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Galectin-3 Enhances the Malignant Melanoma Phenotype by Regulating Autotaxin

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GALECTIN-3 ENHANCES THE MALIGNANT MELANOMA PHENOTYPE BY REGULATING AUTOTAXIN

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Dedication

I would like to dedicate this dissertation to my mother, Sharon Newsom, for her unwavering support and my father, Ronald Braeuer, for instilling a strong work ethic into my life.

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In melanoma patient specimens and cell lines, the over expression of galectin-3 is associated with disease progression and metastatic potential. Herein, we have sought out to determine whether galectin-3 affects the malignant melanoma phenotype by regulating downstream target genes. To that end, galectin-3 was stably silenced by utilizing the lentivirus-incorporated small hairpin RNA in two metastatic melanoma cell lines, WM2664 and A375SM, and subjected to gene expression microarray analysis. We identified and validated the lysophospholipase D enzyme, autotaxin, a promoter of migration, invasion, and tumorigenesis, to be down regulated after silencing galectin-3. Silencing galectin-3 significantly reduced the promoter activity of autotaxin. Interestingly, we also found the transcription factor NFAT1 to have reduced protein expression after silencing galectin-3. Electrophoretic mobility shift assays from previous reports have shown that NFAT1 binds to the autotaxin promoter in two locations. ChIP analysis was performed, and we observed a complete loss of bound NFAT1 to the autotaxin promoter after silencing galectin-3 in melanoma cells. Mutation of the NFAT1 binding sites at either location reduces autotaxin promoter activity. Silencing NFAT1 reduces autotaxin

expression while over expressing NFAT1 in NFAT1 negative SB-2 melanoma cells induces autotaxin expression. These data suggest that galectin-3 silencing reduces autotaxin transcription by reducing the amount of NFAT1 protein expression. Rescue of galectin-3 rescues both NFAT1 and autotaxin. We also show that the re-expression of autotaxin in galectin-3 shRNA melanoma cells rescues the angiogenic phenotype *in vivo*. Furthermore, we identify NFAT1 as a potent inducer of tumor growth and experimental lung metastasis. Our data elucidate a previously unidentified mechanism by which galectin-3 regulates autotaxin and assign a novel role for NFAT1 during melanoma progression.

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CHAPTER 1:

Introduction and Background

Melanoma Incidence

Melanoma is the deadliest form of skin cancer. In 2012 alone, it is estimated that another 76,250 individuals will acquire melanoma and 9,180 deaths will occur [1]. Cutaneous melanoma comprises for 5% of all new male cancer patients, and the fifth most prevalent following prostate, lung, colon, and urothelial cancer respectively. The incidence in women for 2012 is lower and comprises 4% of all cancer patients with only breast, lung, colon, uterine, and thyroid cancer being more prominent [1]. Cutaneous melanoma affects all races and ethnicities; however, in the U.S., Non-Hispanic whites are most likely to acquire the disease over their lifetime, followed by Hispanics, Native Americans, Asians, and African Americans. Interestingly, males have a higher incidence rate compared to females in all ethnicities [2].

Clinical Staging and Survival

In the clinic, melanoma is classified into four distinct stages. The current staging system is based upon a few criteria; tumor thickness, number of lymph node metastasis involved, and the presence of distant metastasis [3]. Primary tumors with no identifiable metastasis are categorized in the first two stages; stage I and II. Patients with stage I melanoma are presented with primary tumors with a thickness of less than 2 mm. Stage I patients are further sub classified into IA and IB. IA tumors are less than 1 mm thick with no ulceration and have less than 1 mitotic cell per mm^2. Although IB tumors are also classified as less than 1mm thick, these tumors are either ulcerated or have greater than 1 mitotic cell per mm^2. Stage IB

includes T2a melanoma which is a tumor with a Breslow thickness of up to 2 mm; however, no ulceration is present. In these tumors, the mitotic rate has become a reliable predictor for patient survival with lower mitotic rates having a better prognosis [4]. Stage II melanoma patients are sub classified into IIA, IIB, and IIC. These sub-stages are classified based on the thickness of tumors ranging from 1mm to greater than 4mm, and whether ulceration is present (Table 1) [3]. The survival rate of patients with primary melanomas varies greatly. Stage IA and IB patients have a 10 year survival rate of greater than 80%. However, Stage II melanoma patients have a 10 year survival rate of approximately 40%-70% with the poorest prognosis being associated with ulcerated primary tumors that are greater than 4 mm thick [3]. Although tumor thickness could be seen as the likely cause for such variation in survival, the mitotic rate and the presence of ulceration are also important in predicting the outcome of patients and are both independent predictors of survival [3, 5]. In 't Hout *et al.* has reported that the Melanoma-specific 10 year survival rate of patients with or without ulceration is 62% and 81% respectively. Patients with ulcerations that are greater than 5 mm in diameter have a 33% chance of survival at 10 years as compared to 69% in ulcerated tumors less than 5mm wide. Interestingly, in their study, the mitotic rate was significantly associated with the presence of ulceration [5]. Stage III melanoma classifies patients with regional lymph node or in transit/ satellite metastasis. These patients are further subgrouped into stage IIIA, IIIB, and IIIC. The criteria for classification include the number of regional lymph nodes involved, the size of the lesion within the node (micro- vs. macrometastasis), and whether in transit metastasis is observed (Table 1). As expected, patients that have macrometastasis or multiple lymph nodes involved have a poorer 10 year survival rate of approximately 25-35% as compared to patients with one or two lymph nodes involved with micrometastasis (approximately 45-65%) [4].

Patients with Stage IV disease are presented with distant metastasis to organs such as the lung, liver, and brain. These patients have the worst prognosis with a 10 year survival rate of less than 20% [4].

Melanoma Development

Melanoma is thought to develop in a stepwise manner. The initial event during this process is the proliferation of normal melanocytes. These benign nevi present slightly raised lesions in the skin with uniform coloration and histology specimens show an increase in the number of melanocytes laying near the basement membrane [6]. Next, aberrant uncontrolled growth of the benign nevus occurs to develop a dysplastic nevus with random atypia. Random atypia is generally classified as sporadic cells with enlarged and abnormal nuclei. Clinically these lesions can appear asymmetric with multiple colors [6]. The cells then acquire the ability to divide and spread throughout the epidermis called the radial-growth phase (RGP). The cells now show continuous atypia throughout the lesion, and clinically can sometimes be observed as raised lesions. Although a few cells can penetrate into the dermis, they fail to form colonies in soft agar *in vitro* [6]. However, as the tumor progresses, more cells invade into the dermis, proliferate, and form a lesion beyond the basement membrane border of the epidermis. This is termed the vertical-growth phase (VGP). These cells can grow in soft agar and are tumorigenic when implanted in nude mice [6]. The final step of the primary tumor is for melanoma cells to enter the lymphatic system and drain to local (sentinel) lymph nodes or intravasate into the vasculature and circulate, survive, and proliferate in distant organ sites termed metastasis.

A.

TNM Staging Categories for Cutaneous Melanoma

Classification	Thickness (mm)	Ulceration Status/Mitoses
T		
Tis	NA	NA
T1	≤ 1.00	a: Without ulceration and mitosis < 1/mm²
		b: With ulceration or mitoses ≥ 1/mm²
T2	1.01-2.00	a: Without ulceration
		b: With ulceration
T3	2.01-4.00	a: Without ulceration
		b: With ulceration
T4	> 4.00	a: Without ulceration
		b: With ulceration
N	No. of Metastatic Nodes	Nodal Metastatic Burden
N0	0	NA
N1	1	a: Micrometastasis
		b: Macrometastasis
N2	2-3	a: Micrometastasis
		b: Macrometastasis
		c: In transit metastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes	
M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

B.

Anatomic Stage Groupings for Cutaneous Melanoma

	Clinical Staging				Pathologic Staging		
	T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Any T	N > N0	M0	IIIA	T1-4a	N1a	M0
					T1-4a	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4a	N1b	M0
					T1-4a	N2b	M0
					T1-4a	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0
					T1-4b	N2c	M0
					Any T	N3	M0
IV	Any T	Any N	M1	IV	Any T	Any N	M1

Table 1. Tumor Staging and Classification

(A) The melanoma TNM categories are tumor thickness (T), lymph node involvement and size of metastasis (N), and location of metastasis (M). (B) Stages I-IV are classified based on the TNM categories. Adapted with permission from Balch CM et al, J Clin Oncol 2009.

Genetic Alterations during Melanoma Progression

Throughout melanoma progression, multiple genetic and epigenetic events occur that allow for the development of cutaneous melanoma and ultimately metastasis. For example, the genetic alterations in genes such as BRAF, NRAS, PTEN, CDKN2A, and cyclin D1 have integral roles in the transition of benign nevi to premalignant lesions (Figure 1). BRAF is a member of the RAF family and acts on the map kinase (MAPK), RAS-RAF-MEK-ERK, pathway [7]. The V600E activating mutation within the kinase activation domain of BRAF occurs in approximately 40-60% of melanoma patients [8, 9]. This is the most prevalent mutation in melanoma and indicates an important role for the MAPK pathway in melanoma progression. Not surprisingly, NRAS, the upstream molecule which activates BRAF, is also mutated in melanoma patients. However, it is only mutated in approximately 20%-30% of melanoma patients [10, 11]. These two mutations are mutually exclusive from each other, and approximately 20-40% of patients do not have either BRAF or NRAS mutations. Although the MAPK pathway seems essential for melanoma development, reports have shown that mutations in BRAF occur in 80% of melanocytic nevi, yet, all of these nevi do not progress into primary melanomas [12]. It has also been reported that the introduction of $BRAF^{v600E}$ in melanocytes can induce cell senescence and apoptosis [13]. This is counterintuitive to the data that overwhelmingly implies $BRAF^{V600E}$ is critical for melanoma progression. This can be explained by acknowledging that other molecules cooperate with BRAF to release cells from senescence and continue with uncontrolled growth. Indeed, the tumor suppressor gene cyclin dependent kinase inhibitor 2A (CDKN2A) has been found to inhibit $BRAF^{V600E}$ induced growth. Through alternative mRNA splicing, this gene encodes both $p16^{Ink4A}$, an inhibitor of the cyclin D/CDK4 complex, and the alternate open reading frame $p14^{ARF}$, an inhibitor of the

p53 regulator MDM2 [7, 14, 15]. The levels of $p16^{Ink4A}$ expression in melanocytic nevi is greatly increased as compared to normal skin, therefore potentially responding to and restricting the proliferative effects of $BRAF^{v600E}$ [16]. Mutations in the $p16^{Ink4A}$ gene have been reported in 7% of primary melanomas and 14% of metastatic lesions [17]. The CDKN2A gene is more frequently associated with mutations in patients with family history of melanoma, and sun exposure. Other genetic events involved in melanoma progression greatly influence their likelihood of developing the disease [18, 19]. These other genetic events could include BRAF and NRAS activating mutations. Without $p16^{Ink4A}$ acting as a "brake" in these patients to induce senescence, melanocytic nevi could potentially respond to MAPK activation and progress to melanoma. One report indicates that this is true in the clinic as promoter methylation of p16 correlated significantly with NRAS mutations [20]. However, another clinical study suggests that there is no correlation between CDNK2A gene deletion and BRAF/NRAS mutations [21]. Yet, the latter study did not indicate the presence of methylation or gene expression of CDNK2A in patients that did not have genetic deletion. Nonetheless, the release of MAPK induced senescence is most likely attributed to other genes as well as CDKN2A.

The development of melanoma has also been associated with the loss of PTEN. PTEN acts as a phosphatase to remove phosphates from lipids such as phosphatidylinositol phosphate (PIP_3) which acts as an intracellular signal induced by growth factors or other extracellular stimuli [6, 22]. PIP_3 then recruits phosphoinositide-dependent kinase 1 (PDK1), which then phosphorylates the survival factor AKT [23]. PIP_3 (PtdIns(3,4,5)) is converted back to PIP_2 (PtdIns(4,5)) by PTEN, thus inactivating the AKT signaling cascade [22]. Initial studies identified that chromosomal deletion on 10q occurred in 30-50% of melanomas [24, 25]. Later

it was discovered that the tumor suppressor PTEN (hence its name; phosphatase and tensin homolog deleted from chromosome ten) was located within this region [26]. Further studies demonstrated that mutation/deletion rates of PTEN occur in 30-40% and 10% of cell lines and primary melanomas respectively [27, 28]. By using immunohistochemistry, it was observed that 90% of the melanomas with no PTEN expression had no mutation or deletion, indicating its loss of expression is also attributed to epigenetic regulation and transcriptional repression [29]. The effect of PTEN loss in melanoma contributes to cell survival and proliferation primarily through AKT activation. Three isoforms of AKT exist with >80% amino acid homology; AKT1, AKT2, AKT3 [30]. Phosphorylation of AKT is increased in the transition from dysplastic nevi to primary melanomas [30]. This phosphorylation affects multiple processes. The up-regulation of N-cadherin and its intracellular interaction with AKT can lead to the inactivation of the pro-apoptotic molecule BAD, thus promoting survival of melanoma cells [31].

The up regulation of NFκB is associated with AKT activation in melanoma. AKT phosphorylates and activates IKKβ which in turn phosphorylates the inhibitor of NFκB, IκB, to allow for NFκB transcriptional activation and subsequent transcription of pro-tumorigenic and angiogenic genes such as IL-8, VEGF, Cox-2, Bcl-2, and MMPs [32-34]. IκBα-transfected melanoma cells decreased tumor size and experimental lung metastasis [35]. The microvascular density was reduced in these tumors as well as the expression of both IL-8 and VEGF [35].

During the transition from the RGP to the VGP, the acquisition of the metastatic melanoma phenotype correlates with the loss of the transcription factor activator protein 2 alpha (AP-2α). In less metastatic melanoma cells, AP-2α is highly expressed, while its

expression is significantly reduced, if not completely lost, in metastatic melanoma cells [36]. The expression of a dominant negative AP-2α in low-metastatic SB-2 cells increased tumor growth and MMP-2 expression *in vivo* and enhanced their migratory phenotype *in vitro* [37]. Its expression is inversely correlated with that of pro-tumorigenic genes such as the membrane adhesion molecule MCAM/MUC18 and the protease activated G-protein-coupled receptor PAR-1 [36, 38]. Indeed, AP-2α binds to the promoters of both genes and suppresses their transcriptional activity [36, 38]. However, as melanoma progresses and the loss of AP-2α occurs, the expression of MCAM/MUC18 and PAR-1 expression increases. The cell adhesion molecule MCAM/MUC18 is an important mediator of melanoma progression and silencing MCAM/MUC18 expression by lentiviral shRNA has shown a significant reduction in melanoma cell migration, invasion, MMP-2 expression, and tumor growth and metastasis [39]. PAR-1 is an important inflammatory molecule that promotes normal platelet aggregation through its cleavage on the extracellular domain by thrombin which acts as a “tethered ligand". In melanoma, PAR-1 signaling is important for tumor growth and metastasis by enhancing vascular endothelial growth factor (VEGF) and MMP-2 expression within the tumor microenvironment, and increasing the expression of another pro-tumorigenic gene, Connexin-43, while suppressing the tumor suppressor gene Maspin [40-42].

Another transcription factor that plays a crucial role during the transition from RGP to VGP is the transcription factor c-AMP response element-binding protein CREB (Figure 1). Although studies in our lab have shown that CREB protein expression does not change significantly in non-metastatic vs. metastatic cells, its phosphorylation and activation is increased in metastatic melanoma [43]. This could be due to multiple factors. One of these factors is the ability to respond to signals within the tumor microenvironment. CREB in the

highly metastatic A375SM cell line is phosphorylated in the presence of the platelet activating factor receptor (PAFR) ligand platelet activating factor (PAF) [44], while PAF did not increase CREB phosphorylation in low metastatic SB-2 cells [45]. PAFR levels were similar in both cell lines, suggesting that intermediate signaling proteins are absent in less metastatic cells which results in reduced CREB activity. Once activated, CREB induces the expression of multiple pro-tumorigenic genes including MUC18 and MMP-2 [46]. CREB also inhibits the expression of genes during melanoma progression. One of these genes is CYR61. Silencing CREB results in increased expression of CYR61 and reduced motility and invasion *in vitro* and tumor growth in metastasis *in vivo*. The over expression of CYR61 resulted in reduced invasion *in vitro*, and decreased tumor growth and metastasis *in vivo* [43]. CREB can act as a survival factor in melanoma as cell. Over expressing a dominant negative form of CREB increased melanoma cell susceptibility to apoptosis [47]. We have also shown that silencing CREB increases the cell cycle inhibitor $p21^{waf1}$. Increased CREB activity during melanoma progression directly suppresses AP-2α expression. AP-2α is a known positive regulator of $p21^{waf1}$. Therefore, CREB has a profound effect on melanoma cells by regulating other transcription factors that regulate multiple genes involved in melanoma progression [48].

Another member of the CREB family, activating transcription factor-2 (ATF-2), has been implicated in melanoma [49, 50]. Once activated, ATF-2 leads to the deregulation of c-Jun, cyclin A, and TGFβ to induce cell growth and melanoma progression [51-54]. Inhibiting ATF-2 can significantly reduce the tumorigenic and metastatic potential of melanoma cells [50].

The deregulation of the transcription factors SNAIL and SLUG also promote melanoma progression. These transcription factors are known to negatively regulate E-cadherin, a

molecule lost during melanoma progression, and their over expression in melanoma cell lines results in reduced levels of E-cadherin [55]. By using siRNA to target SLUG, it was demonstrated that the expression of SLUG is required for melanoma cell invasion [46]. Silencing SLUG also increases melanoma cell susceptibility to chemotherapeutic drugs such as cisplatin and fotemustine [56].

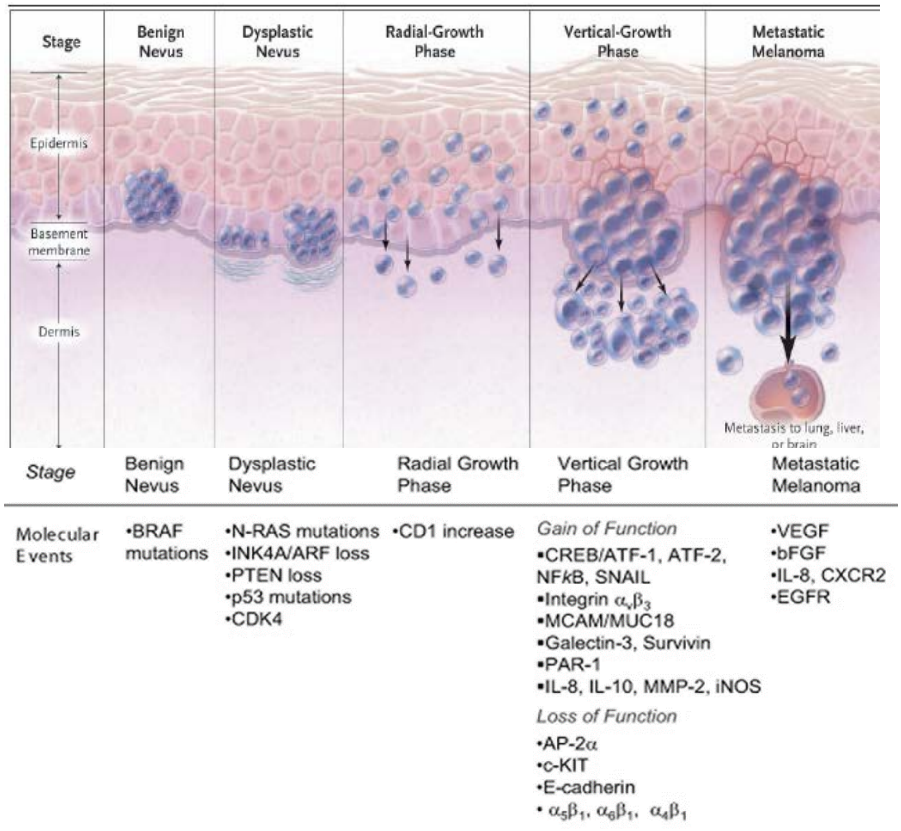


Figure 1. Molecular and Genetic Changes during Melanoma Progression

The progression of melanoma is a stepwise process. From benign nevus to dysplastic nevus genetic mutations occur within the BRAF or NRAS genes. The loss of PTEN or $p16^{INK4A/ARF}$ expression are early events in a subset of melanomas. The tumor then grows radially throughout the epidermis termed the radial growth phase. The acquisition of multiple factors such as CREB and NFκB activation as well as enhanced expression of MCAM/MUC18, PAR1, Il-8, MMP-2 and galectin-3 induce the degradation of the basement membrane and invasion of melanoma cells termed the vertical growth phase (VGP). Finally, a few select melanoma cells intravasate, circulate, and survive in distant organ sites where metastasis forms. Reproduced with permission from Miller AJ and Mihm MC Jr., N Engl J Med 2006, Copyright Massachusetts Medical Society, and Melnikova et al, Cancer Biol Ther 2008.

Status of Current Treatment Modalities for Metastatic Melanoma

The first approved drug for the treatment of malignant melanoma was the DNA damaging compound dacarbazine (DTIC) in 1975 [57]. DTIC or its oral analogue temozolamide remains the standard of care for malignant melanoma. However, the response rate is low at approximately 5-12%. Long term response occurs in less than 2% of patients [58]. High dose IL-2 therapy is another Food and Drug Administration (FDA) approved option for patients with inoperable disease. Unfortunately, only a 16% response rate is observed; however, 6% of the patients have a complete response [59]. The treatment of patients after all melanoma is surgically removed, termed adjuvant therapy, with stage II and III disease is another technique used to reduce the likelihood of disease progression. After primary tumor resection, thick tumors (>4mm) with no sentinel lymph node involvement (Stage II patients) may receive adjuvant therapy such as high dose interferon-α [59]. This therapy may include unwanted side effects. Therefore, the risk of metastasis, potential benefits, and side effects must be included in the decision process. If regional lymph node involvement is found, stage III patients undergo lymphadenectomy followed by consideration of adjuvant therapy.

Recently, clinical trials focusing on intratumoral T-cells and boosting their antitumor activity by targeting the T-lymphocyte associated antigen 4 (CTLA-4) with the blocking antibody, ipilimumab, have shown promise. CTLA-4 expression on T-cells acts as a “break” by recognizing self and inhibiting the autoimmune response. Blocking CTLA-4 results in a more robust T-cell reaction towards melanoma cells [60]. A phase III clinical trial conducted with previously untreated stage IIIC and IV melanoma patients with ipilimumab plus DTIC increased two year survival rate to 28.5% as compared to 17.9% with DTIC alone [61]. These results have led to the FDA approval of ipilimumab for the treatment of advanced melanoma

[62]. Inhibiting another T-cell checkpoint molecule, PD-1, has shown promise in a phase I clinical trial that included melanoma, non-small-cell lung cancer, prostate, or colorectal cancer [63]. Treatment with the anti-PD-1 antibody BMS-936558 generated an objective response rate of 28% (26 of 94 patients). Of these 26 patients, 13 have had a sustained response for over 1 year [63]. Interestingly, the phase 1 study also showed that melanomas that do not express the PD-1 ligand on tumors, PD-L1, have no response to anti-PD-1. Only patients with the disruption of the PD-L1 ligand on tumors cells with PD-1 on T-cells had a response to therapy [63]. A small subset of patients qualify for this treatment regimen (PD-L1 expressing tumors), and according to the trial, only a small percentage of those patients have an objective response. Although modulation of the T-cells are showing modest responses, they are still a step in the right direction in regards to the immunotherapeutic potential for malignant melanoma treatment.

Recently, with the identification of mutated and activated genes in melanoma progression, compounds targeting specific molecules have found their way into clinical trials. Among the compounds garnering the greatest attention are those that target the mutant $BRAF^{V600E}$ mutation. In 2011 the FDA approved the first BRAF inhibitor, vemurafenib (PLX-4032), for the treatment of advanced metastatic melanoma [64]. In 2011, a study was conducted on unresectable stage III and distant metastatic stage IV patients. The response rate was 48% and a median progression free survival of 5.3 months as compared to 1.6 months with DTIC alone [65]. In a more recent study of patients with stage IV disease, vemurafenib had an overall response rate of 53%, progression free survival of 6.8 months, and the overall survival rate at 16 months was 50% [66].

Brain metastasis has been considered a terminal prognosis for melanoma patients with few treatment options. An intact blood-brain barrier would complicate the transfer of drugs into the metastatic lesion. Fortunately, for the treatment of melanoma brain metastasis, tumors tend to create a "leaky" vasculature. Indeed, using another BRAF inhibitor, dabrafenib, in a phase II trial, 20% of melanoma patients had an intracranial response with a duration of 20 and 28 weeks in previously untreated or previously treated brain metastasis patients respectively. Median overall survival of patients with brain metastasis was still rather low at 33 weeks [67]. Nonetheless, targeted therapy for brain metastasis is a promising method of treatment.

