Galectin-3 Contributes to Melanoma Growth and Metastasis via Regulation of NFAT1 and Autotaxin

Russell R. Braeuer1, Maya Zigler1, Takafumi Kamiya1, Andrey S. Dobroff1, Li Huang1, Woonyoung Choi2, David J. McConkey2, Einav Shoshan1, Aaron K. Mobley1, Renduo Song1, Avraham Raz3, and Menashe Bar-Eli1

Abstract
Melanoma is the deadliest form of skin cancer in which patients with metastatic disease have a 5-year survival rate of less than 10%. Recently, the overexpression of a β-galactoside binding protein, galectin-3 (LGALS3), has been correlated with metastatic melanoma in patients. We have previously shown that silencing galectin-3 in metastatic melanoma cells reduces tumor growth and metastasis. Gene expression profiling identified the protumorigenic gene autotaxin (ENPP2) to be downregulated after silencing galectin-3. Here we report that galectin-3 regulates autotaxin expression at the transcriptional level by modulating the expression of the transcription factor NFAT1 (NFATC2). Silencing galectin-3 reduced NFAT1 protein expression, which resulted in decreased autotaxin expression and activity. Reexpression of autotaxin in galectin-3 silenced melanoma cells rescues angiogenesis, tumor growth, and metastasis in vivo. Silencing NFAT1 expression in metastatic melanoma cells inhibited tumor growth and metastatic capabilities in vivo. Our data elucidate a previously unidentified mechanism by which galectin-3 regulates autotaxin and assign a novel role for NFAT1 during melanoma progression. Cancer Res; 72(22); 5757–66. ©2012 AACR

Introduction
Galectins are a family of carbohydrate-binding proteins that bind with a high affinity to β-galactoside sugars (1). Of these carbohydrate-binding proteins, galectin-3 has a unique chimeric molecular structure, which consists of 3 domains: an NH2-terminal domain, a proline, glycine, and tyrosine-rich domain, and a COOH-terminal carbohydrate-binding domain (1). The NH2-terminal domain is posttranslationally modified via phosphorylation at Ser6 by casein kinase 1 and this controls its cellular compartmentalization, transcriptional regulation of specific genes, antiapoptotic function, and its carbohydrate-binding properties (2, 3). Both MMP-2 and MMP-9 cleave extracellular galectin-3 in the intermediate domain, and a NWGR "antideath" motif within its carbohydrate-binding domain inhibits cytochrome c release and apoptosis in cancer cells (4, 5). Along with binding to N-glycosylated surface proteins, galectin-3 has been reported to form intracellular protein complexes with different molecules to induce K-Ras nanoclustering, WNT/β-catenin signaling, or mRNA spliceosome complexes (6–8).

In cancers, increased expression of galectin-3 has been correlated with the progression of glioma, melanoma, thyroid, pancreatic, and breast cancer (9–13). Its expression in breast cancer cells contributes to the upregulation of cell cycle molecules such as cyclin D1, and chemotherapeutic insults in breast cancer cells induces phosphorylation of galectin-3, which translocates it to the cytoplasm to inhibit cytochrome c release and apoptosis (13, 14). Cleavage of galectin-3 by matrix metalloproteinase 2 (MMP-2) within the tumor microenvironment has been reported to induce angiogenesis, and exogenous galectin-3 increases capillary tube formation of endothelial cells in a carbohydrate-binding dependent manner (15). As we reported previously, this phenomenon has also been identified in the C8161 melanoma cell line by a different mechanism, i.e., by regulating VE-Cadherin expression (16). Silencing galectin-3 in C8161 cells also reduced the proangiogenic chemokine interleukin-8 (IL-8) and MMP-2, indicating its role in invasion and metastasis (16). Indeed, C8161 melanoma cells transduced with galectin-3 short hairpin RNA (shRNA) significantly decreases experimental lung metastasis (16). Furthermore, mice fed with Modified Citrus Pectin, a carbohydrate-binding inhibitor of galectin-3, significantly reduced spontaneous metastasis of MDA-MB-435 breast cancer cells (17).

