum adenocarcinoma (READ) tumor histologies for TEK, GMPS, RUVBL1, TIMELESS, and MAPK3 genes, and [>.5] in LUSC and READ tumor histologies for TEK, GMPS, and ZEB1 genes. Within endodermal tumors, associations > [.4] were noticed in cholangiocarcinoma (CHOL), esophageal carcinoma (ESCA), lung adenocarcinoma (LUAD), pancreatic adenocarcinoma (PAAD), stomach adenocarcinoma (STAD), and uterine corpus endometrial carcinoma (UCEC) tumor histologies for CDK2, TEK, TIMELESS, ZEB1, RUVBL1, EIF4G1 genes, and [>.5] in colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), PAAD, STAD, thymoma (THYM) and UCEC tumor histologies for ZEB1, TEK, and TIMELESS. The most significant association of R = .7643 was noted in endodermal tumor, STAD with gene ZEB1 **(Figure 9)**. Within mesodermal tumors, associations > [.4] were noticed in kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), mesothelioma (MESO), and testicular germ cell tumors (TGCT) tumor histologies for ZEB1, NOS2, RUVBL1, EIF4G1, TEK, and TIMELESS genes, and [>.5] in KICH tumors for genes TEK and GMPS.

			ECTODERM								
	All	Ectoderm	BRCA	CESC	GBM	HNSC	LGG	LUSC	READ	SKCM	UVM
MAPK3	0.3120	0.3537	0.0964	-0.0017	0.2263	0.1392	**0.4552**	0.2770	0.2033	-0.1715	-0.1609
ZEB1	0.3621	**0.4341**	0.3440	0.3611	-0.0398	0.1550	-0.1824	0.3842	**0.5468**	0.3226	0.1933
NOS2	0.3611	0.3186	0.1027	-0.0353	-0.0376	-0.0352	0.3182	0.0518	-0.1816	0.1315	-0.1148
TEK	0.3113	0.3336	**0.4936**	0.3813	0.3704	0.1800	0.3062	**0.5188**	**0.4457**	0.2351	0.1458
PTCH1	0.3031	**0.4040**	0.2230	0.0095	-0.0992	0.0040	-0.0869	0.0707	0.0146	-0.0923	0.2382
EIF4G1	-0.3216	-0.4268	-0.3901	-0.1558	-0.2865	-0.1893	-0.3336	-0.3456	-0.2330	-0.2772	-0.0213
GMPS	-0.3074	**-0.4698**	**-0.4385**	-0.2199	-0.3172	-0.3239	-0.2850	**-0.5018**	-0.1909	0.0158	-0.0620
CDK2	-0.3186	**-0.4687**	-0.3478	-0.2001	-0.2826	-0.1947	-0.4429	-0.3372	-0.1683	-0.2561	0.0487
RUVBL1	-0.3135	-0.3270	**-0.4263**	-0.1571	-0.1067	-0.2456	-0.4090	-0.4065	-0.3015	-0.0388	-0.0612
TIMELESS	-0.3036	**-0.4284**	**-0.4488**	-0.3193	-0.2292	-0.3152	-0.3093	**-0.4516**	-0.3431	-0.0534	-0.0042