Resistance to BRAF inhibitors is almost certain. Usually this is due to the up-regulation of the MAPK pathway by alternative means, such as c-RAF, increased activity of RAS, increased expression of receptor tyrosine kinases (RTKs) like insulin like growth factor receptor (IGFR), or the increased activation of the pro-survival PI3K/AKT pathway [68, 69]. Furthermore, 50% of melanoma patients do not harbor the BRAF mutation, and these patients must not be ignored in regards to targeted therapy. BRAF resistance and non BRAF mutant melanomas have led to the development of MEK and AKT inhibitors. Although in their infancy, phase I trials targeting MEK and AKT are showing promise for the treatment of all melanoma patients [70]. Targeting up-regulated molecules in BRAF resistance will undoubtedly be the next step in the treatment of melanoma patients. The investigation of other molecules and their role in melanoma progression could also lead to new therapeutic targets. In our case we will focus on galectin-3 and further evaluate its effect on the metastatic melanoma phenotype.

Galectins

Structural Properties of Galectins

The evolutionarily conserved galectins share a unique structure termed the carbohydrate recognition domain (CRD) [71] The CRD within the galectin family share characteristic amino acid sequences as well as an affinity for β-galactoside sugars (such as lactose), albeit, at a relatively weak affinity [72, 73]. Galectins bind to cell surface and extracellular matrix proteins at a much higher affinity due to the complex glycans present on proteins [73]. For example, galectin-3 preferentially binds to poly-N-acetyllactosamine-containing ([-3Galβ1–4GlcNAcβ1-]n) glycans which are processed by MGAT enzymes [73]. After the initial transfer of Glc3Man9GlcNAc2 by oligosaccharyltransferase in the endoplasmic reticulum, the glyco-protein is further processed sequentially by MGAT 1, 2, 4 and 5, with removal of mannose groups when needed by the mannosidase enzyme ManII. Finally, in the trans-Golgi, β-galactosidases remove GlcNAc and add N-acetyllactosamine (Figure 2A) [74]. Galectin-3 binds well with glycans containing three to four repeating acetyllactosamines [73]. Other galectins recognize slightly different glycans. Galectin-1 binds to poly-N-acetyllactosamine as well, but requires a terminal β-galactose residue to bind at a high affinity. Although galectins have a similar CRD, the overall protein structure can vary. Therefore, they have been subdivided into three unique groups (Figure 2B). The prototypical galectins contain a single CRD and can form homodimers with itself. These galectins include galectin-1, 2, 7, 10, 13, and 14. Tandem-repeat galectins have two CRDs within the same protein. The CRDs are connected by a small peptide domain that can range from 5 to 50 amino acids in length. Galectin-4, 8, 9, and 12 are all members of the tandem-repeat galectins [73]. Interestingly, the two CRDs on the same protein can have different binding properties. In the case of galectin-8,

the amino-terminal CRD binds to sialylated glycans while the c-terminal CRD has no binding affinity to sialylated glycans [73]. The third group of galectins, the chimeric galectins, is unique in that it currently has only one known family member in vertebrates, galectin-3. The group name implies its protein structure; a molecule with multiple domains that represent different structures. The c-terminal end consists of the CRD domain that is present in all galectins; however, unlike other galectins, it also contains a proline rich collagen like domain, and at the n-terminal end can be post-translationally modified which alters its functional properties [75].

Galectin-1 and Galectin-9 in Cancer

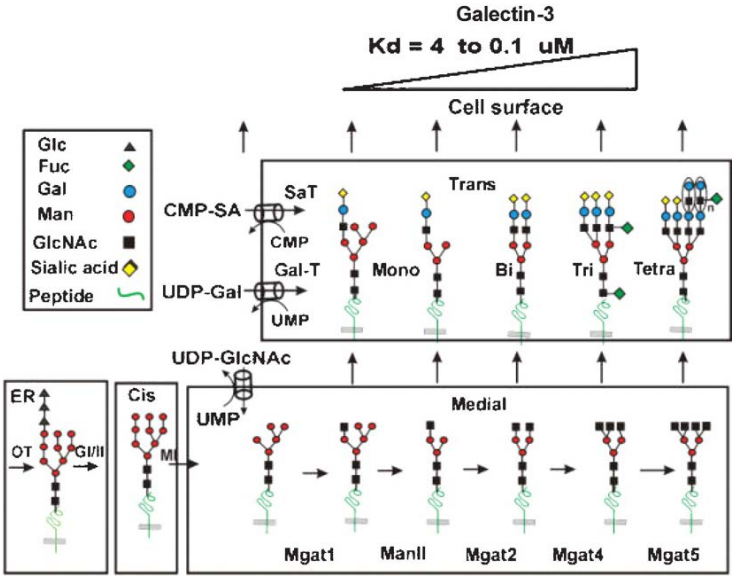
Although there are currently more than 15 galectin family members, relatively few galectins have been extensively studied in cancer. Those that have been studied have shown a variety of effects on tumor development. Besides galectin-3, galectin-1 might be the most studied galectin in cancer. Galectin-1 has been shown to enhance the progression of cervical, lung, ovarian cancer, glioma, and melanoma [76-80]. It can have an effect on tumor growth through intracellular mechanisms or by influencing the tumor stroma and microenvironment. For instance, galectin-1 binds to T-cell membrane glycoproteins. Binding of galectin-1 to the T-cell surface proteins CD7, CD43, and CD45 is necessary for galectin-1 induced apoptosis of activated T cells [81-84]. Silencing galectin-1 in B16F10 murine melanoma cells can significantly reduce tumor growth *in vivo* [85]. However, another group reported that tumor growth is not effected by galectin-1 expression in $Rag^{-/-}$ $Jak^{-/-}$ mice (mice that do not have B- or T-cells) suggesting that galectin-1 affects tumor growth through immunosuppression in this model [86]. Direct interaction with melanoma cells by galectin-1 has also been observed. This

induces melanoma cell aggregation by binding to the 90k/MAC-2BP glycoprotein [87]. Once aggregated, the outer layer of cells act as a barrier protecting this embolic unit in circulation from mechanical and immunological damage. These aggregated cells are more likely to survive and grow in the distant organ parenchyma [88]. Although galectin-1's primary function is carbohydrate binding dependent, intracellular roles have also been established. The interaction of galectn-1 with RAS molecules has been extensively studied by Yoel Kloog and others. For instance, the activated, GTP loaded HRAS briefly binds to galectin-1 at the cellular membrane creating nanoclusters of HRAS. This signal is then propagated to RAF and the downstream MAPK pathway [89]. Galectin-1 is essential for membrane localization of HRAS. Silencing galectin-1 with shRNA results in a dispersed HRAS throughout the cytoplasm and galectin-1 antisense reduced the number of HRAS transformed Rat-1 cells [90]. Although, this has significant implications in other cancers, these studies are less important in melanoma. NRAS is the RAS family member commonly deregulated during melanoma progression and it has been reported that this nanoclustering phenomenon does not occur with NRAS. Furthermore, with 50% of melanoma patients harboring an activating mutation in the downstream target of NRAS, BRAF, it is less likely that galectin-1 contributes to melanoma growth by this manner. Yet other roles such as cell aggregation, immune suppression, and cell survival have been established which could potentially enhance melanoma development and chemotherapeutic resistance. Interestingly, galectin-1 can be found in the nucleus where it binds to the spliceosome complex, and enhances splicing of mRNA substrates [91]. However, currently, no known specific pre-mRNA targets have been established. Identifying whether galectin-1 enhances the expression of pro-tumorigenic genes through mRNA splicing must be further studied in melanoma. Cell survival and the inhibition of apoptosis has become a critical

problem in regards to chemotherapy. Silencing galectin-1 renders B16F10 cells more sensitive to temozolomide treatment *in vitro*, thus, targeting galectin-1 in melanoma could prove useful [92].

Not all galectins have a pro-tumorigenic effect. In melanoma, reduced galectin-9 expression significantly correlates with lymph node metastasis, and galectin-9 expressing primary tumors were less likely to metastasize [93]. Furthermore, the 5 year overall survival was significantly better in patients whose primary melanomas expressed high levels of galectin-9 [93, 94]. *In vitro* galectin-9 induces aggregation of melanoma cells through its CRD [93]. However, *in vivo*, galectin-9 secretion from melanoma cells reduced the number of experimental lung metastasis [93]. Intravenous injection of galectin-9 also resulted in fewer metastatic lung colonies of B16F10 melanoma cells [95]. *In vitro* the authors show that melanoma cell binding to the cell adhesion molecule commonly located on endothelial cells, VCAM-1, is reduced in the presence of exogenous galectin-9. This phenomenon is carbohydrate binding dependent as the melanoma cell binding to VCAM-1 is rescued with the addition of lactose [95]. Galectin-9 also inhibited melanoma cell adhesion to collagen I, IV, fibronectin, and laminin coated plates [95]. Therefore, galectin-9 might prohibit melanoma cell adhesion to endothelial membrane proteins and extracellular proteins rather than "bridge" them together, thus, inhibiting the migratory and metastatic phenotype of melanoma.

A.


Galectin-3
Kd = 4 to 0.1 uM
Cell surface
Glc
Fuc
Gal
Man
GlcNAc
Sialic acid
Peptide
CMP-SA
SaT
CMP
UDP-Gal
Gal-T
UMP
Trans
Mono
Bi
Tri
Tetra
UDP-GlcNAc
UMP
ER
OT
GI/II
Cis
MI
Medial
Mgat1
ManII
Mgat2
Mgat4
Mgat5

B.

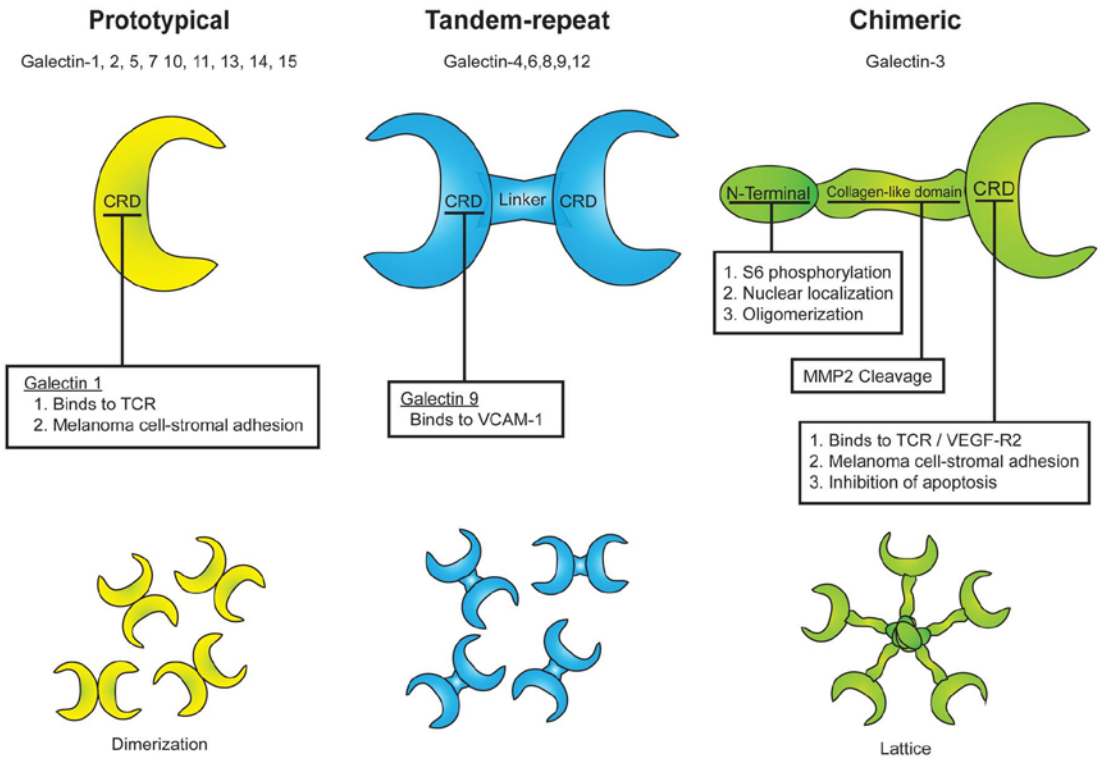

Prototypical
Galectin-1, 2, 5, 7 10, 11, 13, 14, 15
CRD
Galectin 1
1. Binds to TCR
2. Melanoma cell-stromal adhesion
Tandem-repeat
Galectin-4,6,8,9,12
CRD
Linker
CRD
Galectin 9
Binds to VCAM-1
Chimeric
Galectin-3
N-Terminal
Collagen-like domain
CRD
1. S6 phosphorylation
2. Nuclear localization
3. Oligomerization
MMP2 Cleavage
1. Binds to TCR / VEGF-R2
2. Melanoma cell-stromal adhesion
3. Inhibition of apoptosis
Dimerization
Lattice

Figure 2

Figure 2. Carbohydrate Binding and Structural Properties of Galectin-3 and its Family Members

(A) Oligosaccharyltransterase (OT) transfers the glycan to N-X-S/T motifs in the endoplasmic reticulum. The glycol protein is then transported to the Golgi where it is further modified in the cis-, medial- and trans- Golgi. The disassociation constant (K_d) is shown for galectin-3 and the processed glycans. The lower the K_d in μM, the tighter the bond. (B) The three different types of galectins are shown with the corresponding galectins within each group. An example of each group and their functions are given. Prototypical galectins have one CRD and dimerize together. Tandem-repeat galectins have two CRD that are linked by short peptide. Galectin-3 belongs in the chimeric group in which it contains one CRD, a collagen like domain, and an N-terminal domain. Adapted from Lau KS and Dennis JW, Glycobiology 2008 and Braeuer RR et al, Pigment Cell and Melanoma Res 2012.

Galectin-3 in Cancer

Cell Adhesion, Invasion, and Angiogenesis

The glycosylation process by mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase (MGAT) enzymes is critical for galectin-3 binding to β-galactosides. Galectin-3 can either homodimerize with itself at the N-terminal domain, or can form a pentameric structure when binding to multiple glycans [94] Silencing MGAT1 in HeLa and PC-3 cells significantly reduced their migratory phenotype. MGAT1 shRNA also resulted in significant reduction of PC-3 orthotopic tumor growth and lung metastasis in nude mice [96]. MGAT5 silencing in PC-3 cells has shown a marked reduction in their invasive phenotype as well as reduced tumor growth after orthotopic injection in the prostate glands of mice [97]. Studies have shown that highly metastatic B16F10 murine melanoma cells have high levels of branched N-oligosacharides as compared to less metastatic B16F1 cells [98]. When incubated on lung tissue specimens, B16F10 melanoma cells adhered at a much higher affinity as compared to B16F1. The lung specimens also had higher levels of galectin-3, and the authors postulate that galectin-3 in the lung parenchyma is important for the attachment of melanoma cells to the lung endothelium [98]. This hypothesis is supported with galectin-3 knockout mice by which B16F10 lung metastasis is significantly reduced. They reported that host galectin-3 is required for melanoma cell extravasation into the lung and binding of B16F10 melanoma cells to tissue sections from Gal-$3^{-/-}$ mice was also less as compared to wild type mice [99]. Galectin-3 has a profound effect on immunosurveillance of tumor cells. Exogenous galectin-3 can induce T-cell apoptosis [100]. CD45 expression on the surface of T-cells increases the apoptotic sensitivity [101]. Galectin-3 also binds to and reduces the association of T-cell receptors (TCR) with CD4 or CD8 co-receptors. The inhibition of galectin-3 with modified

citrus pectin increased the expression of IFN-γ, IL-2, and tumor necrosis factor (TNF) by tumor infiltrating lymphocytes (TILs) (Figure 3) [102].

The expression of galectin-3 is not limited to the stromal tissue. Its expression is also found in tumor cells. High levels of immunohistochemical staining have been correlated with the progression of melanoma, glioma, breast, colon, and thyroid cancer [103-106], and its expression in melanoma tumors isolated from patients can be found in both the cytoplasm and nuclear compartments [103]. Galectin-3 has a prominent role in cancer cell motility. Silencing galectin-3 in B16F10 murine melanoma, and pancreatic cancer cell lines significantly reduces their migratory phenotype as analyzed by the *in vitro* wound healing scratch assay [107, 108]. The overexpression of galectin-3 in colon cancer DLD-1 cells increased motility, lamellipodia formation, and the rate of wound closure as observed by the scratch assay [109]. MAPK phosphorylation was increased in a KRAS dependent manner in DLD-1 cells when galectin-3 is overexpressed. Silencing KRAS reduced the motility of galectin-3 overexpressed DLD-1 cells [109]. The link between KRAS mediated motility and galectin-3 is not surprising. Galectin-1 has already been shown to nanocluster HRAS and galectin-3 has been reported by the same group to bind with KRAS [110]. The CRD of galectin-3 could potentially bind to the farnesyl group of KRAS. Changing this hydrophobic pocket with a V125A substitution rendered the over expression of galectin-3 ineffective on KRAS nanoclustering. More interestingly, silencing of galectin-3 reduced the amount GTP loaded KRAS [111]. This could be a significant finding for cancers such as thyroid, colon, and pancreatic cancer. However, like galectin-1, galectin-3 has far less of an effect on NRAS nanoclustering [112]. Its effect on motility is not only attributed by RAS signaling. Galectin-3 co-localizes with N-cadherin in cancer cells, and this lattice structure is blocked with the addiction of lactose or MGAT5

siRNA [113]. Therefore, extracellular galectin-3 contributes to N-cadherin localization at lipid rafts and could contribute to N-cadherin turnover and migration [113].

The transition from the radial growth phase to the vertical growth phase requires more than just a migratory phenotype. The cells must degrade the basement membrane and the extracellular matrix in order to invade through the tissue and extravasate into the vasculature. Indeed, galectin-3 has been shown to affect the expression of matrix metalloproteinases, proteins involved in breaking down the extracellular matrix. Silencing galectin-3 in B16F10 melanoma cells reduces MMP-1 expression and results in decreased invasion through Matrigel [107]. Another matrix metalloproteinase, MMP-2, has reduced activity in galectin-3 silenced C8161 melanoma cells as observed by the zymography assay [114].

Interestingly, the collagen like domain of galectin-3 can be cleaved by MMP-2 between G^{32}-A^{33} and A^{62}-Y^{63} resulting in 27 and 22 kDa peptides. This has been considered as a potential tool for distinguishing between pro- or active MMP-2 in patient specimens by staining for cleaved galectin-3 [115]. Moreover, cleavage of galectin-3 by MMP-2 is functionally relevant in tumor growth. Cleavage resistant galectin-3 transfected BT-549 cells resulted in reduced tumor growth and angiogenesis *in vivo* compared to BT-549 with cleavable galectin-3 [115]. The authors identified that cleaved galectin-3, specifically peptides 1-62 and 33-250, acts as a migratory chemoattractant for endothelial cells [116]. Thus, cleavage of galectin-3 might contribute to angiogenesis. Others have shown that exogenous galectin-3 can induce VEGF expression in an AKT dependent manner and this contributes to tube like formation *in vitro*. However, they did not analyze whether whole or cleaved galectin-3 is responsible, nor is the mechanism by which galectin-3 contributes to AKT activity investigated [117]. It has also been suggested that galectin-3 binds to VEGFR2 on endothelial cells to prolong VEGF

signaling and enhance angiogenesis [118]. Galectin-3 can also enhance VEGF and bFGF mediated tube like formation of HUVEC cells *in vitro* by binding to integrin αvβ3 in a carbohydrate dependent manner. Blocking integrin αvβ3 or galectins-3 with lactose reduces the effect of VEGF and bFGF on angiogenesis [119]. Galectin-3 can also affect tube like formation of melanoma cells. As shown with C8161 cells, silencing galectin-3 results in reduced tube like formation on Matrigel coated wells [114].

Anti-Apoptotic Properties of Galectin-3

Targeting cancer cells by cytotoxic drug can be an effective chemotherapeutic approach to treating cancer patients. Unfortunately, the results are temporary or ineffective due to the inhibition of cancer cell apoptosis by various molecules. The intrinsic apoptotic pathway results in increased cytochrome-c release from the mitochondria. Cytochrome-c then binds with APAF1 to generate an apoptosome. Caspase-9 activation occurs and subsequent caspase-3 cleavage initiates cell death [120, 121]. This cell death pathway can be prevented by the inhibition of cytochrome-c release. The BCL-2 family of proteins are known to act as "gatekeepers" to prevent cytochrome-c release from the mitochondria [122]. Galectin-3 shares a similar NWGR motif that is located on the BH1 domain of the BCL-2 family [123, 124]. Phosphorylation of galectin-3 by casein kinase I at Ser6 has a significant effect on its glycan binding and apoptotic properties. Once phosphorylated, galectin-3 binds at a much lower affinity to extracellular proteins [125], and increases its anti-apoptotic function [126]. Under chemotherapeutic stress, endogenous galectin-3 in breast cancer cells is transported from the nucleus to the cytoplasm to inhibit apoptosis, however, Ser6 mutants remained within the nucleus and did not promote cell survival [127]. Phosphorylation at Ser6 is required for export

from the nucleus and into the cytoplasm where it can act as an anti-apoptotic molecule [128]. This suggests that post-translational modification of galectin-3 has a profound effect on its functional role and cellular localization during cancer progression. Galectin-3 located at the surface can also enhance cell survival by inhibiting TRAIL-induced caspase-8 activation. TRAIL resistant cells have higher levels of galectin-3 that co-localize with TRAIL receptors at the plasma membrane [129]. In response to TRAIL, the receptors DR4 and DR5 are internalized. In the presence of galectin-3, the receptors remain located on the cell surface with the addition of TRAIL and apoptosis is less likely to occur [129]. Whether galectin-3 inhibits the initial activation of the death receptors by blocking TRAIL binding or through glycan branching of DR4 and DR5 thus preventing internalization remains unknown. More studies must be performed to identify how galectin-3 inhibits TRAIL induced cell death.

The Potential of Treating Cancer by Targeting Galectin-3

As galectin-3 acts as a pro-tumorigenic molecule, it seems advantageous to develop compounds that selectively target galectin-3 for potential cancer treatment. Targeting extracellular galectin-3 can be achieved by simply sequestering the CRD binding domain with polysaccharides rich in galactoside residues. These polysaccharides include modified citrus pectin (MCP) and okra pectin [130]. Modified citrus pectin, a soluble fiber from citrus fruit, is a non-toxic pectin that can be given orally [131]. MCP has been shown to reduce B16F10 cell aggregation and lung metastasis [130]. Oral delivery of MCP significantly reduced the number of spontaneous lung metastasis in a prostate cancer murine model [131]. The direct binding of galectin-3 to breast cancer cells is drastically reduced in the presence of MCP. MCP can also directly affect angiogenesis by blocking galectin-3 binding to endothelial cells which was

shown to reduce chemotaxic migration of HUVEC cells *in vitro* [132]. The okra pectin, rhamnogalacturonan, has also been shown to bind to galectin-3, and the addition of okra pectin in B16F10 cells induced apoptosis [133]. These non-toxic polysaccharides could prove useful for stage II patients who have no clinical evidence of metastatic lesions, but would prefer a safe adjuvant treatment after resection of the primary tumors with no unwanted side effects. However, this hypothesis need to be further evaluated.

Small peptides have also been found to have an affinity for the galectin-3 CRD [134]. A more promising therapeutic option is using these peptides as homing devices for galectin-3 expressing tumors. Conjugating galectin-3 targeting peptides to "packaged" liposomes containing cytotoxic drugs or siRNA could prove useful. One group has already shown that radiolabeled peptides targeting galectin-3 specifically bind to galectin-3 expressing tumors *in vivo* [135]. However, peptide uptake into the liver and kidneys is currently an obstacle that must be cleared before these treatment modalities could be considered safe and efficient.

Targeting galectin-3 with large molecules like pectins and peptides could however limit their therapeutic potential. These molecules do not readily pass through the plasma membrane, and therefore, they cannot inhibit intracellular galectin-3 functions. However, small molecule inhibitors could be developed to target both intracellular and extracellular functions of galectin-3 with one drug. The amino acid G182A mutation on galectin-3, located in the "NWGR" anti-death domain of the CRD region has been shown to reduce cell survival as well as the carbohydrate binding to Galβ1-3glcNAc located on cell surface molecules [136]. Therefore, targeting amino acid G182 and the surrounding structure with a small molecule could potentially inhibit cell survival as well as prevent cell adhesion mediated metastasis.

Galectin-3 in WNT/β-Catenin Signaling

The canonical Wnt signaling pathway begins with a Wnt family ligand binding to the frizzled receptor. The frizzled receptor then recruits dishevelled to the membrane for activation. Once activated, dishevelled blocks GSK3β from phosphorylating β-catenin. This results in stable β-catenin and allows for its translocation from the cytoplasm to the nucleus to transcribe target genes [137]. The role that the Wnt/β-catenin pathway plays in malignant melanoma is controversial. This pathway has been reported to promote or antagonize melanoma progression depending on the cells, patient samples used, or context of the genes involved [138]. Studies have shown that nuclear beta catenin correlates with improved survival and benign melanoma express higher levels of β-catenin as compared to metastatic melanomas [139-142]. Another report indicates that β-catenin expression in melanocytes inhibited their migratory phenotype, but the over expression of β-catenin in melanoma cells increased the number of experimental lung metastasis [143]. β-catenin independent Wnt signaling by ligands such as WNT5A are associated with melanoma metastasis [144]. Galectin-3 is a binding partner of β-catenin and contains a GSK3β phosphorylation consensus sequence of $S_{92}XXXS_{96.}$ Indeed GSK3β phosphorylates galectin-3 [145, 146]. Casein kinase I (CK1) is also implicated in the WNT/Beta catenin pathway and CK1 mediated phosphorylation at Ser6 of galectin-3 occurs. However, unlike proteasomal degradation of β-catenin, phosphorylation of galectin-3 might have different effects such as nuclear localization or biological functions. Nevertheless, this implicates galectin-3 within the WNT signaling pathway. Its role on cancer progression has not been fully understood beyond the anti-apoptotic properties and nuclear localization resulting from phosphorylation at Ser6 by CK1.