In the present study, we sought out to identify novel downstream molecules that are regulated by galectin-3, which contribute to melanoma growth and metastasis. To that end, we stably transduced WM2664 and A375SM metastatic...
melanoma cell lines with lentiviral-based shRNA targeting galectin-3. These cells were subjected to gene expression profiling. Of the genes deregulated by galectin-3 shRNA, we identified a downregulation of autotaxin. Autotaxin was originally identified as a motility factor in A2058 melanoma cells (18). The mechanism in which autotaxin induced motility remained elusive until it was identified that its phosphodiesterase catalytic site was required to enhance cell migration (19). It was later realized that autotaxin was identical to lysophospholipase D purified from FBS, which catalyzes lysophosphatidylcholine (LPC) to the bioactive lysophosphatidic acid (LPA; ref. 20). LPA acts as a ligand for 3 of the endothelial differentiation gene (EDG) family G-protein coupled receptors (GPCR), LPA1, LPA2, and LPA3, or the non-EDG GPCRs that belong in the purinergic receptor family such as LPA4, LPA5, LPA6 to induce downstream signaling, which promotes chemotaxis, migration, invasion, angiogenesis, and tumorigenesis of multiple types of cancers (21–23). A strong enhancer of autotaxin expression in cancer is the transcription factor nuclear factor of activated T cells (NFAT1). Here we report that silencing galectin-3 decreases the protein expression of NFAT1, which reduces the transcriptional activation of autotaxin in melanoma cells, thus decreasing melanoma growth and metastasis. Our data unravel a novel mechanism by which galectin-3 contributes to the acquisition of the melanoma metastatic phenotype by enhancing autotaxin expression. Our studies also assign a mechanistic role for NFAT1 in melanoma growth and metastasis.

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. The WM2664 melanoma cell line was purchased from the American Type Culture Collection, and is highly metastatic in nude mice. The nonmetastatic SB-2 melanoma cell line was isolated from a primary cutaneous lesion. The culture conditions for the melanoma cell lines and the 293T cells were previously described (25). A volume of 25 μL supernatant and 25 μL 2× 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.

Lentiviral expression vectors, shRNA, and siRNA

Galectin-3 shRNA was prepared as previously described (16). In brief, galectin-3 targeting shRNA 5′-GACCATCATTCCGGT- 

\[ \text{CTAA-3′} \]

and nontargeting shRNA 5′-TTCTCCGAACGTGTCGACGT-3′ were designed with a hairpin and inserted into a pLVTHm lentiviral vector. Galectin-3, autotaxin, and NFAT1 genes were cloned from A375SM cDNA. Either gene was cut with the designated restriction enzymes (Supplementary Table S1), inserted into a PcDH vector and packaged within the lentiviral system as described above.

Western blot analysis

To detect galectin-3 and NFAT1, 20 μg of whole cell protein lysate was loaded on SDS-PAGE and transferred to polyvinylidene difluoride membranes. To detect autotaxin protein expression, 1.5 million cells were plated in a 10-cm dish and were incubated with 8 mL of serum-free Minimum Essential Media for 48 hours. The supernatant from cell culture was concentrated to 100 μL, was protein precipitated as previously described, and resuspended in 6 M urea lysis buffer (24). 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. Replicates of Westerns were analyzed by densitometry and the mean and SD are shown as bar graphs underneath the corresponding blots.

Quantitative RT-PCR

Isolation of RNA was conducted with the RNAqueous kit (Ambion) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR (RT-PCR) was conducted with the autotaxin Taqman Gene Expression Assay and standardized to 18s (Applied Biosystems).

Autotaxin activity assay

To analyze autotaxin lysophospholipase D activity, the fluorescent compound FS-3 (L-2000; Echelon) was used as previously described (25). A volume of 25 μL supernatant and 25 μL 2× 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

Chromatin immunoprecipitation (ChIP) assays were conducted with the ChIP-IT Express Enzymatic kit (53009; Active Motive) according to the manufacturer’s protocol and as previously described (24). 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 carried out surrounding both NFAT1 binding sites.

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. Direct site mutagenesis of NFAT1 binding sites were carried out using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer’s instructions. Transfection with Lipofectin (Invitrogen)
were used. Mice were then sacriﬁced. For galectin-3 and NFAT1 shRNA studies. Ten mice per group and 8 mice per group for galectin-3 shRNA and 32 days for NFAT1 shRNA melanoma cells. RNA was converted into cRNA using the Illumina TotalPrep Ampliﬂex Kit (Ambion) and hybridized in triplicate to the HT-12 Version 3 Illumina chip. Gene expression analysis was carried out between the 2 samples.