			ENDODERM											
	All	Endoderm	BLCA	CHOL	COAD	ESCA	LIHC	LUAD	PAAD	PRAD	STAD	THCA	THYM	UCEC
MAPK3	0.3120	0.3380	0.0840	-0.0316	0.1961	-0.0779	0.0403	0.0931	-0.3481	0.1184	-0.0387	0.0807	0.1247	0.0712
ZEB1	0.3621	0.3348	0.2797	0.1345	**0.5624**	**0.5958**	0.0564	0.3695	**0.4272**	0.2290	**0.7643**	0.3922	-0.0217	**0.5233**
NOS2	0.3611	0.3428	0.1233	-0.1647	-0.0377	-0.2033	0.0929	0.2647	0.0010	-0.0464	-0.0723	0.1557	0.1176	0.1501
TEK	0.3113	0.2600	0.3414	0.2356	**0.5555**	**0.4071**	0.1877	**0.4777**	**0.5500**	0.1593	**0.5571**	0.3425	**0.5010**	**0.4318**
PTCH1	0.3031	0.2879	0.1454	0.0122	0.1493	0.0856	0.0861	0.2215	0.3214	0.0207	0.2563	0.0323	0.3173	0.1815
EIF4G1	-0.3216	-0.2064	-0.2672	-0.0939	-0.2033	-0.2011	-0.1787	-0.2377	-0.1502	-0.0686	-0.3292	-0.4659	0.0418	**-0.4116**
GMPS	-0.3074	-0.0928	-0.2056	-0.2998	-0.2770	-0.2878	-0.1402	-0.4512	-0.1608	-0.0396	**-0.4315**	-0.4152	-0.3923	-0.2285
CDK2	-0.3186	-0.0563	-0.0686	**-0.4479**	-0.2305	-0.2869	-0.0854	-0.2531	0.0384	0.0259	-0.3158	-0.2506	-0.2479	-0.0819
RUVBL1	-0.3135	-0.1886	-0.1966	-0.3331	-0.3985	-0.3879	-0.1112	-0.2898	-0.3082	-0.0105	**-0.4446**	0.1271	-0.4574	-0.3865
TIMELESS	-0.3036	-0.1505	-0.0694	-0.3932	-0.3433	**-0.4430**	-0.1009	-0.3678	-0.1605	-0.0656	**-0.5386**	-0.2404	-0.3190	-0.3652

			MESODERM									
	All	Mesoderm	ACC	DLBC	KICH	KIRC	KIRP	MESO	OV	SARC	TGCT	UCS
MAPK3	0.3120	0.2252	0.0519	-0.1208	0.3498	0.1308	0.0869	-0.1559	0.0297	0.1396	0.0525	0.2202
ZEB1	0.3621	**0.4901**	0.0255	0.0504	**0.4232**	**0.4696**	0.2750	0.3801	0.2475	-0.0212	0.1890	-0.0998
NOS2	0.3611	**0.4849**	0.3165	0.2317	0.3432	0.2776	0.2337	**0.4952**	0.2452	0.2850	0.1633	0.1646
TEK	0.3113	**0.5131**	0.3589	0.1020	**0.5893**	0.1107	0.4243	**0.4955**	0.2401	0.2486	-0.0536	0.2982
PTCH1	0.3031	-0.0061	-0.0768	0.0589	-0.1956	0.0015	0.0873	0.1630	-0.0347	-0.0427	-0.0070	0.2592
EIF4G1	-0.3216	-0.3779	-0.1728	-0.2419	**-0.4865**	-0.2194	-0.2494	-0.1347	-0.1163	0.0257	-0.1888	0.2371
GMPS	-0.3074	-0.3005	-0.0245	0.0562	**-0.5384**	-0.3468	-0.0905	-0.1695	-0.1779	-0.0992	-0.0794	-0.1211
CDK2	-0.3186	-0.2345	-0.1798	0.1525	0.0186	0.1549	-0.1156	-0.0387	-0.1533	-0.1246	-0.2531	0.0317
RUVBL1	-0.3135	**-0.4716**	0.0553	0.0502	-0.0667	-0.3439	-0.3142	-0.2208	-0.2133	0.0859	-0.3611	-0.1995
TIMELESS	-0.3036	-0.3717	-0.1704	0.0553	-0.1223	-0.1824	-0.2077	-0.1352	-0.1376	-0.0970	**-0.4431**	0.0721

Figure 8 shows associations of VIP with "primary" genes by the cancer types that fall into either ectoderm, endoderm, or mesoderm.