Galectin-3 and its Regulation of Downstream Genes

Galectin-3 can affect multiple pathways by performing different functions such as carbohydrate binding on cell surface proteins, intracellular protein binding, and nuclear localization. These functions can have a profound effect on signaling pathways that affect the expression of multiple genes that could enhance tumor progression. Early research on galectin-3 identified the cell proliferation gene cyclin D1 as a downstream transcriptional target of galectin-3. Initial studies showed that the over expression of galectin-3 in breast epithelial cells enhanced the promoter activity of cyclin D1 [147]. Later, it was found that galectin-3 can bind directly with β-catenin and colocalize together within the nucleus. This resulted in up regulation of cyclin D1 and c-Myc in the breast BT549 epithelial cell line [145]. Interestingly, another group overexpressed galectin-3 in BT549 cells and observed the same increased expression of cyclin D1. Furthermore, by gene expression array, they identified a large group of genes that are deregulated when galectin-3 is over expressed and confirmed the up regulation of cyclin D1, insulin-like growth factor binding protein 5, protease serine 3, and dual specificity phosphatase 6 by western blot [148]. The over expression of galectins-3 with a Ser6 mutation to Glu did not have the same effects, showing the important nature of phosphorylation at Ser6 to induce the expression of select genes [148]. Moreover, injection of BT549 cells with the galectin-3 expression vector in nude mice generated tumors while the empty vector and Ser6 galectin-3 mutant remained tumor free for greater than 40 days [148].

Our lab has also reported that galectins-3 can differentially regulate genes that promote melanoma progression. Silencing galectin-3 in A375SM and C8161 results in reduced expression of the inflammatory cytokine interleukin-8 (IL-8) and fibronectin-1 [114]. The endothelia cell adhesion molecule VE-cadherin is highly expressed in C8161 melanoma cells.

Silencing galectin-3 in this cell line resulted in ~70% reduction of VE-cadherin. The transcription factor EGR-1 was found to bind to both the IL-8 and VE-cadherin promoter after silencing galectin-3. Over expressing EGR-1 significantly reduced VE-cadherin and IL-8 protein expression [114]. Therefore, it is likely that galectins-3 suppresses EGR-1 activity during melanoma progression. Galectin-3 silenced C8161 melanoma cells were injected into mice and showed a significant reduction in tumor growth and metastasis. Immunohistochemical staining confirmed the down regulation of IL-8, VE-cadherin, and MMP-2, and less vasculature was observed by CD31 staining [114]. Although, the expression of MMP-2 is significantly reduced in galectin-3 silenced C8161 cells, there was no observed change in A375SM cells. Therefore, galectin-3 regulation of select genes could be cell line dependent. These data clearly indicate that galectin-3 could regulate multiple genes during melanoma progression. Yet, the majority of the genes have yet been identified, and how they enhance the metastatic melanoma phenotype will have to be elucidated.

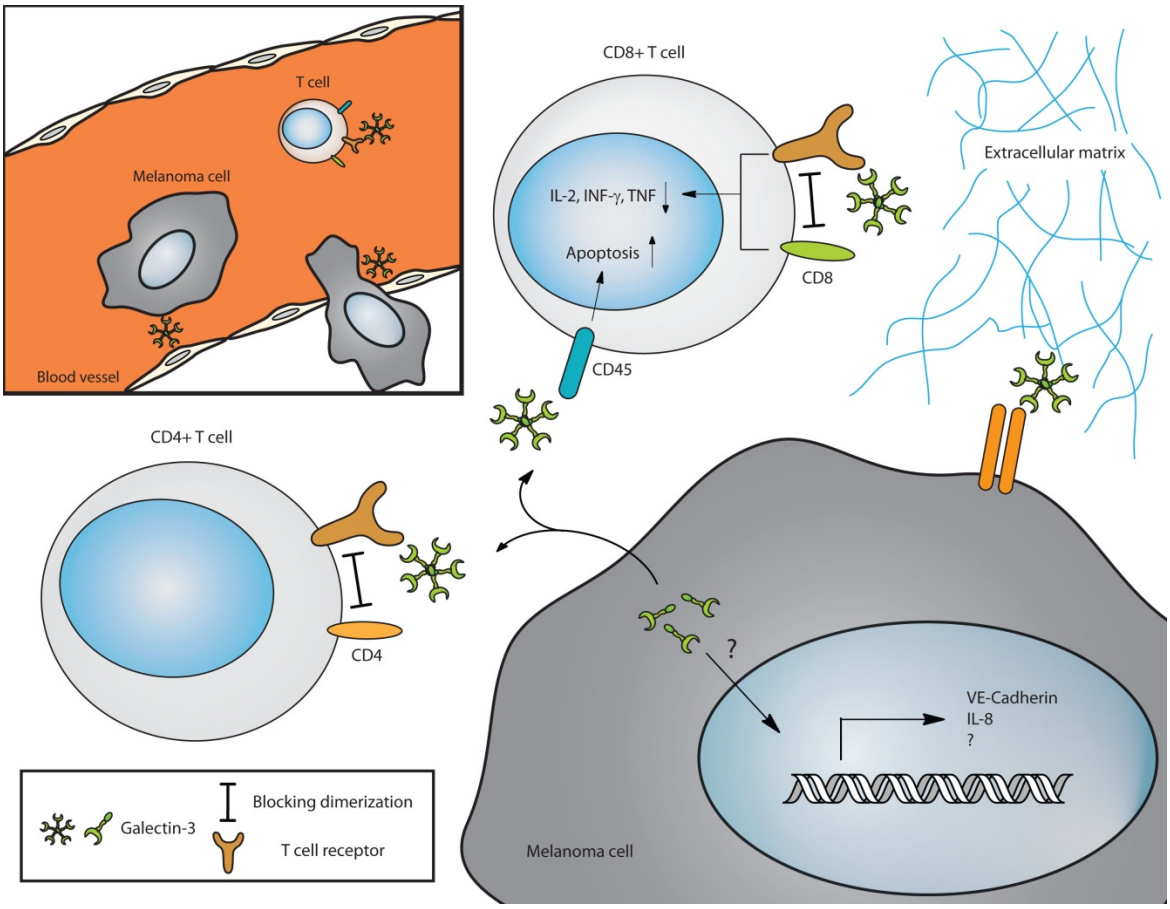


Figure 3. The Contribution of Galectin-3 to Melanoma Growth and Metastasis

Galectin-3 expression can enhance tumor cell binding with endothelial cells and potentially enhance melanoma cell extravasation. Galectin-3 inhibits immunosurveillance by inhibiting T-cell receptor and either CD4 or CD8 activation. In CD8 T-cells, this results in reduced IL-2, INF-γ, and TNF levels. Binding of galectin-3 on CD45 in T-cells activates T-cell apoptosis. Intracellular galectin-3 can induce the expression of metastatic genes such as IL-8 and VE-cadherin. Adapted from Braeuer RR et al, Pigment Cell and Melanoma Res 2012.

Specific Aims

Galectin-3 is highly expressed in primary and metastatic melanoma patient specimens as compared to benign nevi [103]. Others have shown that galectin-3 increases the metastatic phenotype of B16F10 murine melanoma cells. We have previously shown that silencing galectin-3 can differentially regulate specific genes such as IL-8, VE-cadherin, and fibronectin [114]. In breast epithelial cells galectin-3 drastically changed their gene expression profile. Therefore, in melanoma, it is likely that novel, previously unidentified genes could be deregulated by galectin-3 in malignant melanoma cells. Our study will identify these novel downstream targets, characterize their regulation by galectin-3, and evaluate their role during melanoma progression.

Therefore, we hypothesize that galectin-3 differentially regulates the expression of genes that promote the metastatic melanoma phenotype.

To test this hypothesis we developed the following aims:

1. **Determine the *In Vitro* Migratory, Invasive, and Colony Formation Potential of Melanoma Cell Lines after Silencing Galectin-3 Expression with Lentiviral-Based shRNA**
2. **Identification of Novel Downstream Target Genes of Galectin-3 that Contribute to the Metastatic Melanoma Phenotype**
    - **2.1. Galectin-3 as a Potential Regulator of Autotaxin Expression in Melanoma Cells**
    - **2.2. Autotaxin and NFAT1 Contribute to Melanoma Growth and Metastasis**

CHAPTER 2: Materials and Methods

Cell Culture

The A375SM melanoma cell line was established through intravenous injection of A375-P in which the pooled lung metastasis were collected and grown [149]. The WM2664 melanoma cell line was purchased from the American Type Culture Collection, and are highly metastatic in nude mice [150]. The SB-2 melanoma cell line was isolated from a primary cutaneous lesion and is non-metastatic and poorly tumorigenic in mice [151]. All cell lines except WM902B were cultured in Eagles minimum essential media (MEM) supplemented with 10% FBS. WM902B was culture in RPMI-1640 with 5% FBS. The human embryonic kidney cells (293FT) used for lentiviral shRNA and over expression vectors were maintained in DMEM supplemented with 10% FBS.

Lentiviral shRNA and siRNA

Galectin-3 targeting shRNA 5'-GTACAATCATCGGGTTAAA-3' and Non Targeting shRNA 5'-TTCTCCGAACGTGTCACGT-3' were designed with a hairpin and inserted into a pLVTHm lentiviral vector. The lentivirus was then produced by transfecting 293FT cells with the pLVTHm vector containing either the Galectin-3 or NT shRNA sequence, the packaging plasmid (MD2G), and the envelop plasmid (PAX2) to produce a viable virus. The NT shRNA has no homology to any known human genes. The supernatant was collected containing a mature virus and was concentrated 10x. WM2664 and A375SM cells were plated at 70% confluence on a six well plate and were transduced with 500ul MEM / 500ul of supernatant containing the virus and were incubated overnight. The cells were then grown and the top 30%

GFP expressing cells were cell sorted by FACS. NFAT1 siRNA was purchased from Sigma and transfected into WM2664 and A375SM melanoma cells by using HiPerFect Transfection Reagent (QIAGEN) according to the manufacturer's instruction. The siRNA sequence from Sigma targeting 5'-CTGATGAGCGGATCCTTAA-3' was used to stably silence NFAT1 by inserting it or NT shRNA into a PcDH vector and packaged within the lentiviral system as described above.

Nontargetable Galectin-3 Expression Vector

The Galectin-3 gene was amplified from A375SM cDNA with the following primers; gal-3AsclF- TTGGCGCGCCAAATGGCAGACAATTTTTCGCTCC and gal-3EcoR1R- CGGAATTCCGTTATATCATGGTATATGAAGCAC, cut with AscI and EcoR1 restriction enzymes, and inserted into the OG2 puromycin resistant vector. The Galectin-3 shRNA targeting site was mutated to ATATAACCACCGTGTCAA (underlined nucleotide designates mutated sites) with the following primers; gal-3mutF-GAATGATGCTCACTTGTTG-CAATATAACCACCGTGTCAAAAAACTCAATGAAATCAGC and gal-3mutR- GCTG-ATTTCATTGAGTTTTTTGACACGGTGGTTATATTGCAACAAGTGAGCATCATTC. The virus was then produced with the OG2 Empty vector or OG2-Gal-3 Rescue, MD2G, and PAX2 plasmids as previously described. WM2664 and A375SM Gal-3 shRNA cell lines were then transduced with 800ul MEM / 200ul supernatant containing virus overnight and were selected with MEM containing 1ug/ml puromycin.

Autotaxin and NFAT1 Expression Lentiviral Vector

Autotaxin and NFAT1 genes were cloned from A375SM cDNA. Autotaxin was cloned with the following primers; ATXXbaIF-TGCTCTAGAGCCACCATGGCAAGGAGGAGCTC-GTTCC and ATXBamHIR- CGGGATCCTTAAATCTCGCTCTCATATG. NFAT1 was cloned with the following primers; NFAT1XbaIF-GCTCTAGAGCCACCATGCAGAGA-GAGGCTGCGTTCAG and NFAT1NotIR-ATAAGAATGCGGCCGCTCATAATATGTTTT-GTATCCAG. Either gene was cut with the designated restriction enzymes, inserted into a PcDH vector and packaged in a lentiviral virus as previously described.

Western Blot Analysis

To detect Galectin-3 and NFAT1, 20ug of whole cell protein lysate was loaded on SDS-PAGE and transferred to PVDF membranes. To detect Autotaxin protein expression, 1.5 million cells were plated in a 10cm dish and were incubated with 8ml of serum free MEM for 48hrs. The supernatant from cell culture was concentrated to 100ul, was protein precipitated as previously described, and resuspended in 6M urea lysis buffer [43]. A total of 10ug of protein from the supernatant was loaded onto SDS-PAGE. Blots were incubated with primary antibodies rabbit polyclonal anti-Galectin-3; anti-NFAT1 Santa Cruz Biotechnology; anti-Autotaxin Abcam. To confirm equal loading of the supernatant, the membrane was coomassie blue stained and destained with 40% methanol, 50% water, and 10% acetic acid until protein bands were visible.

Invasion and Migration Assays

Matrigel invasion assays were performed with Biocoat Matrigel invasion chambers (BD Biosciences) as previously described [43]. Boyden chambers were plated and assayed in the same manner. Wells were repeated in triplicate and the invaded/migrated cells were quantified per field of view and statistically analyzed.

Soft Agar Colony Formation Assay

A 0.6% agar in MEM bottom layer is plated in 6 well plates and allowed to solidify. The cells are then suspended in 0.8% agar/MEM and plated at $5x10^3$ cells per well in triplicate. Following 30 days incubation, the number of colonies is quantified in triplicate wells.

Semi Quantitative RT-PCR

Isolation of RNA was performed with the RNAqueous kit (Ambion) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR was performed with the Autotaxin Taqman Gene Expression Assay and standardized to 18s (Applied Biosystems). LPAR1-6 Taqman Gene Expression Assays were acquired from Applied Biosystems. All six receptors were detectable by qRT-PCR and standardized to one with the SB-2 melanoma cell line. Autotaxin and NFAT1 Taqman Gene Expression Assays were acquired from Applied Biosystems and qRT-PCR was performed on WM2664 and A375SM melanoma cells. Each probe was standardized to one with NT shRNA.

mRNA Stability Assay

Melanoma cells were subjected to 2, 4, 6, 8, or 12 hours of actinomycin D treatment at 10 ug/ul concentration followed by RNA isolation by the RNAqueous kit (Ambion). cDNA was generated and qRT-PCR for autotaxin was performed.

Autotaxin Activity Assay

To analyze Autotaxin lysophospholipase D activity, the fluorescent compound FS-3 (L-2000; Echelon) was used as previously described [152]. Briefly, cells were plated for supernatant collection as described previously. The supernatant was then concentrated to a volume of 250ul. A total of 50ul of supernatant from WM2664 and A375SM NT and Gal-3 shRNA cell lines were plated in triplicate in a clear bottom white walled plate. The volume for each well was brought up to 100ul with reaction buffer (Final concentrations in the assay: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Tris, pH 8.0), and 5 μM FS-3. Serum Free MEM was used as a control to confirm that there is no lysophosholipase D activity within unconditioned media (data not shown). The plate was then placed in the Spectra Max Gemini EM (Molecular Devices) fluorescence reader at 37°C and read every two minutes for six hours with an excitation at 485 nm and emissions reading at 538 nm. A volume of 25 ul supernatant and 25 ul 2x loading buffer for each sample were run on SDS-PAGE and silver stained with Silverquest™ Silver Staining Kit (LC6070; Invitrogen) to confirm equal total protein concentration.

Chromatin Immunoprecipitation Assay

ChIP assays were performed with the ChIP-IT Express Enzymatic kit (53009; Active Motive) according to the manufacturer's protocol and as previously described [43]. Fixed protein DNA complexes were pulled down with anti-NFAT1 antibody (sc-7296; Santa Cruz Biotechnology), were Protein-DNA reverse cross-linked, and prepared for PCR. PCR was performed surrounding both NFAT1 binding sites with the following primers; NFATF-GCTCAAACTGCCAGCAAAAT and NFATR- CACAGGGTGTTCACAAATCG. The PCR product was run in a 1.5% agarose gel.

Reporter Constructs and Luciferase Activity Analysis

The Autotaxin promoter was cloned from A375SM melanoma cells to encompass 930 base pairs upstream of the transcriptional initiation site with the following primers; 930KPN1F-GGGGTACCCCCACAATAGCCTCAAAGG and 50BglIIR-GAAGATCTTCTCTTTGCCTT-CACGGAG. PGL-3 basic was cut with kpn1 and bglII restriction enzymes and the Autotaxin promoter was inserted. Direct site mutagenesis of NFAT1 binding sites were carried out using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Cells were plated in a 24 well plate with 2.0×10^4 cells/ well. After 48 hours, transfection with Lipofectin (Invitrogen) was performed according to manufacturer's instructions. Briefly, each well was transfected with 0.8 μg of the basic pGL3 expression vector with no promoter sequence or with 0.8 μg of pGL3 with the inserted Autotaxin promoter, single mutation, or double NFAT1 mutation sites. As a control, 2.5 ng of cytomegalovirus (CMV) driven renilla luciferase construct (pRL-CMV, Promega) was included per well. Each group was plated in replicates of six. After 48 hours the cells were lysed, and

luciferase activity was assayed with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The ratio of PGL3 firefly to CMV-driven renilla luciferase activity was used to normalize each sample.

Nuclear Run-On Assay

Nuclear run-on was performed as previously described [153]. Briefly, the nuclei fractions of A375SM NT shRNA and Galectin-3 shRNA melanoma cells was collected and RNA synthesis was performed *in vitro* with ATP, CTP, GTP, and biotin-16-UTP. Reaction was stopped after 30 minutes and 50ul of Dynabeads M-280 (Invitrogen) were added to capture biotin labeled RNA. The beads were then washed with 2x standard saline citrate and resuspended in H_2O. RNA was synthesized into cDNA with the High Capacity cDNA Reverse Transcription Kit, and qRT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems). Freshly transcribed Autotaxin was amplified in triplicate with ATXF-GTTCACTTTTGCCG-TTGGAG and ATXR-ACCTTCCTCCCATCCTTCTG and standardized to GAPDH primers; GapdhF- AAGGTCATCCCTGAGCTGAA and GapdhR- AGGTCCACCACTGACACGTT. The Rq Max is shown.

Immunoprecipitation of FLAG-tagged NFAT1

NFAT1 was inserted into the pFLAG CMV 5.1 plasmid. WM2664 and A375SM melanoma cells with either NT shRNA or galectin-3 shRNA were plated into a 10cm dish. After 24 hrs, the pFLAG-NFAT1 CMV5.1 plasmid was transfected with FuGENE 6 (Roche). The proteasome inhibitor Bortezomib at 20 nM was added to the media. Following 24hrs, the cells were lysed and FLAG immunoprecipitated with the FLAG Immunoprecipitation Kit (Sigma)

according to the manufacturer's instructions. Western blot analysis of NFAT1 with anti-NFAT1 was then performed. Parental cells were used as a negative control for immunoprecipitation of FLAG.

Immunohistochemistry and Immunofluorescence

Mice were sacrificed and tumors were removed. Half of each tumor was formalin fixed and paraffin embedded while the other half was placed in optimum cutting temperature and frozen at -80°C. Mouse anti-Galectin-3 antibody (Santa Cruz Biotechnology) was used in combination with citrus buffer antigen retrieval and mouse anti-Autotaxin (Abcam) and anti-VEGF (Santa Cruz Biotechnology) with pepsin antigen retrieval in paraffin sections. Fragment blocking was performed overnight prior to addition of secondary HRP conjugated antibody. Anti-CD31 staining was used in mouse frozen sections to identify tumor blood vessels. TUNEL staining was performed using the DeadEnd Fluoremetric TUNEL system (Promega) with paraffin sections according to manufacturer's instructions.

Tumor Growth and Metastasis

Female Athymic Balb/c nude mice were purchased from Tanomics and were housed in pathogen free conditions. All studies were approved and supervised by The University of Texas MD Anderson Cancer Center, Institutional Animal Care and Use Committee (IACUC). For the tumor growth model, $1x10^6$ cells were injected subcutaneously and tumor size was monitored twice a week for 27 days for Galectin-3 shRNA and 32 days for NFAT1 shRNA studies. Ten mice per group and eight mice per group for Galectin-3 and NFAT1 *in vivo* studies respectively were used. Mice were then sacrificed and tumors were collected. For

experimental lung metastasis, eight mice per group were sacrificed four weeks after $5x10^5$ cells were injected intravenously as previously described [43].

Expression Microarray

Total RNA was isolated from WM2664 NT and Galectin-3 shRNA melanoma cells. RNA was converted into cRNA using the Illumina TotalPrep Amplificatin Kit (Ambion) and hybridized in triplicate to the HT-12 Version 3 Illumina chip. Gene expression analysis was performed between the two samples.

Statistical analysis

Student's t-test was performed for the analysis of *in vitro* assays. The Mann-Whitney U test was performed for statistical analysis of the *in vivo* tumor growth and metastasis results.

CHAPTER 3: Specific Aim 1

Determine the *In Vitro* Migratory, Invasive, and Colony Formation Potential of Melanoma Cell Lines after Silencing Galectin-3 Expression with Lentiviral-Based shRNA

Introduction

During the transition from the RGP to VGP, melanoma cells must acquire the ability to invade through the basement membrane and migrate into the dermis where they enter the vasculature and travel to distant sites of metastasis. Galectin-3 could be a critical gene involved in this phenotype. Previous studies have shown that the addition of carbohydrate recognition domain competitors such as lactose or MCP can reduce the motility and invasion of breast cancer cells [154, 155]. Interestingly, in the murine B16F10 melanoma cell line, MCP reduced their ability to grow in soft agar [156]. Our lab has previously silenced galectin-3 in C8161 cells and showed a significant reduction in their ability to invade through Matrigel coated membranes, and silencing galectin-3 reduced the activity of MMP-2 [114]. Other labs have over expressed galectin-3 in non-cancerous cells and have shown increased invasive and migratory properties [157]. Furthermore, imunohistochemical analysis of patients' specimens identified galectin-3 to be highly overexpressed in primary and metastatic lesions as compared to benign nevi [103]. By evaluating previous evidence, we hypothesize that galectin-3 is a key player in the migratory and invasive phenotype of melanoma cells. Therefore, we stably

silenced galectin-3 with a lentiviral construct to determine the *in vitro* effect on migration, invasion, and soft agar colony formation.

Results

Expression Analysis of Galectin-3 in a Melanoma Cell Panel

We sought to determine the expression pattern of galectin-3 in our melanoma cell lines. We confirm that our more metastatic cell lines expressed higher levels of galectin-3 as compared to the less tumorigenic and metastatic melanoma cells. As displayed in figure 4, our highly metastatic cell lines (TXM18, WM2664, WM902B, 451-Lu, and A375SM) express higher levels of galectin-3 as compared to the less tumorigenic and metastatic melanoma cells (SB2, DX3, and DM4). Interestingly, C8161 melanoma cells, which are highly metastatic, invasive cells, express low levels of galectin-3. Although lower than other metastatic cells, its expression was enough to deregulate IL-8, VE-cadherin, and fibronectin as our lab has previously shown. Why galectin-3 is expressed less in c8161 is not completely understood. However, one brief study was performed. Our cell panel can potentially be separated into three groups. Those with NRAS, $BRAF^{V600E/D}$, or other BRAF mutations. If analyzed in this manner, a striking observation was found. $BRAF^{V600E/D}$ mutant melanoma cells had higher levels of galectin-3. However, this is only preliminary data. A much larger scale analysis of galectin-3 corresponding to mutational status should be performed. Therefore, this observation could be due to our panel lacking enough highly tumorigenic NRAs and non $BRAF^{V600E}$ mutant cell lines.

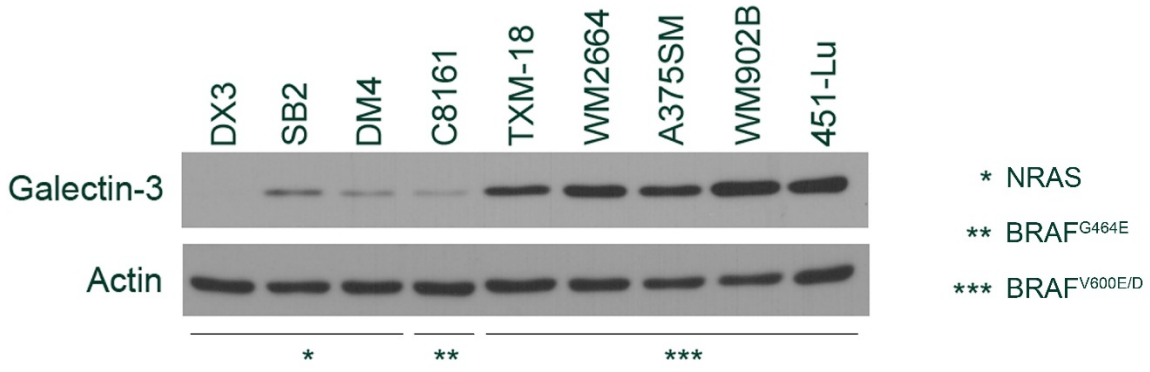


Figure 4. Galectin-3 is Expressed at Higher Levels in Metastatic Human Melanoma Cell Lines

Western blot analysis in melanoma cell lines was performed. The less tumorigenic DX3, SB2, and DM4 melanoma cell lines express far less galectin-3 than the more tumorigenic and metastatic TXM-18, WM2664, A375SM, WM902B, and 451-Lu cells. C8161 melanoma cells express less galectin-3; however, are still highly metastatic melanoma cells. The NRAS and BRAF mutation status is shown for each cell line.

Silencing Galectin-3 in WM2664 and A375SM Metastatic Melanoma Cell Lines

To establish the role that galectin-3 melanoma cells have on melanoma progression, we chose to stably silence galectin-3 in two metastatic and invasive melanoma cell lines that have high levels of galectin-3 expression. Of our cell panel, the cells that met these criteria were the WM2664 and A375SM melanoma cell lines. The two melanoma cell lines were then stably transduced with non-targetable (NT) or galectin-3 shRNA packaged lentivirus. The non-targeting shRNA sequence has no known homology to any known human gene, and will be used as a control throughout the study. The packaged vector utilizes the green fluorescent protein (GFP) based lentiviral system. After transduction, the cells for both NT and galectin-3 shRNA melanoma cells were sorted for the top thirty percent of GFP fluorescence (based of GFP expression) by Fluorescent Activated Cell Sorting (FACS).

After cell sorting, both WM2664 and A375SM melanoma cell lines transduced with NT or galectin-3 shRNA were grown. Western blot analysis was performed to determine the silencing efficiency of galectin-3 shRNA. By utilizing densitometry to normalize galectin-3 with actin, it was observed that both melanoma cell lines with galectin-3 shRNA have almost a complete knock down in galectin-3 expression as compared to the NT shRNA control (Figure 5). These cells were then used for our studies.

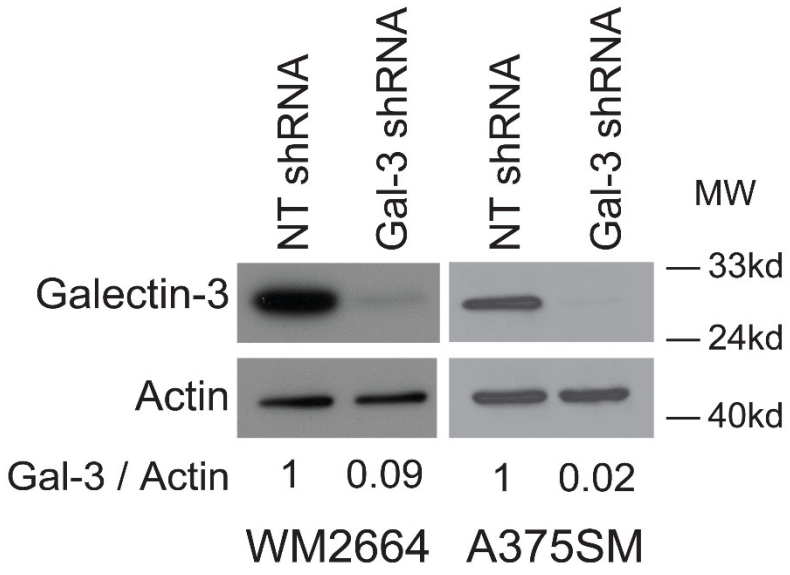


Figure 5. Stable Transduction of Galectin-3 shRNA is Efficient at Reducing Galectin-3 Expression in both WM2664 and A375SM Melanoma Cell Lines

Galectin-3 expression is almost completely lost in both melanoma cell lines with the stable lentiviral based transduction of galectin-3 shRNA. Densitometry analysis for WM2664 cells confirms that approximately 90% of galectin-3 expression is lost. For A375SM cells, galectin-3 expression is almost completely lost.

The *In Vitro* Migratory and Invasive Phenotype of Melanoma Cells After Silencing Galectin-3

To corroborate that galectin-3 reduces the migratory phenotype of melanoma cell lines, galectin-3 silenced WM2664 and A375SM melanoma cells were subjected to the modified Boyden chamber migration assay. The cells were plated with serum free media in the top chamber and were incubated for 24 hours. The bottom chamber contained MEM media with 20% fetal bovine serum (FBS) to act as a chemoattractant. The number of migrated cells through the Boyden chamber was then evaluated. A significant reduction in the number of migrated melanoma cells was observed after silencing galectin-3 in both WM2664 and A375SM cell lines,*$p < 0.01$ (Figure 6). A more than 5 fold reduction is observed in WM2664 cells and more than 2 fold reduction with A375SM.

The lab has previously shown that silencing galectin-3 in C8161 cell lines reduce their invasive phenotype and reduced MMP-2 activity is observed [114]. To confirm that galectin-3 plays a role in the invasive potential of both WM2664 and A375SM melanoma cells were plated in Matrigel invasion chambers with serum free media. As with the migration assay, 20% FBS was used as a chemoattractant. As seen in Figure 7, the invasive capacity of melanoma cells is significantly reduced after silencing galectin-3, $p < 0.01$, with more than a 10 fold reduction in WM2664 and 3 fold reduction in A375SM. Therefore, our data supports the idea that galectin-3 is critical for the invasive phenotype of malignant melanoma cell lines.

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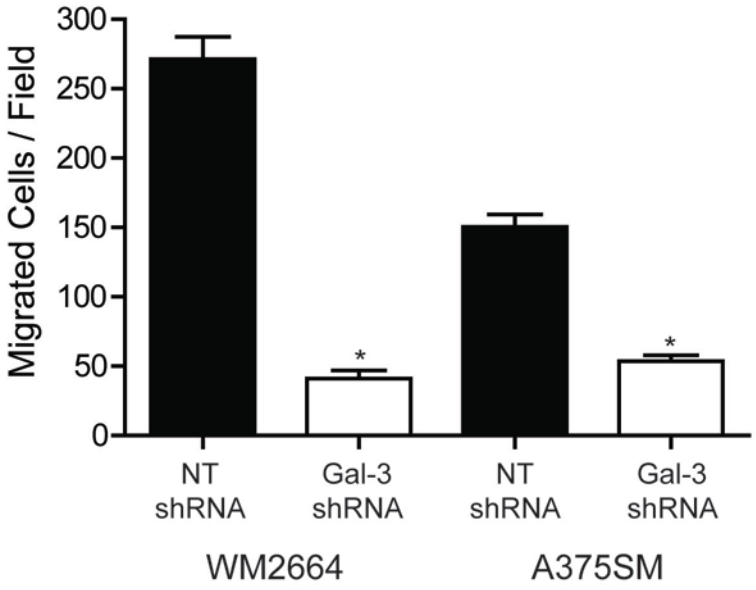
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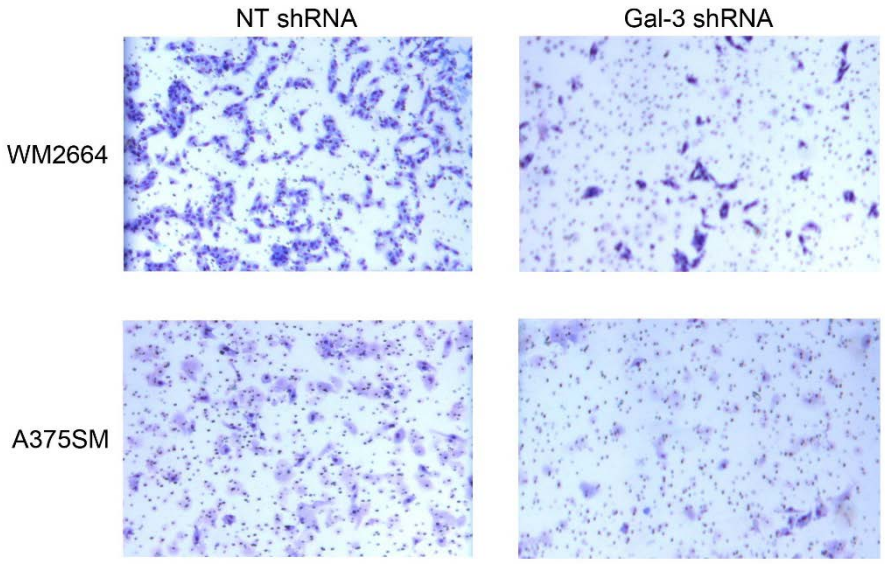
NT shRNA
Gal-3 shRNA
WM2664
A375SM

Figure 6

Figure 6. The Migratory Phenotype of Melanoma Cells after Silencing Galectin-3

The migratory phenotype of melanoma cells were analyzed by the Boyden chamber assay. (A) The number of migrated cells was counted per field. Silencing galectin-3 in both melanoma cell lines significantly reduced the number of migrated cells as compared to NT shRNA (*P < 0.01). (B) Representative images are shown for the number of migrated cells for each cell line transduced with NT or galectin-3 shRNA.

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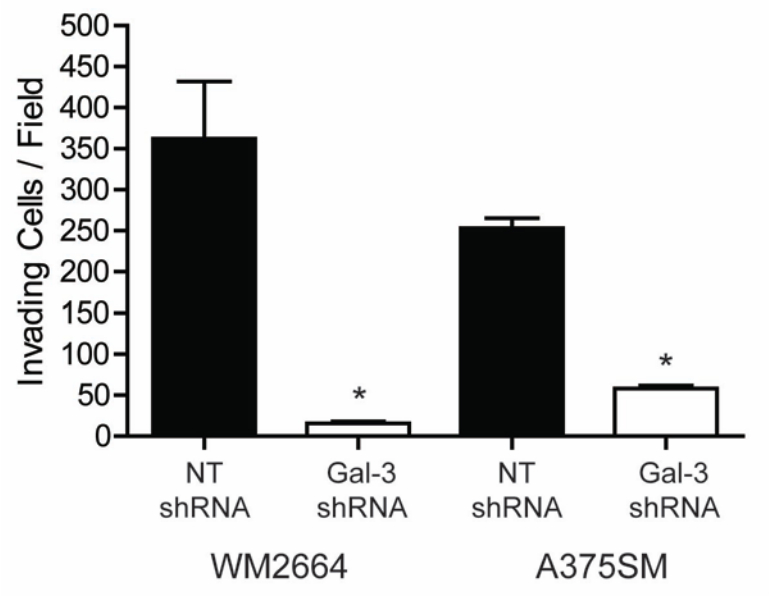
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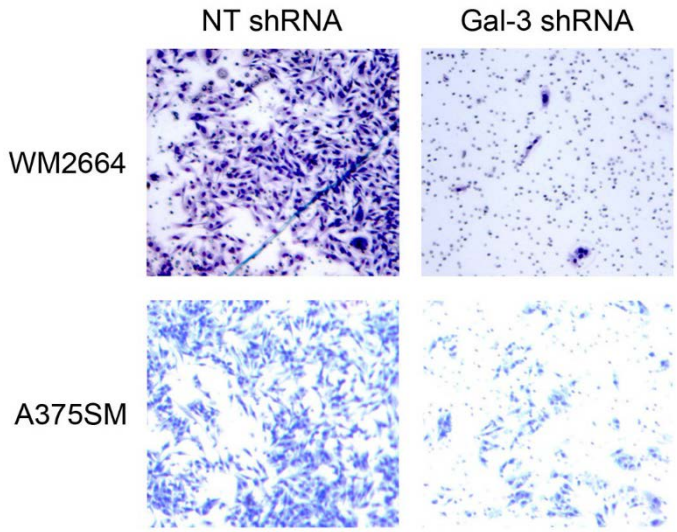

NT shRNA
Gal-3 shRNA
WM2664
A375SM

Figure 7

Figure 7. The Invasive Potential of Melanoma Cells after Silencing Galectin-3

(A) The number of invaded cells through Matrigel is significantly reduced after silencing galectin-3 in both WM2664 and A375SM melanoma cells as compared to NT shRNA (*P < 0.001). (B) A representative image for each cell line with either NT or galectin-3 shRNA is shown.

Soft Agar Colony Formation of Galectin-3 Silenced Melanoma Cells

The soft agar colony assay is a stringent method that has been widely used for the identification of anchorage independent growth and transformed cancers cells. This method has also been used to isolate circulating melanoma cells from periphery blood samples of patients with metastatic disease [158]. Studies with murine fibrosarcoma cells identified that metastatic cell clones were able to grow colonies in 0.6% soft agar while non-metastatic clones were almost completely restricted in growth [159]. Selection of breast cancer cells in 0.9% agar created cell clones that were very similar in their molecular phenotype as those from *in vivo* brain metastasis [160]. This method could give us a good indication on whether galectin-3 affects anchorage independent growth, a key indicator for the metastatic potential of melanoma cells. To that end, WM2664 and A375SM NT and galectin-3 shRNA melanoma cells were mixed with 0.6% soft agar and plated. After thirty days of incubation, the number of soft agar colonies was quantified. As shown by Figure 8, silencing galectin-3 significantly reduced the number of colonies formed in soft agar from from 191 to 50 in WM2664 and from 131 to 40 in A375SM cells ($P < 0.001$).

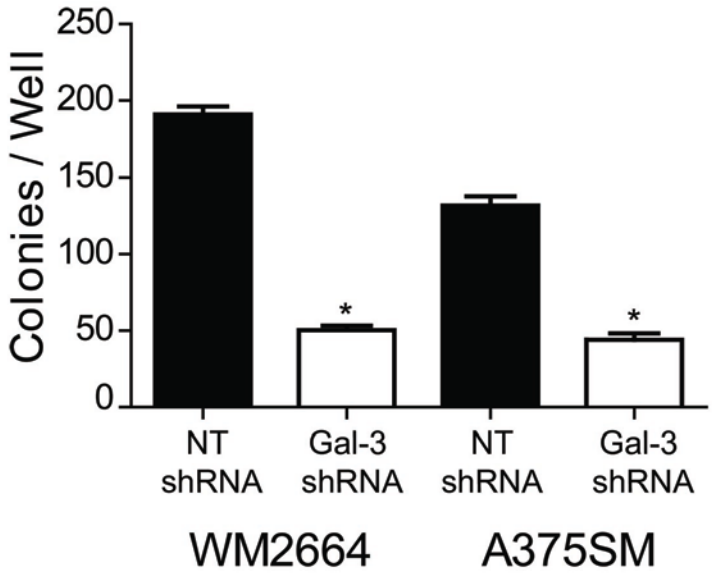


Figure 8. Colony Formation of WM2664 and A375SM Melanoma Cells in 0.6% Agar

After incubation in soft agar for 30 days, the mean number of colonies was counted in triplicate. Silencing galectin-3 in WM2664 melanoma cells significantly reduced the number of soft agar colonies by more than threefold, while a greater than two fold reduction in the number of colony formation was observed in A375SM cells (*$P < 0.001$).

Discussion

Herein, we report that silencing galectin-3 in WM2664 and A375SM melanoma cells significantly reduces their migratory and invasive potential. Galectin-3 has previously been shown to induce the migratory and invasive phenotype of cancer cells. For example, silencing galectin-3 in B16F10 murine melanoma cells reduced MMP-1 expression and *in vitro* migration and invasion [107]. In sarcoma cells, galectin-3 increases the migratory phenotype in a carbohydrate dependent manner by activating PI3K and disrupting adhesion plaques [161]. The exogenous expression of β1 integrin increases galectin-3 expression and the epithelial to mesenchymal transition (EMT) phenotype in GE11 cells [162]. We have previously shown that silencing galectin-3 in C8161 melanoma cells reduces their ability to invade through Matrigel coated membranes [114]. Therefore, our data corroborate previous studies.

The metastatic potential of melanoma cells relies heavily on the ability for cells to survive and proliferate in anchorage independent conditions. The survival of melanoma cells is dependent on their ability to withstand the harsh microenvironment of the metastatic site. The soft agar assay is a good prognostic tool for identifying the metastatic potential of melanoma cells as it too represents a harsh microenvironment in which the cells must survive and grow in an anchorage independent manner. Guo et al have even suggested that the ability of cancer cells to grow in increasingly higher concentrations of soft agar, from 0.3% to 0.9% selects for more invasive breast cancer cells that are more likely to metastasize and grow in the brain of nude mice [160]. Metastatic fibrosarcoma clones are more likely to grow in 0.6% soft agar as compared to non-metastatic clones [159]. Silencing galectin-3 in both metastatic melanoma cell lines significantly reduced their ability to grow in 0.6% soft agar. Therefore, we can predict that silencing galectin-3 reduces the metastatic potential of melanoma cells.

CHAPTER 4: Specific Aim 2

Identification of Novel Downstream Target Genes of Galectin-3 that Contribute to the Metastatic Melanoma Phenotype

Sub-Aim 2.1: Galectin-3 as a potential regulator of Autotaxin Expression in Melanoma Cells

Introduction

Previous data has indicated that galectin-3 can have a profound effect on the transcriptional regulation of genes. The overexpression of galectin-3 in breast epithelia cell lines changed the genomic signature that included genes such as cyclin D1. The change in expression of these unique genes caused cell transformation of BT549 breast epithelial cells [148]. Our lab has previously identified IL-8, VE-cadherin, and fibronectin as downstream targets of galectin-3 [114].

In aim 2, we sought out to identify novel downstream molecules regulated by galectin-3 that contribute to melanoma growth and metastasis. To further evaluate the effect galectin-3 has on the gene expression profile of melanoma cells, a gene expression microarray was performed (Illumina). Our microarray identified many potential targets including autotaxin (*ENPP2)*. The regulation of autotaxin by galectin-3 was previously unknown. Therefore, we chose to elucidate this mechanism and identify how the interplay between these two molecules enhances melanoma progression.

Autotaxin was first identified as a pro migratory molecule in A2058 melanoma cells. However, the mechanism by which autotaxin enhanced their motility was unclear [163]. Although secreted, autotaxin was not considered a ligand for any known receptors. Only later was it realized that autotaxin contains a phosphodiesterase catalytic site that is required for the migratory phenotype [164]. Further evaluation matched the structure of autotaxin with a lysophospholipase D enzyme purified from fetal bovine serum which catalyzes lysophosphatidylcholine (LPC) to the bioactive lipid, lysophosphatidic acid (LPA) [165]. LPA then acts as ligand for two types of G-protein-coupled receptors. This includes three from the endothelial differentiation gene (EDG) receptor family termed LPA1, LPA2, and LPA3. Three other receptors that respond to LPA are structurally similar to the purinergic receptor family and are termed LPA4, LPA5, and LPA6 [166, 167]. Activation of LPA receptors enhances G-protein signaling and downstream targets such as PI3K/AKT, PKC, cAMP, and Ca+ influx [167]. This results in enhanced chemotaxis, migration, invasion, angiogenesis, and tumorigenesis in mice [168-170].

Results

Silencing Galectin-3 Changes the Gene Expression Profile of WM26644 Melanoma Cells

To identify novel downstream targets of galectin-3, a cRNA microarray was performed with the Illumina HT-12 Version 3 chip. RNA from three separate 10cm dishes for both WMM2664 NT shRNA and galectin-3 shRNA cells were used to confirm reproducibility. The gene expression profile was randomly grouped. As expected, the three NT shRNA samples grouped with each other while the three galectin-3 shRNA samples generated another group with a similar gene expression profile (Figure 9). Our initial data suggest that silencing galectin-3 does indeed deregulate multiple genes in melanoma cells. We then analyzed our data to identify the genes with the greatest fold change in gene expression. We focused our attention on genes down regulated after silencing galectin-3 as they could likely be tumor promoting genes. The top identified genes were then sorted with ingenuity software based on their phenotypic function such as invasion, cell cycle, and tumor malignancy. The top potential candidate genes are shown in Table 2. Many pro-tumor genes such as endothelin receptor B, cathepsin K and B, cyclin dependent kinases, and l-plastin had reduced expression after silencing galectin-3 according to the gene microarray. Of the several potential genes, we focused our attention on autotaxin. Autotaxin was chosen due to previously published data that suggest autotaxin can enhance invasion, migration, and tumorigenicity. These same phenotypes are observed with galectin-3. Therefore, we hypothesize that galectin-3 might contribute to melanoma progression through its regulation of autotaxin.

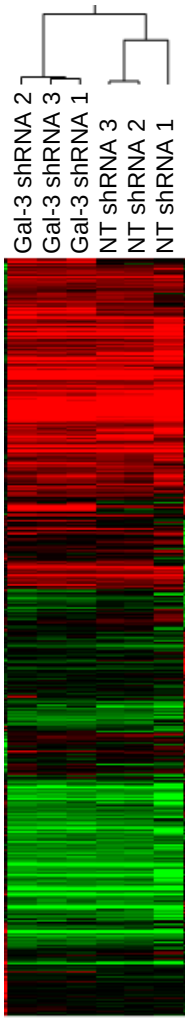


Figure 9. The Heat Map Comparing WM2664 NT shRNA with Galectin-3 shRNA Transduced Melanoma Cells

Total mRNA was isolated from three separate 10cm dishes for both WM2664 NT shRNA and galectin-3 shRNA. The cRNA from each sample was hybridized on an HT12 chip, Illumina. The three NT shRNA grouped together in a distinctly different gene expression profile from the galectin-3 silenced melanoma cells.

Phenotype	Gene	Fold Decrease
Invasion	Autotaxin (ENPP2)	2.33
	Osteopontin (SPP1)	2.05
Malignancy	Galectin-3 (lgals3)	7.14
	Stearoyl-CoA desaturase (SCD)	4.07
	Osteopontin (SPP1)	2.05
	Endothelin receptor B (EDNRB)	2.00
Cell Cycle	Cyclin A2 (CCNA2)	1.88
	Cyclin C (CCNC)	1.66
	CDC25B	1.90
Other	L-plastin (LCP1)	4.506
	Endothelin receptor like b	2.08
	Cathepsin B	1.59
	Cathepsin k	1.93
	MAPKKK1	1.74
	HIF-2α (EPAS1)	1.57

Table 2. Top Potential Genes Down Regulated after Silencing Galectin-3

The top potential candidate genes are shown. These genes were down regulated after silencing galectin-3 in WM2664 melanoma cells as compared to the non-targeting (NT) control. The candidate genes were further subgrouped by ingenuity pathway analysis into known phenotypes caused by each gene. Note that our gene expression array confirmed that galectin-3 was silenced by more than seven fold. Autotaxin was reduced by more than 2 fold.

Autotaxin Expression and Activity is Reduced in Melanoma Cells after Silencing Galectin-3

To corroborate our initial gene expression microarray, qRT-PCR and Western blot analysis was performed to measure autotaxin expression in WM2664 and A375SM melanoma cells transduced with NT or Galectin-3 shRNA. Indeed, the mRNA expression of autotaxin was reduced by more than two fold after silencing galectin-3 in both melanoma cell lines (Figure 10A). Interestingly, intracellular autotaxin protein expression by whole cell lysis was unidentifiable. However, autotaxin is primarily secreted from cells were it performs its biological function. Therefore, the supernatant of melanoma cells was collected from non-targetable or galectin-3 shRNA melanoma cells after 48hr incubation in serum free media. After methanol precipitation and isolation of the protein within the supernatant, a western blot was performed to identify the amount of autotaxin. As shown in Figure 10B, silencing galectin-3 drastically reduces the amount of autotaxin within the supernatant by more than tenfold. The disparity between mRNA and protein expression is not fully understood. One possibility is that silencing galectin-3 additionally reduces the translation rate or protein stability of autotaxin by unknown mechanisms. Another is that our method of precipitation and isolation of the supernatant results in low yields of protein. Five to ten μg of total lysate is loaded per well. Our autotaxin antibody might not be able to read such low levels of autotaxin expression in our gal-3 shRNA cells and thus, the amount of protein between groups appears greater. Nevertheless, these data confirm our initial microarray studies by which autotaxin expression is reduced after silencing galectin-3.

Although we identified that autotaxin protein levels are indeed reduced, we have yet to determine whether this has any relevance on tumor cell biology. Our foremost objective was to

determine whether autotaxin from our melanoma cells is indeed enzymatically active within the supernatant. To that end, we generated a protocol based on the autotaxin activity assay from Echelon. In this assay, the compound FS-3 is used. FS-3 is similar in structure as the endogenous lipid LPC. However, FS-3 contains a quencher where choline is located on LPC, and a fluorescent labeled R group (Figure 11A). If autotaxin is present in the system, it will cleave the quencher in the same manner it cleaves choline from LPC. This results in a fluorescent signal that is quantified by a fluorescent plate reader. To analyze FS-3 fluorescence in our study, $1x10^6$ melanoma cells were grown over a 24 hour period in serum free conditions followed by concentrating the supernatant to a total volume of 200ul. The media was then split into triplicate wells (50ul per well) with 50ul of 2x reaction buffer containing FS-3. As shown in Figure 11B, the amount of fluorescence is increased over a period of six hours in both WM2664 and A375SM NT shRNA melanoma cells. However, after silencing galectin-3, the rate of fluorescent activity is significantly reduced in both cell lines (Figure 11B). Autotaxin is the primary lysophospholipase D enzyme that converts LPC to LPA, and FS-3, and LPC analog, is considered to have a high affinity for autotaxin. Therefore, we conclude that the reduced rate of FS-3 activity is contributed by reduced levels of autotaxin. This can be translated to the biological system. Lower levels of autotaxin should result in lower levels of LPC conversion to the active LPA. Less LPA results in lower LPA receptor signaling.