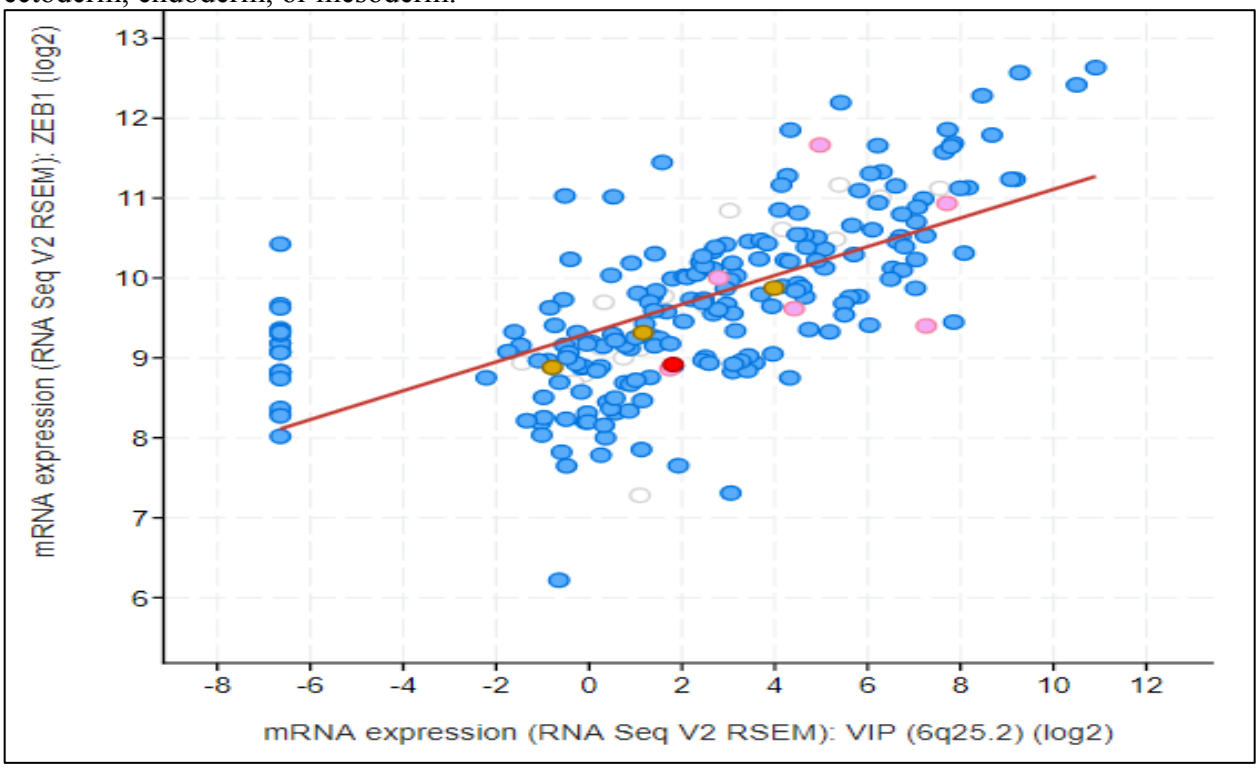


Figure 9 shows the most significant association found in the TCGA dataset, R-value of 0.7643. This association is between VIP and ZEB1 in Stomach Adenocarcinoma (STAD).

In Vitro Modeling: In vitro experiments using Jurkat T cells confirmed expression of VIP and ZEB1 proteins. Jurkat T-cells were used as a model organism and treated with the VIP-R inhibitor ANT308 and VIP agonist at concentrations of 0µM, 1µM, 3µM , 5µM, and 10µM. Ant 308 reduced VIP and ZEB1 protein expression while VIP agonist increased VIP expression. **(Figure 10)**.

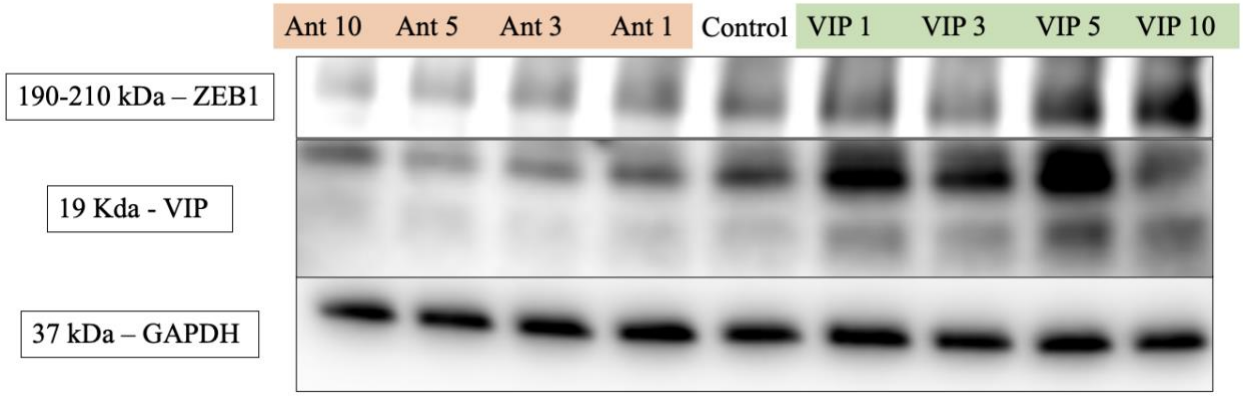


Figure 10 shows western blot of Jurkat T-cells treated with VIP antagonist (ANT 308) and agonist.

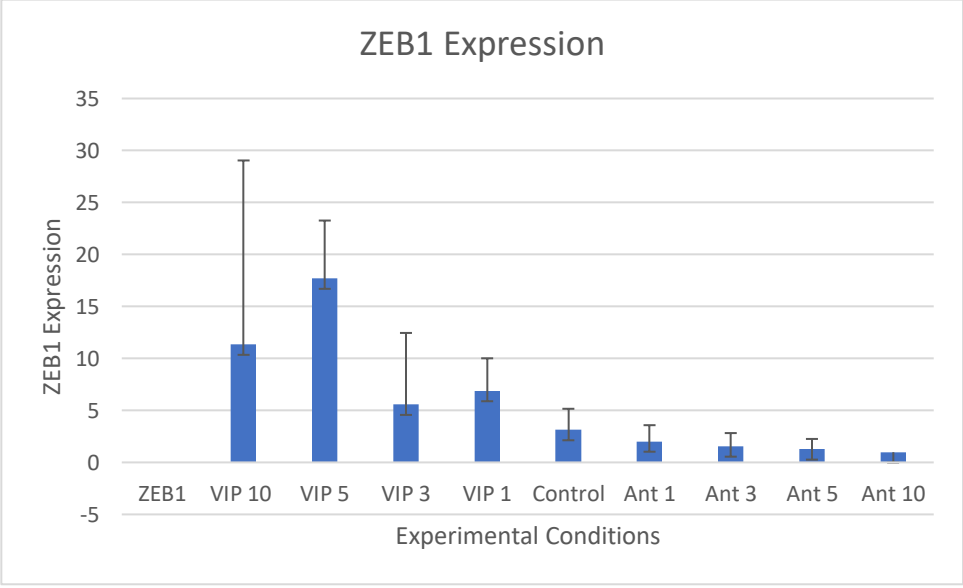


Figure 11 shows ZEB1 expression after quantification of nine western blots of Jurkat T-cells treated with VIP antagonist (ANT 308) and agonist.

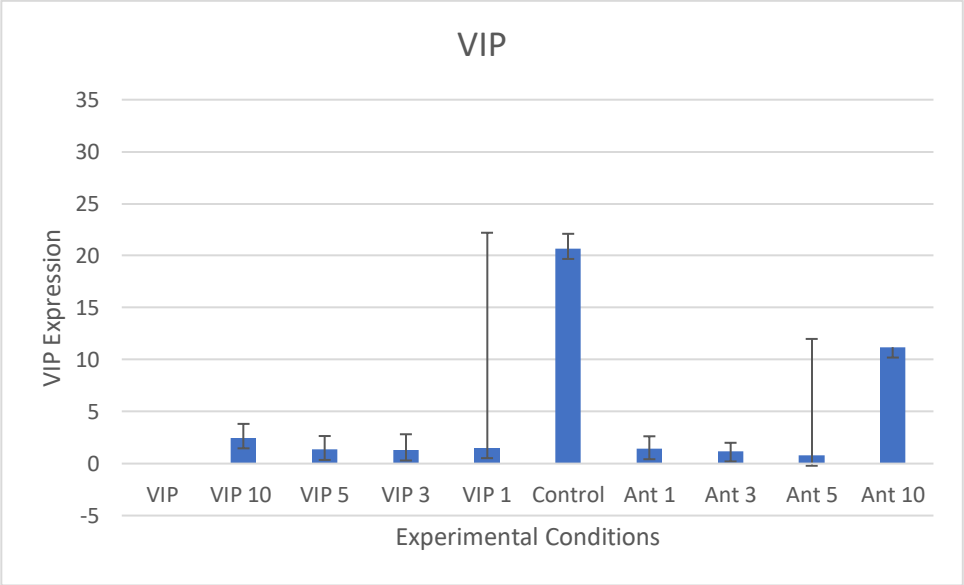


Figure 12 shows VIP expression after quantification of nine western blots of Jurkat T-cells treated with VIP antagonist (ANT 308) and agonist.