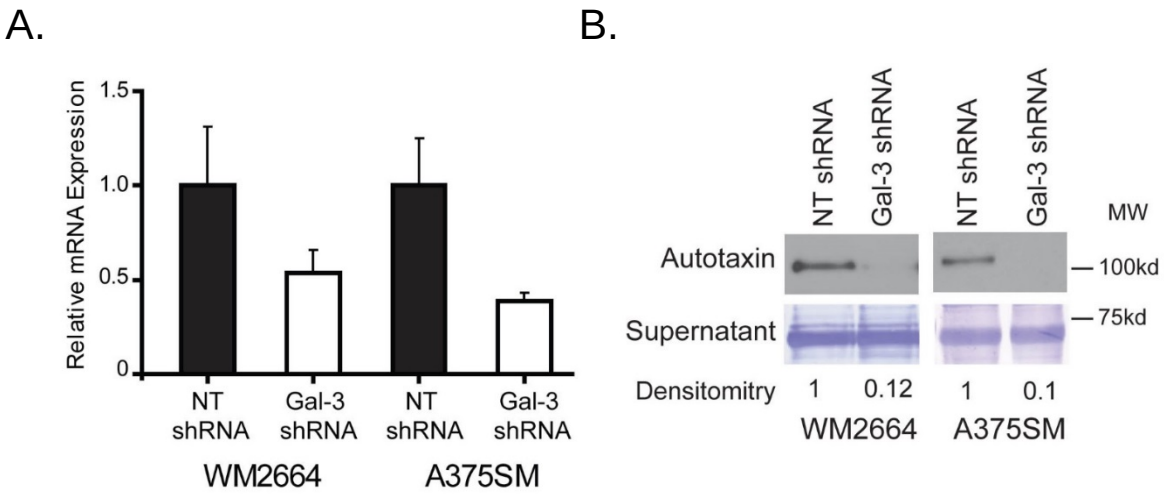
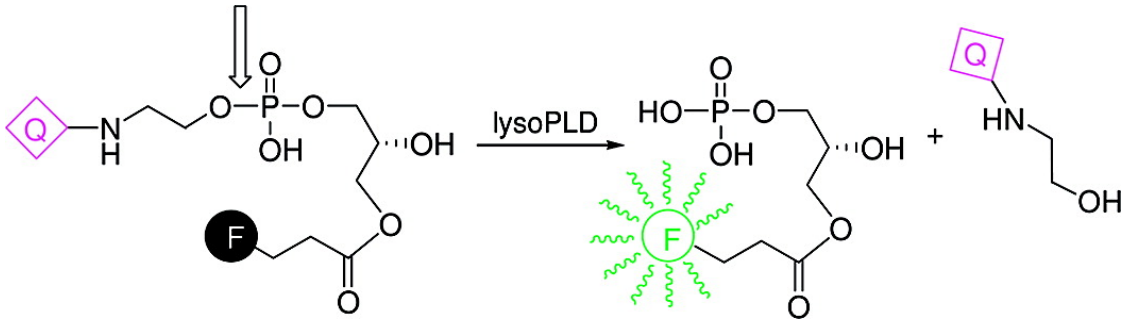


Figure 10. Autotaxin Expression after Silencing Galectin-3

(A) The mRNA expression of autotaxin was analyzed after silencing galectin-3. A more than two fold reduction was observed in both WM2664 and A375SM melanoma cells. The error bar represents the Rq-Max of triplicate reactions. (B) The supernatant is collected from NT shRNA and Gal-3 shRNA transduced WM2664 and A375SM melanoma cells, concentrated, methanol precipitated, and suspended in 6M urea lysis buffer. By western blot analysis, we observe a reduction of autotaxin expression after silencing galectin-3 by approximately tenfold in both cell lines. Coomassie blue membrane staining was used as a loading control.

A.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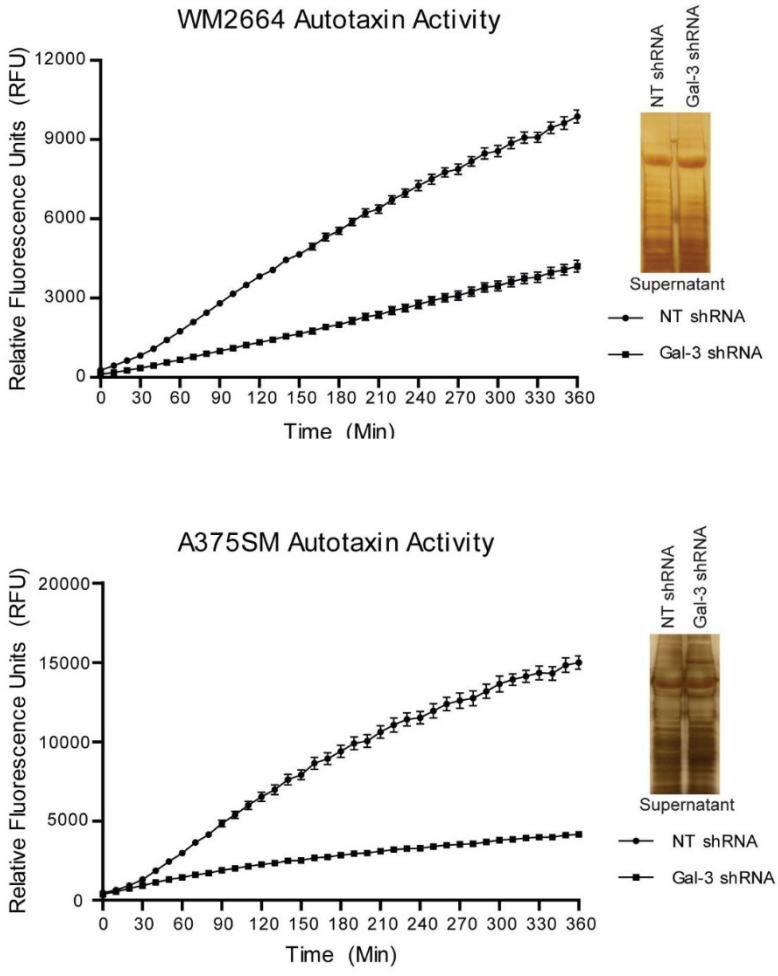

WM2664 Autotaxin Activity
Relative Fluorescence Units (RFU)
12000
9000
6000
3000
0
0 30 60 90 120 150 180 210 240 270 300 330 360
Time (Min)
NT shRNA
Gal-3 shRNA
Supernatant
NT shRNA
Gal-3 shRNA
A375SM Autotaxin Activity
Relative Fluorescence Units (RFU)
20000
15000
10000
5000
0
0 30 60 90 120 150 180 210 240 270 300 330 360
Time (Min)
NT shRNA
Gal-3 shRNA
Supernatant
NT shRNA
Gal-3 shRNA

Figure 11

Figure 11. Autotaxin Activity After Silencing Galectin-3

(A) FS-3 is composed of both a quencher and fluorescent tag. The quencher mimics the choline site on LPC while the fluorescent tag replaces the R group. A fluorescent reading is achieved when cleavage of the quencher occurs by autotaxin. Adapted from Ferguson CG et al, Organic Letters 2006. (B) Mean fluorescence is plotted for each sample every two minutes for six hours (360 min). WM2664 and A375SM NT shRNA cell lines have a higher rate of fluorescent activity as compared to galectin-3 shRNA transduced cells. Equal volumes of the supernatant were run on a gel and silver stained to confirm equal protein loading.

Autotaxin is Regulated by Galectin-3 at the Transcriptional Level

Our initial microarray and qRT-PCR studies confirm that galectin-3 regulates autotaxin expression at the mRNA level. However, we have yet to elucidate whether this is transcriptional or post-transcriptional regulation. To that end, we adopted the nuclear run-on method from Patron et al [153]. The intact nucleus is collected and incubated with ATP, GTP, CTP, and biotinylated UTP. The final mRNA transcript is transcribed by RNA polymerase II with the incorporation of biotinylated UTP. Pull down of the freshly transcribed mRNA by streptavidin beads, followed by qRT-PCR allowed us to identify real time transcription of autotaxin from A375SM NT shRNA and galectin-3 shRNA melanoma cells. As observed by qRT-PCR, the amount of autotaxin mRNA that was actively transcribed in galectin-3 silenced A375SM melanoma cells was reduced by more than threefold (Figure 12A). MicroRNA's can have a profound effect on mRNA expression. We have yet to rule out that galectin-3 could differentially regulate genes such as microRNAs that could post-transcriptionally regulate autotaxin. To rule out any added post-transcriptional regulation of autotaxin by galectin-3, the rate of mRNA degradation was analyzed after the addition of actinomycin D, an inhibitor of mRNA synthesis. After standardizing both samples to one at time zero, there was no significant change in the degradation rate of mRNA (Figure 12B). Therefore, the reduced levels of autotaxin after silencing galectin-3 is through its transcriptional regulation and our data suggest that microRNAs are not involved.

With the nuclear run-on assay we were able to determine that autotaxin is regulated at the transcriptional level. However, it was still unclear how silencing galectin-3 results in transcriptional repression. To that end, the first 988 base pairs of the promoter prior to the autotaxin mRNA start site (~1Kb) were cloned and inserted in front of the luciferase gene in

the PGL3 vector. After transfection of the PGL3 vectors in WM2664 and A375SM NT or galectin-3 shRNA melanoma cells, luciferase activity was analyzed. Luciferase activity was significantly reduced in both melanoma cell lines after silencing galectin-3 (Figure 13). The 1Kb promoter in front of the luciferase gene is less active in galectin-3 shRNA transduced melanoma cells. Our data now confirm that the transcriptional regulation of autotaxin occurs within 1Kb of the mRNA start site.

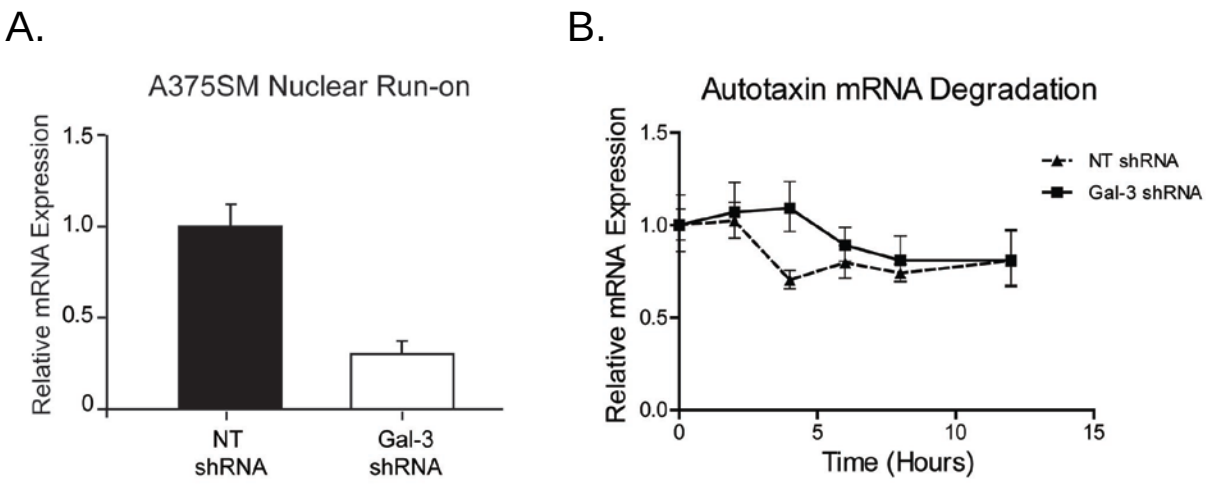


Figure 12. Silencing Galectin-3 Reduces mRNA Transcription as Observed by the Nuclear Run-On Assay

A375SM melanoma cells tranduced with NT or Galectin-3 shRNA were used to confirm that silencing galectin-3 reudces the transcriptional activation of autottaxin. (A) The nuclear run-on assay was used with biotin labeld UTP. Pull down of biotinylated mRNA follwed by qRT-PCR shows a reduced amount of freshly transcribed autotaxin mRNA by aproximately 3 fold. (B) The rate of mRNA degradation is shown in the presense of actinomycin D over 12 hours. Time zero was standardized to one for both A375SM NT and galectin-3 shRNA samples. The rate of degradation does not change after silencng galectin-3. A drop at 4 hours is noticed in NT shRNA cells, however, this appears to be an outlier in our data.

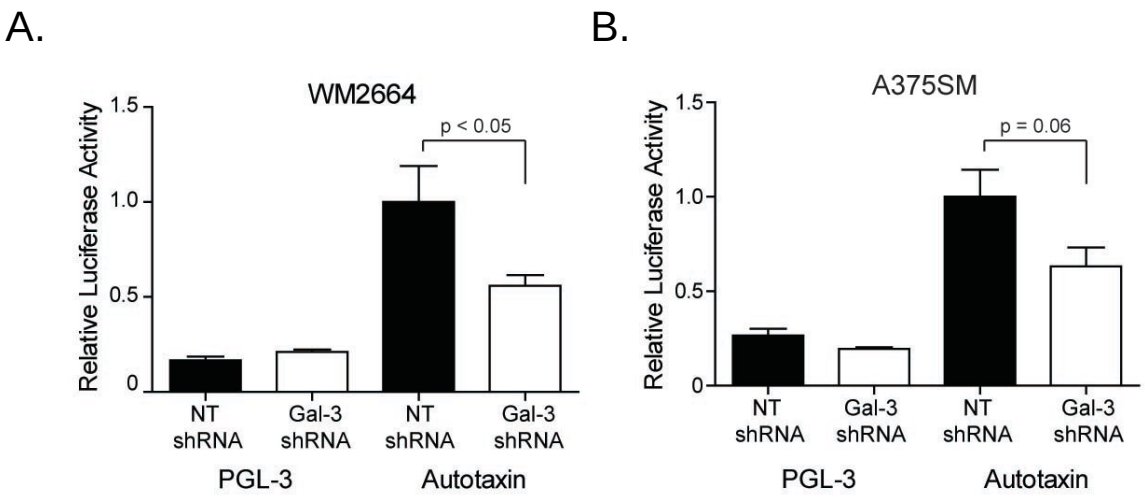


Figure 13. Dual Luciferase Activity of the Autotaxin Promoter is Reduced After Silencing Galectin-3

PGL-3 basic (no promoter) or the autotaxin 1Kb-PGL-3 vector were transfected into (A) WM2664 or (B) A375SM melanoma cells. Silencing Galectin-3 resulted in a significant reduction of luciferase activity in WM2664 melanoma cells ($p<0.05$) and was almost significant ($p=0.06$) in A375SM cells.

Silencing Galectin-3 Reduces the Protein Expression of the Transcription Factor NFAT1, a Known Regulator of Autotaxin

Galectin-3 is not considered to have a direct effect on transcriptional activation. In our system, it is likely that galectin-3 affects a signaling pathway that leads to the increased activation of specific transcription factors that enhance autotaxin expression. Therefore, we analyzed the first 1Kb of the autotaxin promoter to identify potential transcription factor binding sites. Through online Genomatix promoter analysis, more than 300 candidate transcription factors binding sites were found. Two of these binding sites were for the transcription factor Nuclear Factor of Activated T-Cells 1 (NFAT1). Both locations were within 500bp of the autotaxin mRNA site (Figure 14A).

NFAT1 belongs to the NFAT family of proteins that include NFAT1, 2, 3, 4, and 5. The DNA binding domain is highly conserved within the NFAT1 family which can interact with Fos/Jun to initiate transcriptional activation [171]. NFAT1 was originally identified in T-cells as an inducible transcription factor that bound to the antigen receptor response element (ARRE-2) of the interleukin-2 (IL-2) promoter [172]. NFAT1 was found to be a downstream transcription factor of the canonical calcium signaling pathway and is tightly regulated by the Ser/Thr phosphatase calcineurin. Once Ca^{2+} is released within the cytoplasm from compartments such as the endoplasmic reticulum, Ca^{2+} and the protein calmodulin bind to the calcineurin regulatory domain. This generates a conformation shift to activate phosphatase activity. Calcineurin then binds to NFAT1 and dephosphorylates it's nuclear localization sequence (NLS). "Unmasking" the NLS of NFAT1 enhances its transport into the nucleus and increases transcriptional activity of target genes [171, 173]. NFAT1 activation by calcium influx can be blocked with calcineurin inhibitors such as cyclosporine A (CsA) and tacrolimus

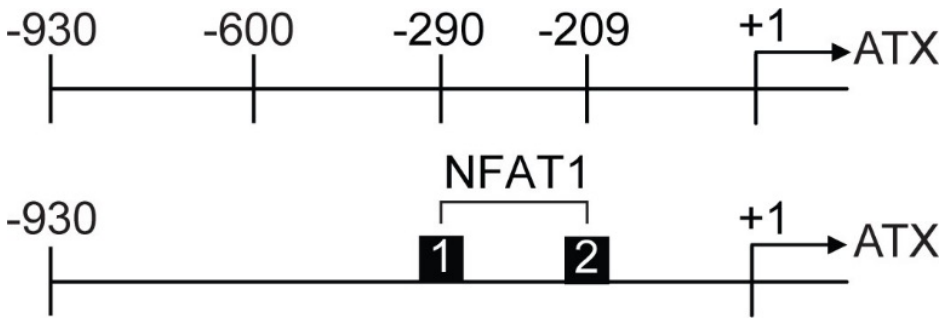
(FK506) [174]. Inhibition of Ca^{2+} mobilization by removing the stimulus or using calcineurin inhibition results in re-phosphorylation of NFAT1 within 5-15 min [175, 176]. Interestingly, this phenomenon in NFAT proteins can be repeated many times with the addition and removal of inhibitors. This suggests that constant regulation of NFAT by calcineurin and active kinases maintains the homeostatic balance in T-cells [175, 177].

NFAT1 expression has also been implicated in cancer. In breast cancer patient specimens, high expression of NFAT1 was identified in primary tumors and lymph node metastasis as compared to normal adjacent tissue [178]. Another group identified that NFAT1 enhances breast cancer cell invasion by up regulating the pro-inflammatory gene cyclooxygenase-2 (COX-2) [179]. The forced expression of integrin $\alpha 6\beta 4$ in breast cancer cells has been shown to activate NFAT1. This resulted in increased binding of NFAT1 to the autotaxin promoter as observed by the electrophoretic mobility shift assay and increased cellular motility of breast cancer cells[180]. Little is known about NFAT1 in melanoma. However, both NFAT2 and NFAT5 activity are increased in a $BRAF^{V600E}$-dependent mechanism in melanoma [181], and interestingly, NFAT1 knockout mice have fewer metastatic lesions when injected with B16F10 murine melanoma cells [182]. Recently, it has been reported that silencing NFAT1 or adding CsA resulted in increased cleaved caspase-3 and the number of apoptotic cells [183].

Previous evidence connecting NFAT1 with autotaxin expression and cancer progression caused us to generate the hypothesis that galectin-3 regulates autotaxin potentially by regulating NFAT1. Our initial study was to first identify the protein expression status of NFAT1 in our melanoma cells before and after silencing galectin-3. To our surprise, a significant reduction in NFAT1 protein expression is observed when galectin-3 is silenced in

both WM2664 and A375SM melanoma cells (Figure 14B). Therefore, it is likely that galectin-3 regulates autotaxin expression through NFAT1.

A.



B.

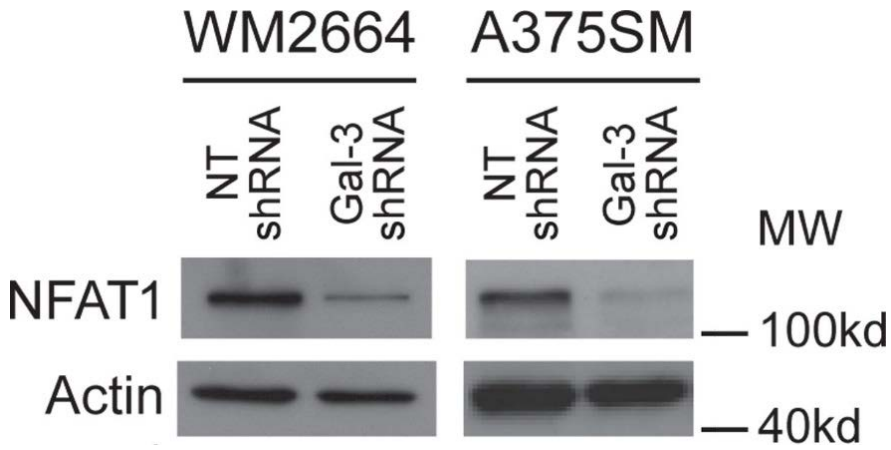


Figure 14. NFAT1 Protein Expression is Reduced After Silencing Galectin-3 in Melanoma Cells

(A) Two NFAT1 binding sites are located on the autotaxin promoter, both of which located within 300 bp of the transcriptional start site. (B) The protein expression of NFAT1 was analyzed. A significant reduction in NFAT1 was observed after silencing galectin-3 in both WME2664 and A375SM melanoma cells.

NFAT1 Enhances the Promoter Activity and Expression of Autotaxin

We expect that reduced NFAT1 protein expression after silencing galectin-3 will result in less bound NFAT1 to the autotaxin promoter. To that end, we next tested whether galectin-3 silencing affects the binding of NFAT1 by utilizing the chromatin immunoprecipitation (ChIP) assay. We used an anti-NFAT1 antibody to IP DNA-NFAT1 complexes in melanoma cells. End point PCR was performed surrounding both NFAT1 binding sites. As shown in Figure 15, NFAT1 binds to the promoter of autotaxin in both WM2664 and A375SM melanoma cells. When galectin-3 is silenced, no NFAT1 was bound to the autotaxin promoter in both melanoma cell lines (Figure 15).

Chromatin immunoprecipitation confirms that NFAT1 binds to the autotaxin promoter. NFAT1 binding is lost after silencing galectin-3. However, it is unclear whether bound NFAT1 on the promoter has an effect on transcriptional activation. To further establish the role NFAT1 has, the dual luciferase promoter assay was used with mutations in the autotaxin promoter at site 209, 290, or dual mutation. Silencing galectin-3 results in an ~50% reduction of luciferase activity with the wild type promoter (Figure 16) corroborating our initial luciferase assay (Figure13). When the mutated promoters are inserted into NT shRNA melanoma cells, the luciferase activity is also reduced to ~50% of the wild type promoter. The mutations have no effect on luciferase activity in galectin-3 silenced melanoma cells as compared to the wild type promoter (Figure 16). Therefore, it is likely that our reduced promoter activity after silencing galectin-3 is a direct result of reduced NFAT1 protein expression and binding to the autotaxin promoter. Interestingly, the dual mutation did not have an additive effect. This result suggests that both sites are equally critical for transcription.

Whether NFAT1 affects endogenous autotaxin expression has yet to be determined. Thus, we sought to test whether silencing NFAT1 reduces autotaxin expression in melanoma. To that end, NFAT1 was transiently silenced with NFAT1 siRNA in WM2664 and A375SM cell lines. After 48 hours incubation in serum free media, total cell lysate was collected and the supernatant was concentrated, methanol precipitated, and suspended in 6M urea lysis buffer. As shown in Figure 17A, transient NFAT1 siRNA efficiently knocks down NFAT1 expression in the total protein lysate, and this resulted in reduced autotaxin expression within the supernatant. The overexpression of NFAT1 in SB-2 (low metastatic, NFAT1 negative) melanoma cells resulted in a significant increase of autotaxin expression within the supernatant (Figure 17B). However, there are two bands present when probed for autotaxin. The larger band consists of isoform 1. It is likely that the lower molecular weight band is another isoform of autotaxin (isoform 2 or 3), or is a non-specific band. Interestingly, this band is not seen in our more metastatic cell lines. These data concluded that the transcription factor NFAT1 drives autotaxin expression in melanoma.

A more efficient, stable knockdown of NFAT1 was achieved in A375SM with lentiviral shRNA targeting NFAT1 (Figure 18A). Autotaxin expression within the supernatant was significantly reduced, corroborating our siRNA results (Figure 18A). Since the conversion of LPC to LPA is the primary function of autotaxin, the autotaxin activity assay was performed with the compound FS-3 to determine whether reduced expression of NFAT1 results in less FS-3 fluorescence *in vitro*. Indeed, A375SM melanoma cells with stable NFAT1 silencing results in a reduced rate of fluorescent activity (Figure 18B).

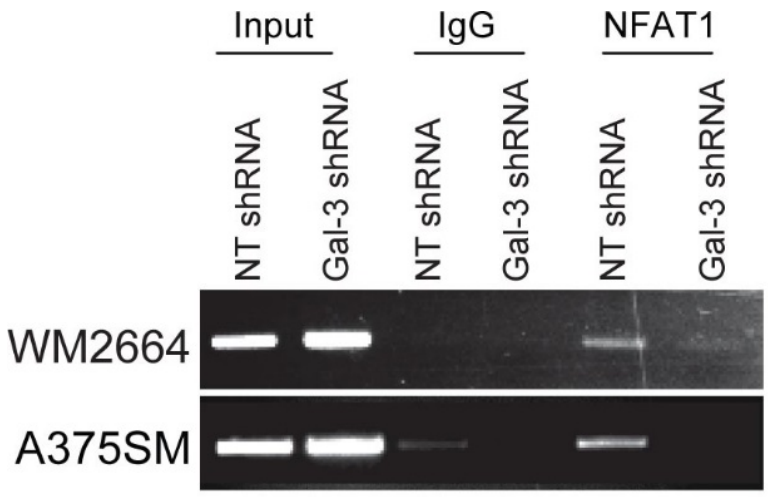


Figure 15. Chromatin Immunoprecipitation of NFAT1 on the Autotaxin Promoter is Lost after Silencing Galectin-3

Endpoint PCR was performed surrounding both NFAT1 binding sites on the autotaxin promoter. Chromatin immunoprecipitation of NFAT1 with anti-NFAT identifies a PCR product in NT shRNA transduced WM2664 and A375SM cells. No PCR product is amplified in galectin-3 shRNA transduced melanoma cells. Nonspecific IgG was used as a negative control. Sheered chromatin prior to immunoprecipitation was used as a positive control.

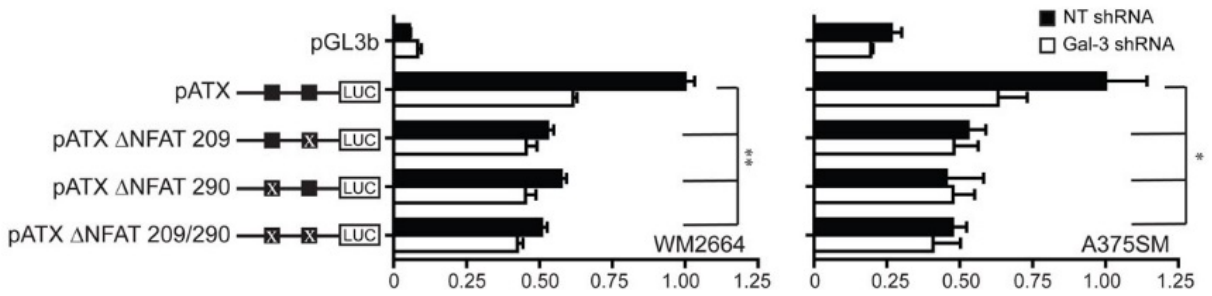


Figure 16. Dual Luciferase Promoter Activity is Reduced in the Presence of NFAT1 Binding Site Mutations

Silencing galectin-3 in both WM2664 and A375SM significantly reduced the luciferase promoter activity of the wild type promoter by approximately 40% (white bar,) as compared to NT shRNA (black bar); **P < 0.01 and *P < 0.05 respectively. Mutating either NFAT1 binding site at location 209 or 290 resulted in reduced promoter activity to approximately 50% of the wild type promoter; **P < 0.01 and *P < 0.05. Dual mutations were not significantly different from single mutations.

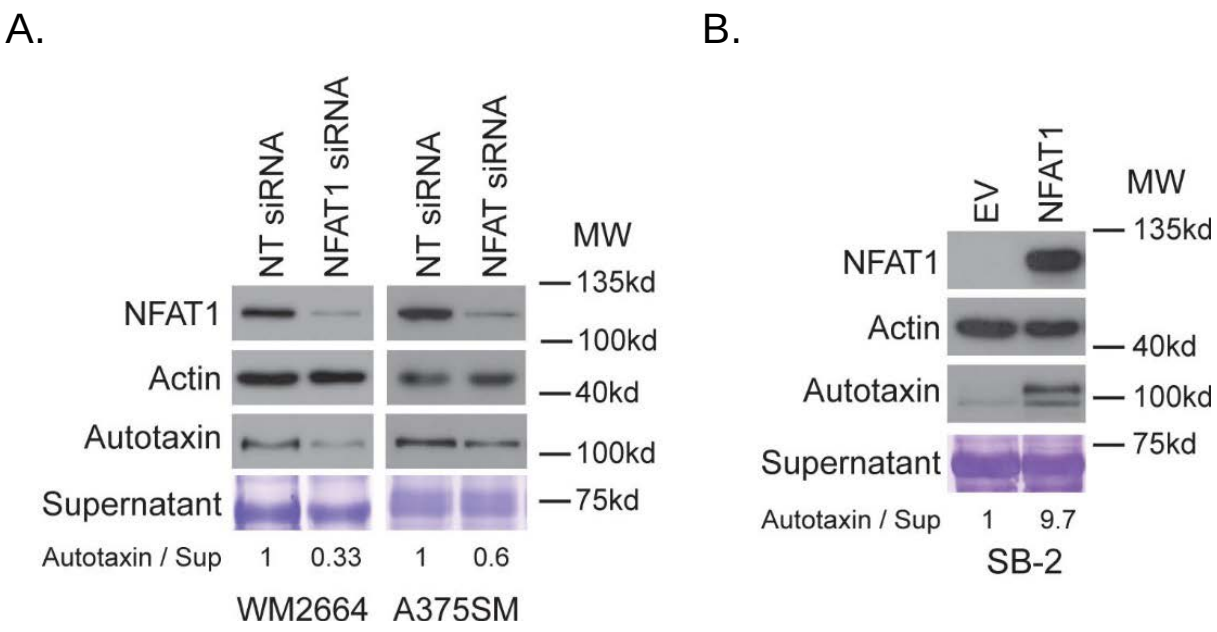


Figure 17. Silencing NFAT1 Decreases Autotaxin Expression in Melanoma Cells

(A) The addition of NFAT1 siRNA effectively reduces NFAT1 expression in both WM2664 and A375SM melanoma cell lines. The resulted in approximately a 70% reduction of autotaxin expression within the supernatant in WM2664 and a 40% reduction of autotaxin in A375SM cells. Coomassie blue staining of the membrane was used to confirm equal loading of the supernatant. (B) Over expression of NFAT1 in low metastatic non NFAT1 expressing melanoma cells increases the expression of two bands around the same molecular weight as autotaxin by 9.7 fold. The upper band likely isoform 1 and the lower band potentially being one of the smaller isoforms 2 or 3.

A. B.

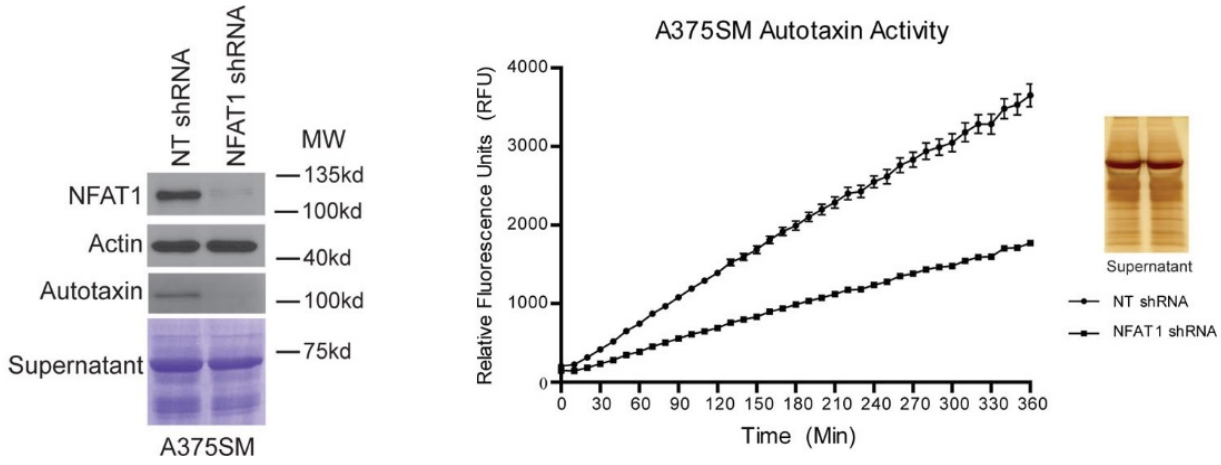


Figure 18. Silencing NFAT1 in A375SM Melanoma Cells Reduces Autotaxin Expression and Activity

(A) Stable knockdown of NFAT1 was achieved with lentiviral base NFAT1 shRNA as shown in whole cell lysate. NFAT1 shRNA effectively reduces autotaxin expression isolated from the cell culture supernatant. Coomassie blue staining of the membrane is shown for equal loading of the supernatant. (B) The fluorescent readout of FS-3 was obtained every two minutes for 6 hours (360 min). A375SM melanoma cells transduced with NFAT1 shRNA cleaved FS-3 at a lower rate than NT shRNA transduced A375SM cells. Silver staining of a gel with equal volumes of the supernatant that were used for the autotaxin activity assay were ran to confirm equal loading.

Rescue of Galectin-3 in Melanoma Cells Results with the Rescue of NFAT1 and Autotaxin.

Lentiviral based shRNA is a powerful tool for silencing the expression of target genes. However, this method can cause unwanted, nonspecific effects due to targeting the wrong genes or by causing an antiviral response to double stranded mRNA. To rule out these effects, galectin-3 was rescued in both WM2664 and A375SM galectin-3 shRNA melanoma cells. We were able to rescue galectin-3 in A375SM melanoma cells and partially rescue galectin-3 in WM2664. This resulted in the re-expression of NFAT1 and autotaxin in both melanoma cell lines while the empty vector control had no effect (Figure 19). Therefore, the deregulation of autotaxin and NFAT1 is not generated by off target effects of galectin-3 shRNA. These downstream events occur through galectin-3.

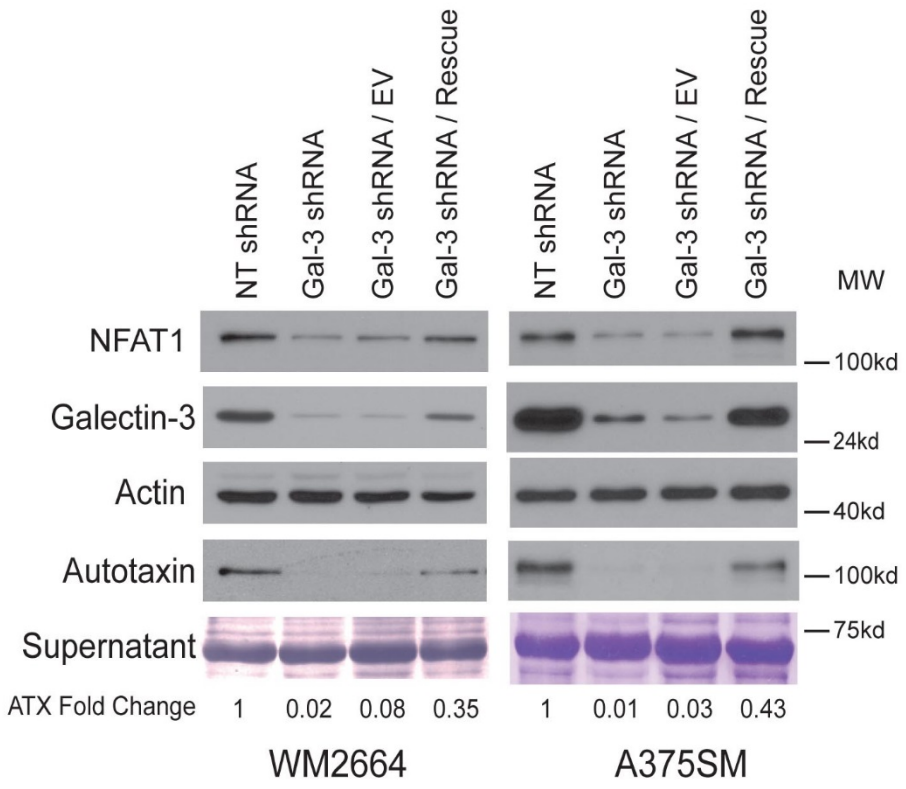


Figure 19. The Rescue of Galectin-3 Rescues NFAT1 and Autotaxin Protein Expression

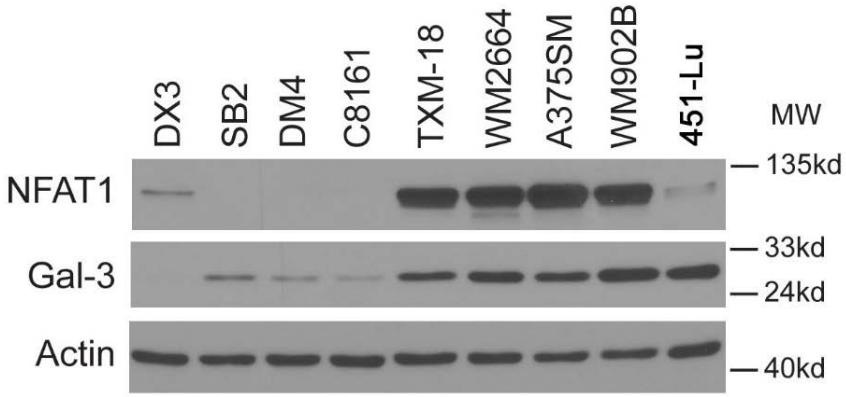
The Rescue of Galectin-3 is shown for both WM2664 and A375SM melanoma Cells. An increased expression of NFAT1 is observed after rescuing galectin-3 expression as compared to Galectin-3 shRNA cells transduced with an empty vector. The amount of autotaxin expressed in the supernatant of melanoma cells is also increased in galectin-3 rescued cells. The membrane with the supernatant is Coomassie blue stained and shown as an equal loading control.

Galectin-3, NFAT1, and Autotaxin Expression are Positively Correlated in Melanoma Cells

To our knowledge, the expression of NFAT1 has not been established in melanoma. Therefore, we analyzed the expression of NFAT1 by western blot in a panel of melanoma cell lines (Figure 20A). The first observation is that the less tumorigenic and metastatic cell lines (SB-2, DM4, and DX3) express far less NFAT1 than the more metastatic cells (A375SM, WM2664). Second, there is a positive correlation between galectin-3 expression and NFAT1, although, it is not a complete correlation. This is evident in DX3 and 451-Lu melanoma cells. Although low levels of galectin-3 are observed, there is still a slight amount of NFAT1 expression in DX-3 cells. The opposite holds true for 451-Lu. These cells express high levels of galectin-3, however, low levels of NFAT1 are observed.

We then analyzed autotaxin mRNA expression in the same cell panel (Figure 20B). Melanoma cells that express high levels of galectin-3 and NFAT1 express autotaxin. The cells that express low levels of galectin-3 and no NFAT1 do not express autotaxin. What is identified with the DX-3 and 451-Lu cells might shed some light between the correlation of NFAT1 and Galectin-3. Although DX3 cells express a low amount of NFAT1, very low levels of autotaxin are observed. In 451-Lu, there are higher levels of autotaxin mRNA. These cell lines have comparable levels of NFAT1. However, since higher levels of galectin-3 are present in 451-Lu, perhaps galectin-3 enhances the activity of NFAT1 as well as its expression.

A.


DX3
SB2
DM4
C8161
TXM-18
WM2664
A375SM
WM902B
451-Lu
MW
NFAT1
135kd
Gal-3
33kd
24kd
Actin
40kd

B.

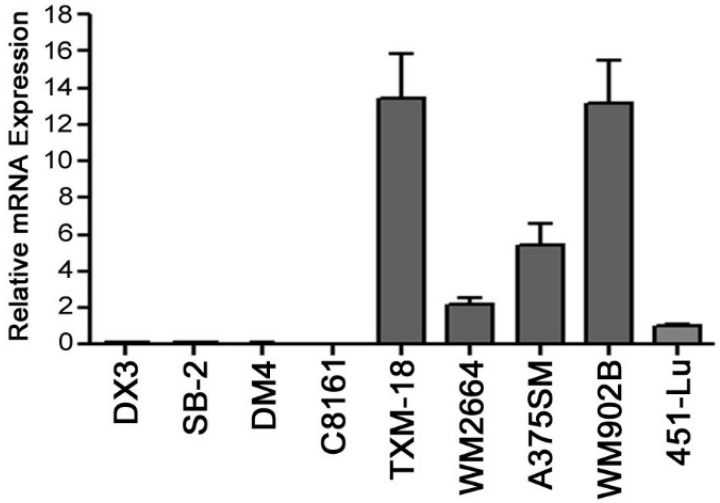

Relative mRNA Expression
18
16
14
12
10
8
6
4
2
0
DX3
SB-2
DM4
C8161
TXM-18
WM2664
A375SM
WM902B
451-Lu

Figure 20

Figure 20. The Expression of NFAT1 and Autotaxin in Melanoma Cell Lines

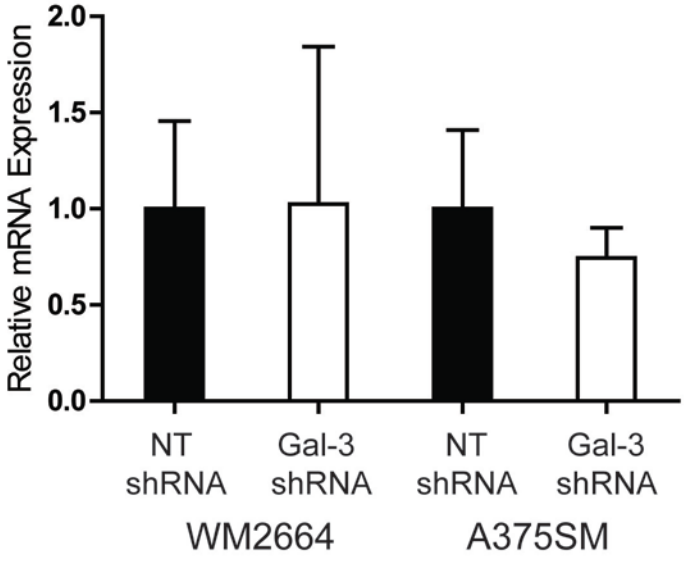
(A) The protein expression of NFAT1 and Galectin-3 was analyzed by western blot in multiple melanoma cell lines. Low expression of galectin-3 correlated with low levels of NFAT1 protein expression. High levels of galectin-3 correlated with high levels of NFAT1 protein expression. The exception to this observation was 451-Lu, as it had high levels of galectin-3 but low levels of NFAT1. (B) The mRNA expression profile of autotaxin was compared between the same melanoma cell lines. High levels of galectin-3 correlated with high levels of autotaxin. Relative expression was standardized to 1 with the 451-Lu melanoma cells. The relative expression of DX3, SB-2, DM4, and C8161 are all less than 0.10.

Galectin-3 Maintains the Expression of NFAT1 at the Post-Translational Level

The mechanism by which galectin-3 regulates NFAT1 remains elusive. We first investigated the mRNA expression of NFAT1 after galectin-3 knockdown. Interestingly, mRNA levels remained the same (Figure 21A). This brought us back to what is observed with the cell panel. The high galectin-3 expressing but with low expression of NFAT1 451-Lu cell line expresses autotaxin while the low galectin-3 expressing DX3 cells have 10 fold lower Autotaxin levels with the same amount of NFAT1. This raises the possibility that galectin-3 might affect NFAT1 activity as well as expression. Perhaps in our melanoma cells, silencing galectin-3 results in reduced activity of NFAT1 which then leads to its degradation. This is a bold hypothesis, however one experiment might suggest this to be correct. NFAT activity can be analyzed by a simple western blot. When NFAT1 is active, it becomes dephosphorylated. The dephosphorylated band is slightly lower in molecular weight. This can even be seen in figure 20A, although the bands are so close they appear as one single band. We therefore tested whether silencing galectin-3 increased NFAT1 protein stability. To that end, we added the protease inhibitor to our cells 6 hours after transfection of a FLAG-tagged NFAT1 vector in WM2664 and A375SM NT and galectin-3 shRNA melanoma cells. The cells were lysed 24 hours later followed by immunoprecipitation with an anti-Flag antibody. A western blot was performed targeting NFAT1. What was observed was striking. Transfection of FLAG-NFAT1 at equal amounts with no protease inhibitor in both NT and galectin-3 shRNA had significant differences in protein expression when lysed 24 hours later (Figure 21B). However, with the addition of Bortezomib, a proteasome inhibitor, NFAT1 levels in galectin-3 silenced cells were higher than without inhibition of the proteasome. However, when protein expression is rescued with the proteasome inhibitor, the lower band representing active NFAT1 is still greatly

reduced in galectin-3 silenced cells. This suggests that galectin-3 drives NFAT1 activity. The loss of galectin-3 results in reduced transcriptionally active NFAT1. Our data suggest that less active NFAT1 might lead to rapid degradation. Further studies identifying how degradation of NFAT1 in this context occurs must be performed (ubiquitilation?, phosphorylation?, etc.).

A.


Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
NT shRNA
Gal-3 shRNA
NT shRNA
Gal-3 shRNA
WM2664
A375SM

B.

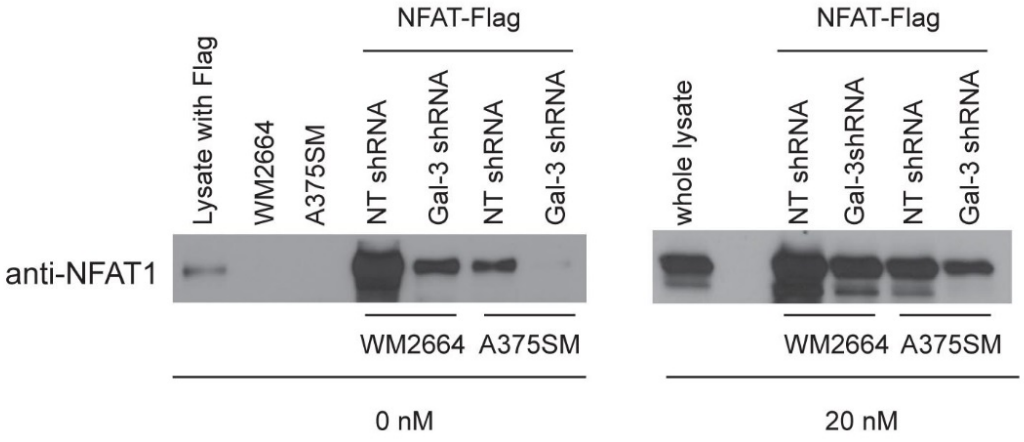

NFAT-Flag
NFAT-Flag
Lysate with Flag
WM2664
A375SM
NT shRNA
Gal-3 shRNA
NT shRNA
Gal-3 shRNA
whole lysate
NT shRNA
Gal-3shRNA
NT shRNA
Gal-3 shRNA
anti-NFAT1
WM2664
A375SM
WM2664
A375SM
0 nM
20 nM

Figure 21

Figure 21. NFAT1 is Degraded at the Protein Level After Silencing Galectin-3

(A) The mRNA expression of NFAT1 in WM2664 and A375SM melanoma cells after silencing galectin-3. NFAT1 expression is similar in WM2664 melanoma cells and is minimally reduced after silencing galectin-3 in A375SM cells. (B) Melanoma cells were transfected with FLAG-NFAT1 and immunoprecipitated with anti-FLAG. Western blot against NFAT1 was performed. Rapid loss of NFAT1 occurs in galectin-3 shRNA melanoma cells as compared to NT shRNA. The addition of the proteasome inhibitor Bortezomib at 20nM inhibits the loss of NFAT1 protein; however, it does not rescue its de-phosphorylation state (lower band). WM2664 uptake higher levels of NFAT1 as compared to A375SM.

Discussion

Out of the galectin family of proteins, galectin-3 could potentially be the most diverse in its function. It can affect biological functions through its carbohydrate binding properties that all galectins possess. However, its unique chimeric protein structure that includes phosphorylation sites at the N-terminal end, a collagen like domain, and an NWGR "anti-death" motif generate a dynamic molecule that performs non-carbohydrate binding functions as well. It has been well established that galectin-3 can modulate the expression of genes involved in cancer progression, whether that be cyclin-D1, insulin like growth factor binding protein 5, IL-8, fibronectin, or VE-cadherin [114, 147, 148]. Phosphorylation of Ser6 on galectin-3 appears to be required for genetic transcription of cyclin-D1 and insulin like growth factor binding protein 5. However, how Ser6 phosphorylation transcriptionally activates these genes remains unclear. Reducing the total expression of galectin-3 has proven to reduce the expression of IL-8, fibronectin, and VE-cadherin. In the present study, we sought to determine novel downstream targets of galectin-3 that have previously been unidentified. By comparing the gene expression profile by cRNA microarray of WM2664 melanoma cells transduced with either NT shRNA or galectin-3 shRNA, we were able to confirm a specific gene expression profile of melanoma cells that is regulated by galectin-3. More importantly, we identified multiple pro-tumorigenic genes that are down regulated after silencing galectin-3. Therefore, galectin-3 might promote the metastatic melanoma phenotype by regulating these genes. L-plastin, osteopontin, autotaxin, hif-2α, and cathepsins B and K have all been implicated in the migratory, invasive or angiogenic phenotype [170, 184-187]. Endothelin receptor B and endothelin like receptor B were also identified in our microarray. Endothelin receptor B stimulation by its ligand endothelin 3 (ET3) induces cell proliferation of A375 melanoma cells,

and endothelin receptor B antagonists can reduce cell viability in both A375 and WM35 cell lines [188, 189]. These are all ideal targets to further study. However, our identified genes are only at the microarray level and must be further confirmed at the mRNA or protein level by qRT-PCR or western blot respectively.