4 Discussion

Across all cancers, VIP was found to be associated positively with pathways related to HIF1 signaling, EMT, MAPK signaling, hedgehog, epigenetic & transcription regulation, FGFR, inflammation, and myeloid immune invasion. Negative associations were seen in pathways related to the cell cycle, mTOR signaling, immortality & stemness, interferon response, WNT signaling, and DNA damage repair. A total of 14 mechanisms of interest were identified. These data also demonstrate that VIP mRNA transcription is differentially associated with the 10 Lead gene pathways based on tumor germ layer origin and histology. In particular, the sub-group analysis showed that of the Lead genes, ZEB1, TEK, GMPS, RUVBL1, and TIMELESS showed the greatest shifts in association with VIP (>[.5]) in certain individual tumor histologies, including rectal adenocarcinoma, pancreatic cancer, stomach adenocarcinoma, esophageal cancer, colon adenocarcinoma, and mesothelioma from the normalized R-values of all cancers

within the respective germinal layer. Based on the above, we propose that VIP signaling is associated with **EMT, Cell Cycle, and DNA Damage repair**, and that further studies interrogating VIP signaling to evaluate the effect on these pathways is necessary.

ZEB1

ZEB1 is a transcription factor that controls epithelial-to-mesenchymal transition (EMT). ZEB1 is expressed in many human cancers where it is thought to be migration, invasion, and metastasis. ZEB1 is a zinc finger and homeodomain protein. ZEB1 and ZEB2 are both apart of zfh transcription factor family (Zhang et al.). The protein-binding domains on ZEB1 and ZEB2 that are important for managing their transcriptional activity include SMAD-, CtBP-, and p300-P/CAF-interaction domains. Unlike other EMT transcription factors, ZEB1 and ZEB2 may have the ability to trigger repression of epithelial genes that encode desmosomes and intermediate filaments which are components of adherens and tight junctions (Drápela et al.). ZEB1 and ZEB2 can upregulate mesenchymal factors such as vimentin, fibronectin, and N-cadherin. Although ZEB1 and ZEB2 are structurally similar, they have different expression domains, activities, and knockout mouse phenotypes.

EMT inducing transcription factors include zinc finger projections (SNAI1 and SNAI2), basic helix-loop-helix transcription factors (TWIST family and E47), and zinc finger and homeodomain proteins (ZEB1 and ZEB2) (Lamouille et al.). An early step in EMT is the loss of E-cadherin function

VIP and Tumor Invasion/Metastasis:

Tumor metastasis is the leading cause of cancer mortality. Tumors start at a primary site. Tumor cells detach from the primary tumor site and initiate the metastatic cascade, which is a multi-step process that involves entry into the vasculature, followed by exit of these dislodged tumor cells from the vasculature into a distant site. These tumor cells proliferate at the distant site, thereby colonizing the distant location (van Zijl, Krupitza and Mikulits).

VIP seems to have varying effects on invasion and metastasis on variety of cancers. Endodermal malignancies such as gastric cancers have increased expression of VIP and VPAC1. High expression of VIP and VPAC1 in the gastric cancer positively correlated with distant metastasis and tumor cell invasion of normal tissue (Tang et al.). VIP and VPAC1 expression cause increased tumor metastasis and invasion by increased Ca2+ entry into the cells mediated by TRPV4. Ca2+ entry in turn contributes to tumor metastasis. VIP/VPAC1/TRPV4/Ca2+ signaling axis forms a positive feedback loop in that increased Ca2+ causes increase in VIP secretion in gastric tumor. In addition, TRPV4 has been recently implicated to inactivate PTEN tumor suppressor gene involved the PI3K pathway, thereby drive oncogenesis in colon carcinoma models (Liu et al.).

VIP has tumor-dependent effects on the tumor metastasis and invasion. VIP has shown to down-regulate metastatic potential in endodermal malignancies while in mesodermal and ectodermal malignancies VIP has shown to increase metastatic potential. However, it is clear that VIP signaling pathway has regulatory effects on tumor metastasis and invasion. Therefore, therapeutically, effects of VIP agonists and antagonists on VIP signaling pathway hold a promise for future research.