Of these genes, we singled out and validated autotaxin. Our choice to selectively focus on the deregulation of autotaxin by galectin-3 is due to a variety of reasons. First, autotaxin was identified as a motility factor for melanoma cells [163]. In our melanoma cells, we see a significant reduction in cell migration after silencing galectin-3. Therefore, these two molecules could be intertwined in regards to the migratory phenotype. Second, autotaxin is a lysophospholipase D enzyme secreted within the tumor microenvironment where it can produce the bioactive ligand LPA from LPC. LPA can have significant effects on cancer cells as well as the surrounding tumor microenvironment. For example, LPA can directly increase cancer cell invasion *in vitro* or can activate lymphatic and endothelial cells to induce tumor lymphangiogenesis and angiogenesis *in vivo* [190-192]. LPA production is the primary function of autotaxin. Autotaxin itself does not directly bind to or activate any known pathways. The phenotypic relevance of autotaxin is linked to LPA production. Genetic instability of melanoma cells does not guarantee that an overexpressed gene is biologically functional. Therefore, we tested the activity of autotaxin in melanoma cells. High autotaxin activity was observed, and silencing galectin-3 in two melanoma cell lines significantly reduced the amount of autotaxin activity *in vitro*. This confirms that autotaxin is functional in our melanoma cells, and galectin-3 could potentially increase the metastatic phenotype by regulating autotaxin.

However, the mechanism by which galectin-3 regulates autotaxin must be elucidated. The nuclear run-on assay was a critical experiment as it can confirm the endogenous transcription rate of autotaxin without the use of artificial promoters. Artificial promoters can be misleading since upstream and downstream transcriptional activators that are required could be eliminated from the inserted promoter. Our nuclear run-on assay suggested that autotaxin expression is reduced at the transcriptional level after silencing galectin-3. The dual luciferase promoter assay in both WM2664 and A375SM melanoma cells confirmed this initial observation. Due to previous publications that identify NFAT1 as a transcriptional activator that binds to the autotaxin promoter, we initially focused on this transcription factor. To our surprise, silencing galectin-3 results in a dramatic reduction of NFAT1 protein expression. ChIP showed that bound NFAT1 to the autotaxin promoter is lost after silencing galectin-3. Mutations at both NFAT1 binding sites within the autotaxin luciferase promoter confirmed that both NFAT1 binding sites are equally critical for autotaxin transcription. Mutating the NFAT1 binding sites reduced promoter activity to that of galectin-3 shRNA cells. These data confirm that reduced levels of NFAT1 after silencing galectin-3 is the main contributing factor to decreased autotaxin expression.

We further investigated the effect of NFAT1 on autotaxin expression by transiently silencing NFAT1 in A375SM and WM2664. This resulted in reduced autotaxin expression within the supernatant. Overexpression of NFAT1 in SB-2 cells up-regulated autotaxin expression. Stably silencing NFAT1 in A375SM melanoma cells not only resulted in the loss of autotaxin expression but also the amount of autotaxin activity within the supernatant as observed by the autotaxin activity assy. The rescue of galectin-3 in WM2664 and A375SM

melanoma cells rescued both autotaxin and NFAT1. Taken together, these data confirm that galectin-3 regulates autotaxin expression via NFAT1.

What remains elusive in our study is the mechanism by which galectin-3 regulates NFAT1 protein expression. We first reviewed our cRNA microarray; however, NFAT1 was not identified as a deregulated gene. This made us lean towards the possibility that NFAT1 is regulated at the protein level. Our results that show little change in mRNA expression of NFAT1 after silencing galectin-3 confirm our hypothesis. Therefore, we sought out to identify how galectin-3 regulates the protein stability of NFAT1. One of our first experiments was to immunoprecipitate NFAT1 and probe for ubiquitin, the posttranslational modification that leads to proteasome targeted degradation. However, our western blots for ubiquitin showed no changes, although, this could have been due to a poorly exposed blot (data not shown). We could not conclude that this was due to our technique or if this was biologically correct. A different approach was then considered. That was to add the proteasome inhibitor, immunoprecipitate NFAT1, and determine whether the proteasome inhibitor rescues the protein expression of NFAT1. By this method, NFAT1 expression was rescued in galectin-3 silenced melanoma cells. This result suggests that NFAT1 is sent for proteasome degradation at a higher rate when galectin-3 is silenced. We also identified another phenomenon with this technique. Even though we rescue NFAT1 protein expression, the dephosphorylation of NFAT1 is not rescued. This is especially evident in A375SM melanoma cell line. We therefore hypothesize that galectin-3 is required for activation of NFAT1, which requires dephosphorylation of the N-terminal regulatory domain. A domain that is tightly regulated by calcineurin, casein kinase 1(CK1), and GSK3β. What immediately stands out is that both CK1 and GSK3β can phosphorylate galectin-3 as well. It must be determined whether this is of

random coincidence or if these molecules are connected within a similar pathway. Galectin-3 binds to and is phosphorylated by GSK3β. Although GSK3β is thought to inactivate NFAT1, Yoeli-Lerner et al. have shown that GSK3β is required to maintain NFAT1 protein stability and its transcriptional activity [193]. GSK3β might play a complex dual role in regulating NFAT1 under certain conditions. The fact that over 14 phosphorylation sites on NFAT1 on the nuclear localization sequence indicate that this could be so. GSK3β could require galectin-3 as a scaffold type protein for its phosphorylation of NFAT1. Immunoprecipitation of galectin-3 followed by blotting for NFAT1 did not identify NFAT1 on the membrane, suggesting that galectin-3 and NFAT1 do not form a complex (data not shown). However, our buffers and antibodies might not be used under the right conditions. Protein stability of NFAT1 by GSK3β requires AKT mediated phosphorylation of GSK3 at Ser9 [193]. Interestingly, silencing galectin-3 has been shown to reduce the phosphorylation of both AKT and GSK3β. This reduced the invasive phenotype of pancreatic cancer cells [108]. Therefore, it is possible that galectin-3 enhances the protein stability of NFAT1 through AKT/GSK3β. Our microarray also identified GSK3 to be down regulated after silencing galectin-3. However, very little change in GSK3β expression was observed at the protein level (data not shown). Casein kinase 1 phosphorylates both NFAT1 and galectin-3. Galectin-3 is highly expressed in our melanoma cell lines that express NFAT1 as compared to those that have no to little NFAT1 expression. Galectin-3 might be the primary phosphorylating partner of casein kinase 1. Silencing galectin-3 then could allow for casein kinase to phosphorylate and inactive other molecules such as NFAT1. However, this is a hypothetical conclusion with no evidence to suggest this occurs. Currently, no conclusive data has been collected to identify the mechanism by which galectin-3 regulates NFAT1 protein expression.

The positive correlation between galectin-3, autotaxin, and NFAT1 is highly prominent in mutant $BRAF^{V600E/D}$ melanoma cell lines as compared NRAS mutants and other BRAF mutations (Figures 4 and 20A from the same western blot). Although this is only correlative, it could be hypothesized that constitutively active $BRAF^{V600E}$ plays a role in driving the expression of galectin-3 and NFAT1 in melanoma. Galectin-3 seems to regulate NFAT1 at the protein level; however, $BRAF^{V600E/D}$ activating mutation could be required for the transcription of NFAT1. If this is the case, then galectin-3 mediated protein stability of NFAT1 would be highly relevant in $BRAF^{V600E}$ melanoma patients, but would have little significance in patients with WT $BRAF^{V600E}$. Observing the promoter activity of NFAT1 in the presence of inhibitors of $BRAF^{V600E}$ could determine whether these pathways are connected. Further, a human tissue microarray comparing NFAT1 and galectin-3 expression in conjunction with the mutation status of BRAF will determine whether there is indeed a correlation between these three molecules in melanoma. Comparing NFAT1 expression in A375 (a low metastatic cell line in mice) with the highly metastatic cell line A375SM will also determine whether NFAT expression is an early event or reliant on a more aggressive metastatic phenotype. Our cell panel contains only a small cohort of melanoma cell lines. Future experiments performing the experiments discussed above will elucidate the contribution of $BRAF^{V600E}$ to the galectin-3/NFAT1/autotaxin pathway.

Sub-Aim 2.2: Autotaxin and NFAT1 Contribute to Melanoma Growth and Metastasis

Introduction

Galectin-3 has been implicated in tumor cell migration, invasion, angiogenesis, tumor growth, and metastasis. Herein, we identify a novel molecule, autotaxin, regulated by galectin-3 via the transcription factor NFAT1. We next sought out to determine whether galectin-3 contributes to the metastatic melanoma phenotype by regulating autotaxin. Autotaxin expression was first identified as a motility and invasive factor for melanoma cells [163]. The conversion of LPC to LPA by autotaxin is a powerful inducer of angiogenesis [194]. In this aim, we will elucidate whether galectin-3 enhances tumor growth, experimental lung metastasis, and angiogenesis in A375SM melanoma xenograft tumors due to its regulation of autotaxin.

The transcription factor NFAT1 is also identified as a novel downstream target of galectin-3. Recently, NFAT1 has been found as a survival factor for melanoma cells under the pressure of MEK and BRAF inhibitors. However, no studies have been conducted in regards to its role in the melanoma invasive and the metastatic phenotype. Therefore, we analyzed the contribution of NFAT1 to the invasive phenotype *in vitro*, and tumor growth and experimental lung metastasis *in vivo*.

Results

The Over Expression of Autotaxin in Galectin-3 Silenced Cells Partially Rescues Tumor Growth and Metastasis *In Vivo.*

To validate the role autotaxin has in melanoma growth and metastasis, rescued autotaxin in our A375SM galectin-3 silenced melanoma cells. We were able to generate a stable cell line with high levels of autotaxin expression and this translated to increased autotaxin activity (Figure 22 A and B). These cells (A375SM galectin-3 shRNA / Autotaxin Rescue) were then injected subcutaneously in nude mice in parallel with A375SM NT shRNA and galectin-3 shRNA / empty vector. Tumor volume was measured for 28 days and the rate of growth was compared between A375SM NT shRNA, galectin-3 shRNA / empty vector, and galectin-3 shRNA/autotaxin rescue. Galectin-3 shRNA / empty vector tumors were significantly smaller than NT shRNA tumors (Figure 23). Immunohistochemistry confirmed that indeed autotaxin expression is increased *in vivo* in autotaxin overexpressing cells and galectin-3 remains silenced (Figure 23). The rescue of autotaxin expression in galectin-3 silenced tumors significantly increased tumor volume as compared galectin-3 shRNA / empty vector xenograft tumors (Figure 23). However, the rescue of autotaxin in galectin-3 silenced melanoma cells did not completely restore tumor growth as compared to NT shRNA. Therefore, autotaxin expression is only one contributing factor. Other factors by galectin-3 most likely affect the remaining difference between tumor growth, such as its anti-apoptotic function and potential regulation of other pro-tumorigenic genes.

Autotaxin and galectin-3 are both reported to enhance angiogenesis [194, 195]. To corroborate those claims, immunohistochemistry was performed on A375SM xenograft tumors with anti-CD31, a marker for blood vessels. NT shRNA melanoma cells have a high number of

blood vessels as compared to galectin-3 shRNA / empty vector. The overexpression of autotaxin in galectin-3 silenced melanoma cells rescued this phenotype (Figure 24). This suggests that reduced angiogenesis after silencing galectin-3 is attributed to the loss of autotaxin. VEGF was also stained within these tumors. A slight reduction in VEGF staining was observed after silencing galectin-3; however, this was only a modest change (Figure 25). Circulating autotaxin is a strong inducer of angiogenesis as well [196]. Therefore we tested whether circulating autotaxin was reduced after silencing galectin-3. Blood serum from tumor bearing mice was taken prior to sacrificing. The autotaxin assay was then used to identify the amount of circulating autotaxin within the blood. Unfortunately, no detectable levels of autotaxin were observed (data not shown). This could have been due to poor isolation of plasma, the majority of autotaxin secreted by the tumor cells stayed within the tumor microenvironment, or the protein was degraded quickly. We also analyzed the number of dead melanoma cells within the tumors by the TUNEL assay. The number of positively stained, dead tumor cells was significantly increased in galectin-3 silenced tumors. The overexpression of autotaxin partially reduced the number of apoptotic cells (Figure 26). This could be attributed by enhanced angiogenesis and access to nutrients and oxygen. Another potential mechanism is that enhanced LPA synthesis in the tumor microenvironment could potentially activate LPA receptors on melanoma cells to increase cell survival. The mRNA expression of six LPA receptors in A375SM melanoma cells were analyzed and compared with WM2664 and SB-2 melanoma cells. High levels of LPAR1 were identified in both A375SM and WM2664 as compared to SB-2 (Figure 27). Therefore, the autocrine loop of autotaxin secretion, LPA production, and LPA receptor signaling is likely to occur within the xenograft tumors.

A375SM melanoma cells transduced with either NT shRNA, galectin-3 shRNA/ EV, or galectin-3 shRNA/Autotaxin over expression were injected intravenously and the number of metastatic lung colonies were counted. Galectin-3 shRNA / EV tumors produced significantly fewer metastatic lesions, 9, as compared to NT shRNA, 68. The re-expression of autotaxin slightly rescued the number of metastatic colonies, 29 (Figure 28). Therefore, the regulation of autotaxin by galectin-3 is an important contributor to the metastatic phenotype. However, the re-expression of autotaxin does not completely rescue the number of experimental lung metastasis. The carbohydrate binding and anti-apoptotic properties of galectin-3 or other metastasis associated genes regulated by galectin-3 likely contribute to the remaining metastatic potential.

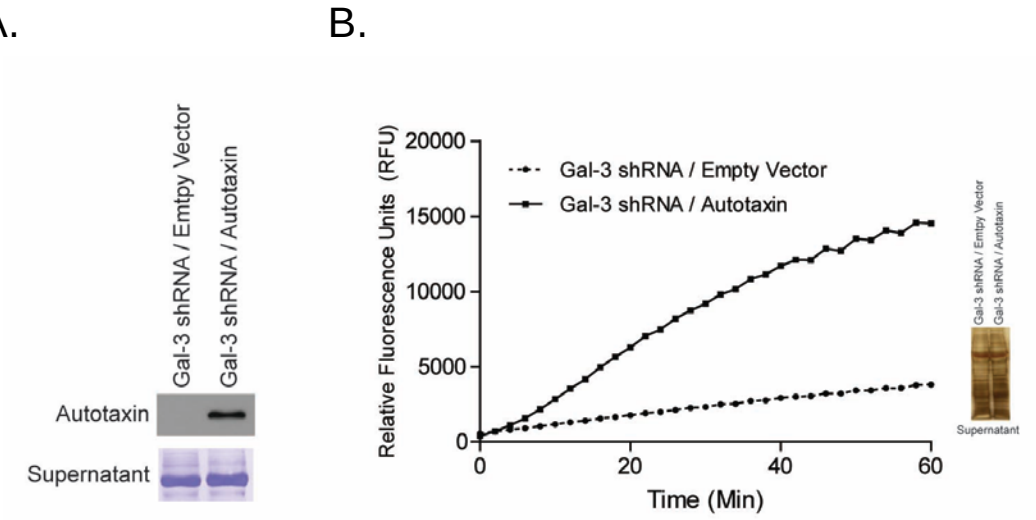
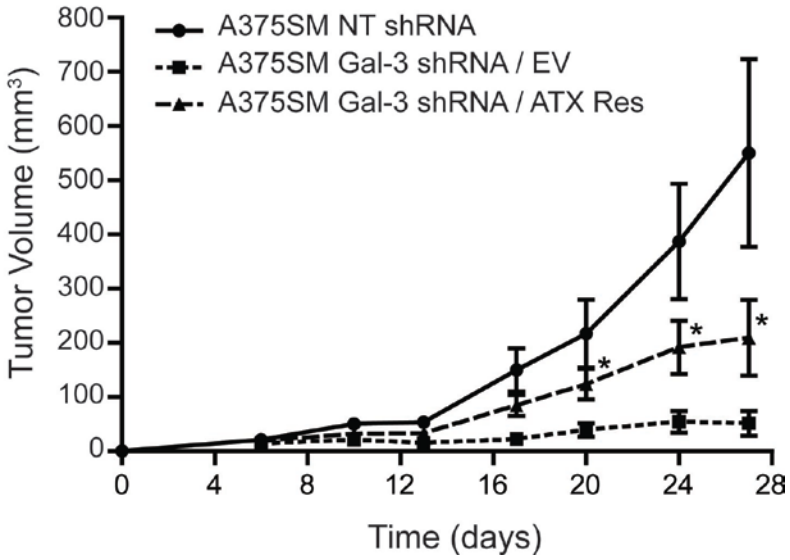


Figure 22. The Over Expression of Autotaxin is Confirmed in A375SM Melanoma Cells

(A) Autotaxin is stably over expressed in A375SM melanoma cells with galectin-3 shRNA. The membrane is stained with coomassie blue for a loading control. (B) The cleavage of FS-3 rapidly occurs after overexpressing autotaxin. Therefore, we a capable of over expressing a functional autotaxin enzyme in A375SM galectin-3 shRNA transduced cells. A silver stain of equal loading supernatant was performed to confirm equal loading.

A.


A375SM NT shRNA
A375SM Gal-3 shRNA / EV
A375SM Gal-3 shRNA / ATX Res
Tumor Volume (mm³)
800
700
600
500
400
300
200
100
0
0
4
8
12
16
20
24
28
Time (days)
*
*
*

B.

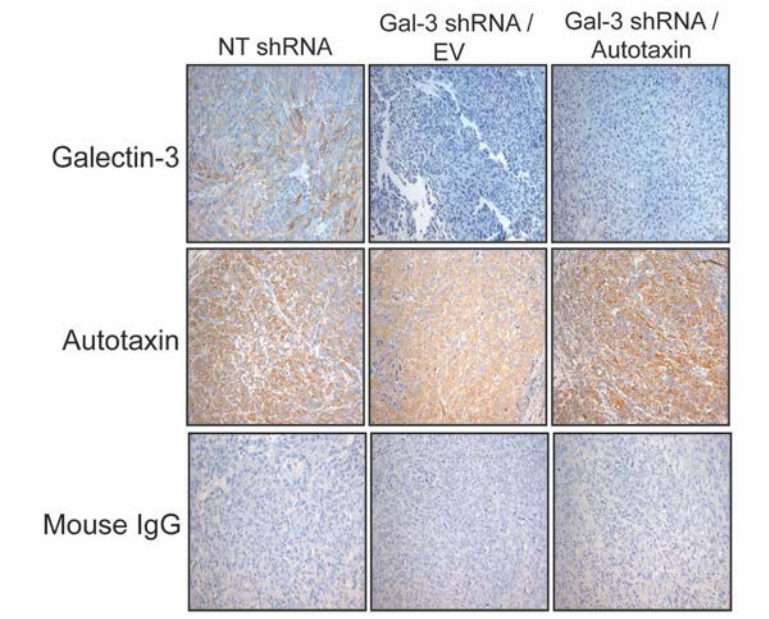

NT shRNA
Gal-3 shRNA / EV
Gal-3 shRNA / Autotaxin
Galectin-3
Autotaxin
Mouse IgG

Figure 23

Figure 23. Re-Expression of Autotaxin in Galectin-3 Silenced Melanoma Cells Partially Rescues Tumor Growth

(A) A375SM melanoma cells with NT shRNA, galectin-3 shRNA / Empty vector, or galectin-3 shRNA / Autotaxin were injected subcutaneously in nude mice. Induced expression of autotaxin partially rescues tumor growth in galectin-3 silenced melanoma cells as compared to the empty vector control, *$P < 0.01$. However, the rescue of autotaxin does not completely rescue tumor growth as compared to the NT shRNA transduced cells. (B) Galectin-3 remains silenced in the xenograft tumors. Autotaxin re-expression is confirmed in galectin-3 shRNA / autotaxin tumors.

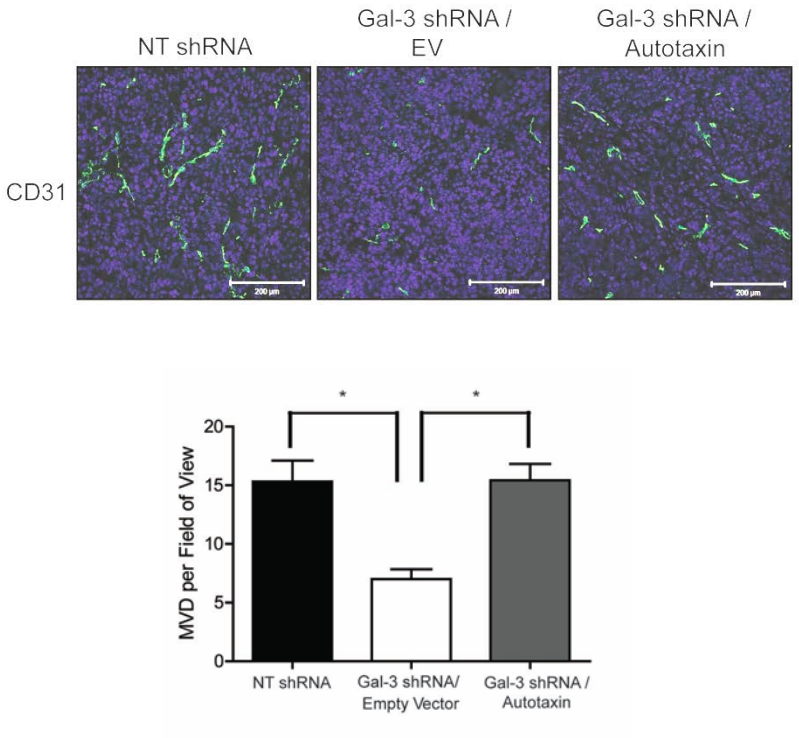


Figure 24. Microvascular Density is Increased in Galectin-3 shRNA Tumors that Have Autotaxin Over Expression

A representative image of the number of CD31 positive endothelial cells is shown from xenograft A375SM tumors. Silencing galectin-3 reduces the number of CD31 stained blood vessels within the tumor. The re-expression of autotaxin in galectin-3 shRNA tumors rescues the microvascular density (MVD), *$P < 0.05$.

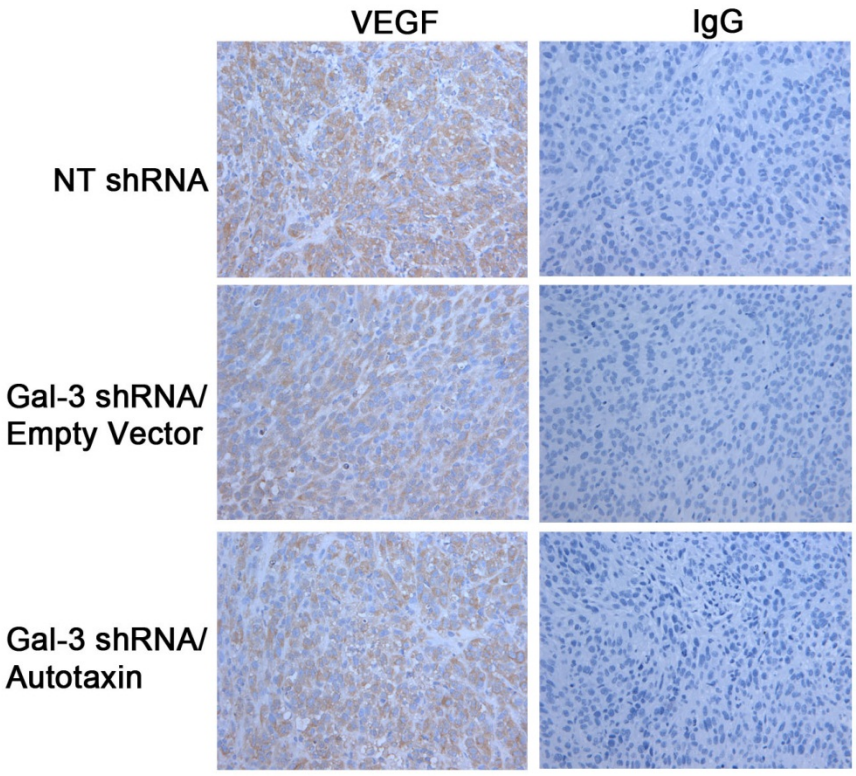


Figure 25. VEGF Expression is Slightly Reduced After Silencing Galectin-3

Immunohistochemistry for VEGF was performed. Silencing galectin-3 slightly reduces the amount of staining for VEGF. The rescue of autotaxin does not rescue VEGF expression within the tumor microenvironment.

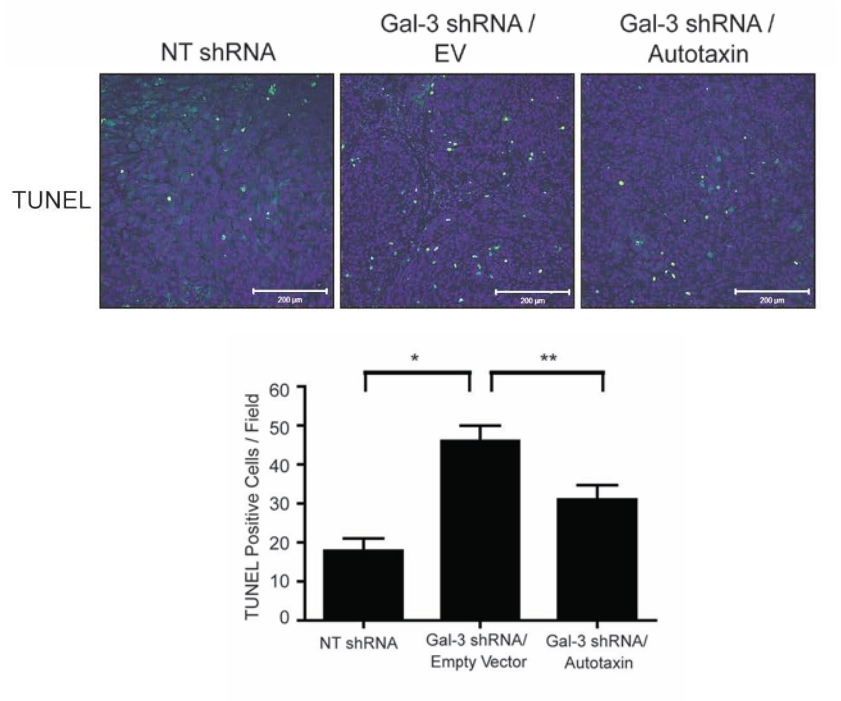


Figure 26. The Number of TUNEL Positive Cells in A375SM Xenograft Tumors

Silencing galectin-3 significantly increases the number of apoptotic TUNEL positive cells within the subcutaneous tumor, *P < 0.01. The rescue of autotaxin within these tumors significantly reduces the number of apoptotic cells, **P <0.05.

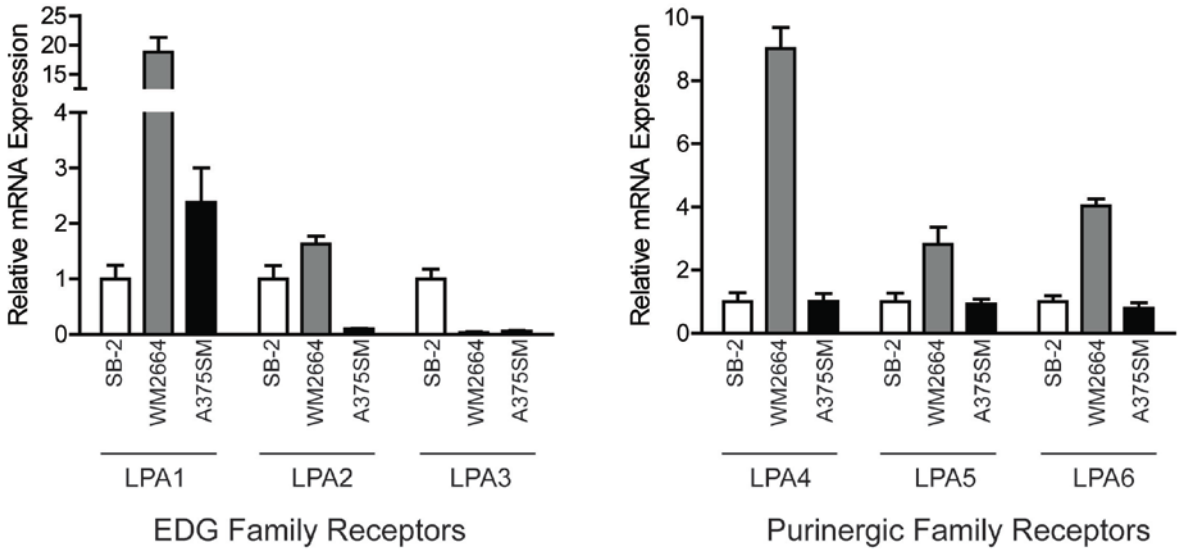
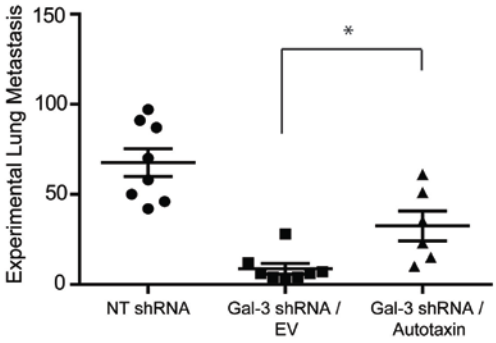


Figure 27. Relative mRNA Expression of LPA Receptors in Melanoma Cell Lines

Six LPA receptors were analyzed by qRT-PCR and standardized to one with the low tumorigenic SB-2 cell line. High levels of LPA1 are expressed in both WM2664 and A375SM as compared to SB-2 melanoma cells. High levels of LPA1, 2, 4, 5, and 6 were seen in WM2664. A375SM only express high levels of LPA1. SB-2 melanoma expressed more LPA3 than both WM2664 and A375SM melanoma cells.

A.



B.

A375SM	Experimental Lung Metastasis		
	Median	Range	Incidence
NT shRNA	68	42-97	8/8
Gal-3 shRNA / EV	9	3-28	8/8
Gal-3 shRNA / ATX	29	10-61	6/6

Figure 28. Reduced Lung Metastasis by Silencing Galectin-3 is Partially Rescued with the Re-Expression of Autotaxin

A375SM melanoma cells transduced with NT shRNA, galectin-3 shRNA / Empty vector, or galectin-3 shRNA / autotaxin were injected intravenously in nude mice. The number of metastatic lung colonies were then quantified. (A) The number of experimental lung metastasis is reduced after silencing galectin-3 as compared to NT shRNA. The re-expression of autotaxin partially rescues the metastatic phenotype of melanoma cells, *$P < 0.01$. (B) The median number of metastatic colonies in the NT shRNA group was 68. Silencing galectin-3 reduced the median to 9 metastatic colonies, while re-expression of autotaxin increased the median number of metastatic colonies to 29.

NFAT1 Expression is Required for the Malignant Melanoma Phenotype

Interestingly one group reported that B16F10 cells generate fewer lung metastasis in NFAT1 knockout mice, thus, NFAT1 could play a critical role in the tumor microenvironment [182]. However, how NFAT1 expression within melanoma cells contributes to the malignant phenotype has not yet been studied. In breast cancer NFAT1 expression has been shown to enhance invasion through Matrigel [179]. To test whether the same phenotype is observed in melanoma, our SB-2 melanoma cells transduced with either an empty expression vector or NFAT1 from sub-aim 2.1 were subjected to the Matrigel invasion assay. Interestingly, the overexpression of NFAT1 significantly increased the number of invaded SB-2 cells as compared to the empty vector control (Figure 29). This invasive phenotype is contributed by many factors. One of them is the induction of autotaxin expression (Figure 17) which has previously been shown as a pro-invasive molecule [197]. Other factors are most likely contributed to other downstream target genes that NFAT1 regulates. These potential genes are now being investigated in our lab.

The *in vitro* invasive phenotype suggests that NFAT1 can contribute to a more aggressive and metastatic tumor. However, the role of NFAT1 on melanoma growth and metastasis has not yet been established. Therefore, we decided to inject our highly metastatic A375SM melanoma cells stably transduced with NT or NFAT1 shRNA into nude mice (Figure 30). Tumor volume was measured for 32 days after subcutaneous injections of A375SM NT or NFAT1shRNA melanoma cells. Indeed, silencing NFAT1 significantly reduced tumor growth *in vivo*, *P <0.05 (Figure 30A). To analyze the metastatic phenotype after silencing NFAT1, A375SM melanoma cells were injected intravenously. A375SM melanoma cells transduced

with NFAT1 shRNA had a dramatic and significantly fewer lung metastasis than the NT shRNA control, *$P < 0.05$ (Figure 30B).

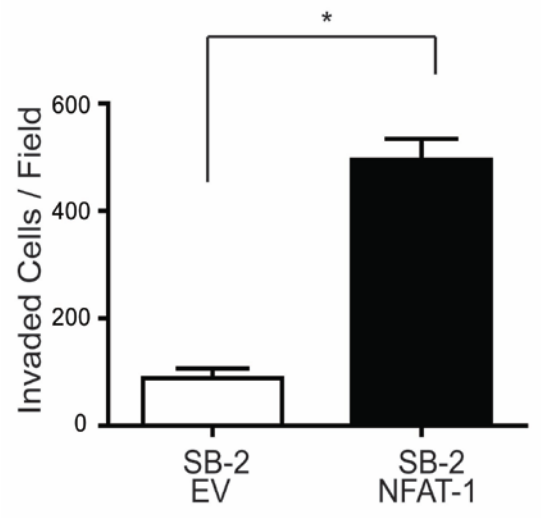


Figure 29. NFAT1 Increases the *in Vitro* Invasive Phenotype

SB-2 melanoma cells were stably transduced with an NFAT1 or empty vector (EV) expression lentivirus (see figure 17). They were then subjected to the Matrigel invasion assay. Expressing NFAT1 significantly increased the number of invaded cells through the Matrigel coated membrane, *$P < 0.001$)

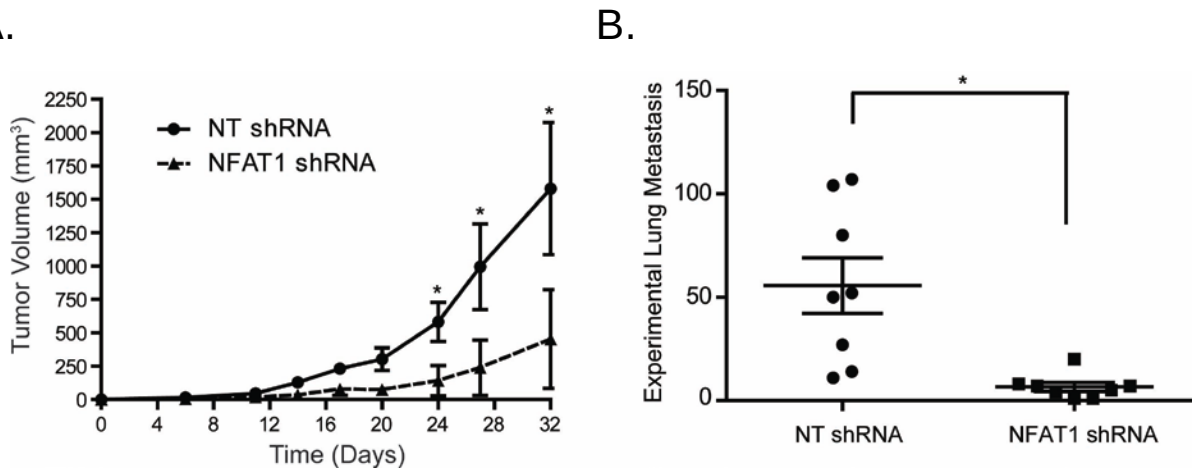


Figure 30. Silencing NFAT1 in A375SM Melanoma Cells Reduces Tumor Growth and Experimental Lung Metastasis in Nude Mice

A375SM melanoma cells were (A) injected subcutaneously in nude mice and tumor growth was monitored for 32 days. Tumor growth was significantly reduce after silencing NFAT1 at day 24, 28, and day 32 (*P < 0.001). (B) Six weeks after intravenous injection of A375SM cells, nude mice were sacrificed and the number of lung metastasis were counted. Silencing NFAT1 significantly reduced the number of experimental lung metastasis (P* < 0.001).

Discussion

Our lab has previously shown that silencing galectin-3 in C8161 melanoma cells could reduce tumor growth and metastasis. In our study we corroborate those results with the A375SM melanoma cell line. We also identify autotaxin as a downstream target of galectin-3. However, we have yet to confirm whether galectin-3 contributes to tumor growth and metastasis through its regulation of autotaxin. To that end, we stably expressed autotaxin in galectin-3 silenced A375SM melanoma cells and injected these cells subcutaneously. Tumor growth was compared with A375SM NT shRNA and galectin-3 shRNA / empty vector transduced melanoma cells. Partial rescue of tumor growth was observed. Interestingly, the microvascular density as shown by CD31 staining was completely rescued in galectin-3 silenced / autotaxin re-expressed melanoma xenograft tumors. Autotaxin is a powerful angiogenic factor. In zebrafish, normal vascular development during embryogenesis requires the expression of autotaxin and LPA receptors [198]. Purified autotaxin mixed with Matrigel plugs generated new blood vessels to the same extent as vascular endothelial growth factor (VEGF). Only a few blood vessels formed in Matrigel without VEGF or autotaxin [199]. Interestingly, VEGF can stimulate autotaxin expression in endothelial cells [200]. Expression of autotaxin can then increase the migratory phenotype of HUVEC cells. This is highly reliant on the conversion of LPC to LPA. The addition of LPC stimulates HUVEC motility; however, silencing autotaxin eliminates the number of migrated endothelial cells in the presence of LPC [200]. Furthermore, silencing autotaxin in HUVEC cells reduced the expression of the VEGF receptor VEGFR2 [200]. This implicates both VEGF and autotaxin in a positive feedback loop by which VEGF induces autotaxin expression, and conversion of LPC to LPA led to the activation of LPA receptors which would promote VEGFR2 expression [199, 200]. In a chick

embryo model however, LPA receptor antagonists did not reduce VEGF mediated angiogenesis while it significantly reduced LPA mediated angiogenesis [192]. Therefore, these two pathways are not completely reliant on each other to induce the angiogenic phenotype.

Our data suggest that autotaxin/VEGF cross talk is not as prominent. Only a slight reduction in VEGF expression is observed after silencing galectin-3, and the amount of VEGF is slightly rescued when autotaxin is overexpressed in our galectin-3 shRNA melanoma cells. VEGF might play a small role in inducing angiogenesis in A375SM xenograft tumors. Most likely, galectin-3 contributes to angiogenesis in A375SM xenograft tumors by inducing autotaxin expression and LPA production. Immunohistochemistry identified decreased expression of autotaxin in A375SM galectin-3 shRNA / empty vector tumors as compared to NT shRNA xenografts. In cell culture, silencing galectin-3 resulted in reduced autotaxin activity within the supernatant as observed by our autotaxin activity assay. Reduced levels of autotaxin are likely to contribute to our reduced autotaxin activity readout. This is expected. Less autotaxin will result in less cleaved LPC to make LPA. Although this is *in vitro*, we can expect this to occur *in vivo* as well. Furthermore, LPA production within the tumor microenvironment can directly bind to melanoma cells and induce cell survival, proliferation, and chemotherapeutic resistance.

Silencing galectin-3 significantly increased the number of TUNEL positive, apoptotic cells. This could be due the reduced number of blood vessels and hence less oxygen and nutrients supplied within the tumor. Melanoma cells express LPA receptors and A375SM cells express high levels of LPA1. Therefore, autotaxin can enhance cell growth and survival by directly affecting melanoma cells. Autotaxin has been shown to increase chemotherapeutic resistance and cell survival of ovarian cancer cells [201]. Autotaxin expression can increase

the proliferation and motility of melanoma cells *in vitro* [165]. Autotaxin can induce the migratory phenotype through LPA activation of PI3kγ [202]. LPA can also enhance migration through p-21 activated kinase 1 (PAK1) phosphorylation and consequent focal adhesion kinase (FAK) activation [203]. Moreover, LPA receptor activation can lead to protein kinase C (PKC), NF-κB, phosphatidylinositol 3 kinase (PI3K), RAC, and RhoA activation. All of which can contribute to cancer progression [200, 204-207]. The re-expression of autotaxin did not completely rescue cell survival. Therefore, galectin-3 contributes to cell survival by potentially regulating other unidentified genes. Also, it is well known that galectin-3 contains the NWGR antideath motif. Galectin-3 can thus inhibit cell death by directly inhibiting cytochrome C release and cancer cell apoptosis [208]. This mechanism and other factors could be the remaining contributing factors by which galectin-3 increases cell survival.

We have also identified the transcription factor NFAT1 to be regulated by galectin-3. NFAT1 has not been intensively studied in melanoma. Only two publications, excluding our report, have discussed the role of NFAT1 in melanoma. The first implicates NFAT1 as an important transcription factor in the stroma for the formation of a favorable metastatic niche. This was shown with NFAT1 knockout mice. When B16F10 murine melanoma cells are injected in $NFAT^{-/-}$ mice fewer lung metastasis are formed as compared to wild type [182]. A similar phenotype was observed with galectin-3 knockout mice [99]. The other manuscript reports that silencing NFAT1 in combination with MEK or BRAF inhibitors sensitizes melanoma cells to apoptosis [183]. Yet, the role NFAT1 plays on the malignant melanoma phenotype has previously not been described. Herein, we show that over expressing NFAT1 in low metastatic SB-2 melanoma cells increases their invasive phenotype. In breast cancer, NFAT1 has already been established as a pro invasive transcription factor by inducing the

expression of COX-2 [179]. We did not determine whether COX-2 expression was affected in SB-2 melanoma cells after over expressing NFAT1. However, autotaxin is significantly increased, and previous reports indicate that autotaxin enhances the invasive phenotype [209]. Our data is the first report to show the importance of NFAT1 on the invasive phenotype of melanoma cells. Other molecules besides autotaxin that are regulated by NFAT1 could contribute to the invasive phenotype as well.

The tumorigenic potential of NFAT1 on melanoma cells has remained elusive. Therefore, we sought to elucidate the potential NFAT1 has on tumor growth and metastasis of melanoma cells. Silencing NFAT1 significantly reduces tumor growth and experimental lung metastasis. This could be due to the regulation of autotaxin by NFAT1. Other genes regulated by NFAT1 could also contribute to tumor growth and metastasis. Future studies need to be conducted to elucidate whether they contribute to the malignant melanoma phenotype.

Summary

In our study, we further establish the contribution by which galectin-3 promotes the metastatic melanoma phenotype. We revealed the following novel findings which are summarized:

1. Silencing galectin-3 by lentiviral shRNA in two metastatic melanoma cell lines significantly reduces their migratory and invasive potential *in vitro*. Silencing galectin-3 significantly reduces the ability of melanoma cells to survive and proliferate in an anchorage independent manner in soft agar.
2. Galectin-3 positively regulates the expression of Autotaxin and NFAT1.
3. Silencing galectin-3 reduces the transcriptional expression of autotaxin via down regulating the protein expression of the transcription factor NFAT1.
4. Galectin-3 promotes Lysophospholipase D enzyme activity as observed by the FS-3 autotaxin assay by regulating autotaxin expression.
5. The rescue of galectin-3 in galectin-3 silenced cells restores the protein expression of both NFAT1 and autotaxin. This confirms that our results are not an off-target effect of galectin-3 silencing.
6. Silencing galectin-3 reduces the tumor growth and metastasis of A375SM melanoma cells. The re-expression partially rescues the proliferative and metastatic properties, indicating that the regulation of autotaxin by galectin-3 contributes to the malignant melanoma phenotype. Angiogenesis is almost completely rescued with the re-expression of autotaxin; therefore, galectin-3 mediated angiogenesis is reliant on its regulation of autotaxin.

7. NFAT1 induces the invasive phenotype *in vitro* and silencing NFAT1 in melanoma cells significantly reduces tumor growth and metastasis.

Taken together, our study identifies a previously unknown role by which galectin-3 promotes melanoma growth and metastasis. We have identified a novel pathway regulated by galectin-3. Galectin-3 positively regulates the protein expression of the transcription factor NFAT1, which in turn, induces the transcription of autotaxin. Autotaxin is then secreted within the supernatant where it performs its lysophospholipase D enzyme activity to convert LPC to LPA. LPA within the tumor microenvironment acts as a ligand for LPA receptors located on both melanoma cells and stroma to induce tumor growth, invasion, angiogenesis, and ultimately metastasis. Figure 31 depicts our model described in this study.

The carbohydrate binding function of galectin-3 and its role in metastasis has previously been established. These studies where the first to implicate galectin-3 with cancer and metastasis. Over time, the intracellular properties of galectin-3 were discovered. Furthermore, these properties have just as significant of a role in tumor progression as do the carbohydrate binding properties of galectin-3. For instance, the anti-apoptotic function of galectin-3 can act as a pro-survival factor under chemotherapeutic stress. The regulation of genes such as cyclin D, IL-8, and VE-cadherin identify an intracellular signaling role by which galectin-3 contributes to cell proliferation, tumor growth, and vasculogenic mimicry. It is easy to understand how galectin-3 earns the chimeric name in both its protein structure and its multiple biological functions. Our study adds another layer to the intracellular network and how galectin-3 mediates its signaling. However, we have yet to fully

understand how galectin-3 regulates NFAT1 protein expression. Whether galectin-3 acts a scaffold protein, dominant negative for casein kinase 1 phosphorylation, or other unknown mechanisms will need to be established.

Therapies targeting galectin-3 and autotaxin have long been studied. Throughout our study, we identify a novel molecule in melanoma, NFAT1, that promotes the malignant phenotype. Targeting NFAT1 could be another potential therapeutic tool. Therapy directed at NFAT1 is clinically feasible as shown with cyclosporine A to inhibit T-cell mediated organ transplant rejection [210]. However, in melanoma, immunotherapy and immunesurveillance promoted by T-cells are considered methods for melanoma treatment. Therefore, systemic therapy directed towards NFAT1 in melanoma could be counterintuitive by reducing T-cell activity. Yet, not all T-cells have anti-tumor function. Regulatory T-cells (Treg) can reduce the immune response towards tumors, and it's been shown that NFAT1 enhances Treg activity as well (Reviewed in [211, 212]). The multiple roles of NFAT1 within the tumor microenvironment create a "double edged sword" in regards to its therapeutic potential. Therefore, directing therapy towards galectin-3 instead of NFAT1 might be the most promising of all these treatments. Modified citrus pectin directly targets galectin-3, reduces metastasis of B16F1 melanoma cells in immunocompetent mice with no toxicity, and potentially could inhibit downstream targets [213, 214]. Whether MCP can target intracellular galectin-3 or reduce the downstream functions, such as NFAT1/Autotaxin has not been studied. The development of a small molecule inhibitor that targets the carbohydrate binding, anti-apoptotic, and cell signaling functions of galectin-3 could be a promising treatment modality for stage II or III melanoma.

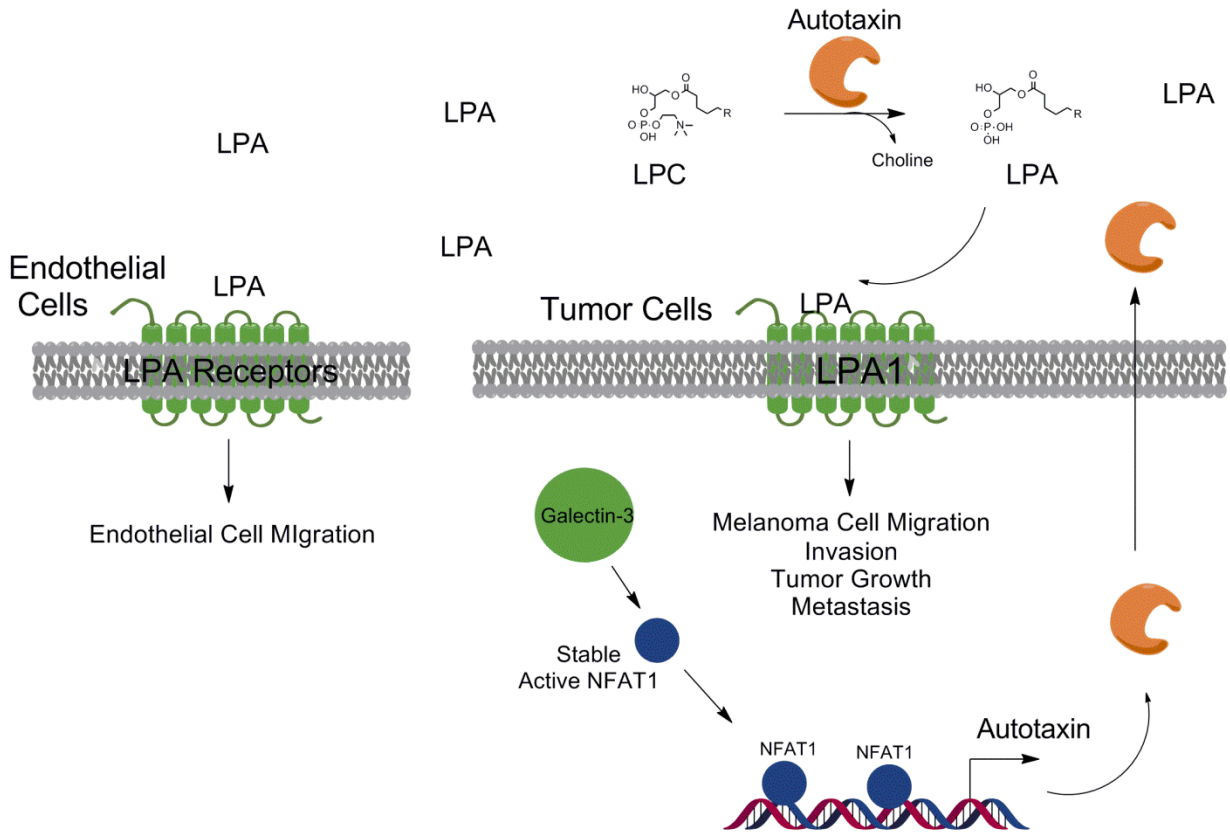


Figure 31. Proposed Mechanism by Which Galectin-3 Contributes to Melanoma progression

Galectin-3 stabilizes NFAT1 protein expression. NFAT1 binds to the promoter at both 209 and 290 bp upstream of the transcription start site of autotaxin and induces its expression. Autotaxin is then secreted into the tumor microenvironment where it converts lysophosphatidylcholine into lysophosphatidic acid (LPA). LPA binds to the LPA receptors located on melanoma cells to induce the metastatic melanoma phenotype. In our melanoma cells, LPA1 is the most likely receptor. LPA binds to endothelial cells and induces angiogenesis.

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Vita

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