Overall, this data strongly supports that VIP has broader mechanistic roles – apart from T-cell suppression and MDSC activation – in cancer that are undiscovered. For instance, it may work upstream of ZEB1 to regulate EMT. The results demonstrate autocrine signaling between VIP expression and cancer associated gene pathways. ZEB1 is a known EMT (epithelial mesenchymal transition) regulator, and our in vitro studies novelly link VIP to EMT and demonstrate that this pathway is therapeutically targetable using a VIPR inhibitor. **Figure 11** shows a proposed mechanism by which VIP and ZEB1 may interact. Additionally, The VIP pathway may implicate other genes such as ZEB1, however this pathway remains largely uncharacterized.

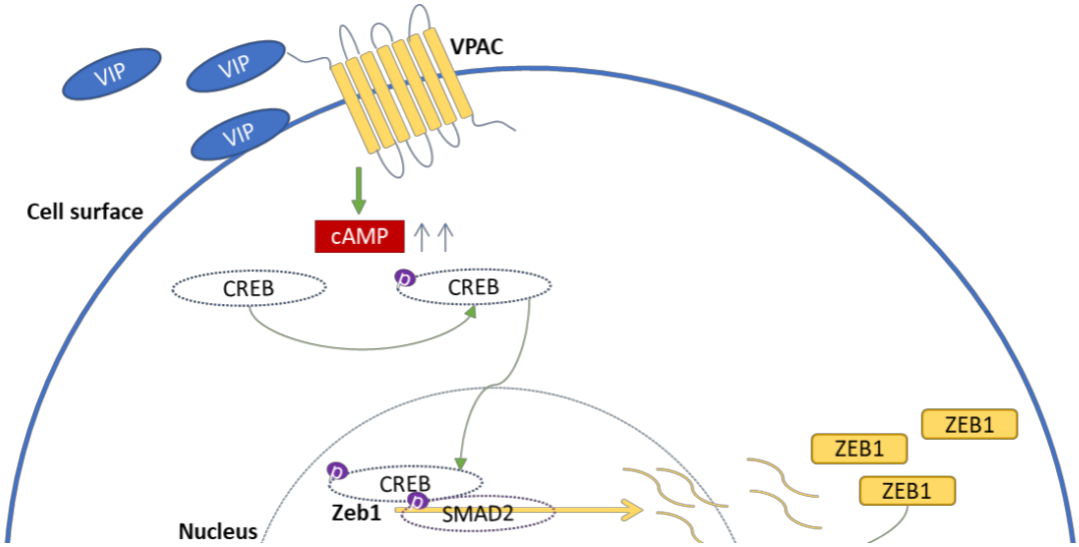


Figure 11 shows a proposed mechanism for VIP and ZEB1 signaling.

Limitations to this work include the pre-defined subset of genes used, which may miss other associations. VIP was also not associated [>.2] for all genes categorized under a pathway per the Nanostring panel. The definition of primary and secondary genes and need for at least 1 primary gene per pathway of interest excludes certain pathways for which associations secondary genes were identified but lacked a primary gene (T-cell exhaustion, Cell adhesion and mobility). However, our methodology represents an initial effort to identify genes of interest, and further

studies are going to look at this more broadly across all mRNA sequenced genes using LinkedOmics. Another limitation of study is that TCGA PANCAN dataset underrepresented certain cancers, such as melanomas. In future studies, we would delve into broader datasets with represent a newer variety of cancers. Finally, repeating this experiment in a GI cancer cell line would be the next step, as restricting the in vitro studies to Jurkat T cells has been another limitation of this work. Of interest are cell lines HGT1 and HT29 as they are GI cancer cell lines that produce quantifiable amounts of both VIP and ZEB1.

As this is hypothesis generating research, there are many directions in which these results can be taken. Further analysis into tumor group may help localize any relationships between primary genes and VIP witnessed by tissue histology. Additionally, studying survival impact, repeating in vitro studies in a GI cancer cell line, delving further into mechanisms in which VIP showed meaningful associations with, and looking into cancer types not studied using the TCGA Pan-Can database are among some next steps.

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