Statistical analysis
Student t test was conducted for the analysis of in vitro assays ± SE. The Mann–Whitney U test was conducted for statistical analysis of the in vivo tumor growth and metastasis results.

Results
Silencing galectin-3 decreases autotaxin expression
We have previously reported that galectin-3 expression is increased in highly metastatic melanoma cell lines and tissue specimens (10). To further investigate how galectin-3 contributes to melanoma progression, we stably silenced galectin-3 by greater than 80% in 2 metastatic human melanoma cell lines, WM2664 and A375SM (Fig. 1A). We then corroborated that silencing galectin-3 reduces the migratory and invasive phenotype of melanoma cells in vitro (Supplementary Fig. S1A and S1B). Furthermore, silencing galectin-3 signiﬁcantly reduced their ability to grow in soft agar, indicating that galectin-3 plays a role in anchorage-independent growth (Supplementary Fig. S1C).

Next, we sought to identify whether galectin-3 can regulate the expression of genes that play a role in migration, invasion, and cell growth. To that end, WM2664 melanoma cells were subjected to Illumina gene expression microarray analysis by which we identiﬁed the protumorigenic gene autotaxin to be downregulated by 2.5-fold after silencing galectin-3. It has previously been reported that autotaxin enhances invasion and induces tumorigenesis in mice (20, 26). Therefore, galectin-3 can have a protumor effect by modulating the expression of autotaxin during melanoma progression. To conﬁrm our initial microarray studies, quantitative RT-PCR was conducted on both WM2664 and A375SM melanoma cells after silencing galectin-3. Autotaxin mRNA expression is decreased by 2- to 2.5-fold in both cell lines, thus corroborating the cDNA microarray analysis (Fig. 1B). Autotaxin is primarily a secreted protein where it conducts its enzymatic function by converting LPC to LPA. Therefore, we collected the supernatant from the galectin-3 silenced melanoma cell lines and we observed less protein secretion of autotaxin by more than 5-fold in both melanoma cell lines as compared with control cells (Fig. 1C). The fold decrease in the secreted autotaxin was much higher than the 2.5-fold decrease observed in the cDNA microarray.

Decreased autotaxin activity is observed after silencing galectin-3
As autotaxin is a lysophospholipase D enzyme, converting LPC to LPA, we next sought to evaluate the effect of silencing galectin-3 on autotaxin activity. To that end, a ﬂuorescent compound, FS-3, was used. FS-3 resembles LPC, the natural substrate of autotaxin, and is cleaved by autotaxin to release the ﬂuorescence from the quencher (27). Therefore, in the presence of higher concentrations of autotaxin, we expect to see a faster rate of FS-3 cleavage and higher ﬂuorescent activity. Indeed, supernatant from NT shRNA transduced WM2664 and A375SM cells have a higher rate of FS-3 cleavage as compared with galectin-3 shRNA transduced cells (Fig. 1D and E). Therefore, we conclude that silencing galectin-3 reduces LPA production within the tumor microenvironment by decreasing autotaxin expression.

Galectin-3 regulates autotaxin expression at the transcriptional level
To determine whether the regulation of autotaxin is transcriptionally or posttranscriptionally regulated, a nuclear run-on assay was conducted with the A375SM melanoma cells after silencing galectin-3. A signiﬁcant decrease in the mRNA expression of autotaxin was identiﬁed after silencing galectin-3 indicating that autotaxin is regulated at the transcriptional level (Supplementary Fig. S2A). To corroborate these results, the promoter of autotaxin was inserted into a PGL3 luciferase reporter vector and transiently transﬁected in WM2664 and A375SM melanoma cells. Luciferase reporter activity was reduced in melanoma cell lines transduced with galectin-3 shRNA (Supplementary Fig. S2B and S2C). These data suggest that galectin-3 shRNA downregulates autotaxin expression at the transcriptional level.

Galectin-3 silencing decreases the protein levels of NFAT1: a transcriptional regulator of autotaxin
The transcription factor NFAT1 has previously been reported to bind to the autotaxin promoter (28). Therefore,
we decided to focus on NFAT1 in our studies. To that end, we first analyzed the protein expression status of NFAT1 in several melanoma cell lines with different metastatic potential. We identified that NFAT1 protein expression is positively correlated with galectin-3 (Fig. 2A). Interestingly, only the highly metastatic melanoma cell lines that have high levels of NFAT1 and galectin-3 express autotaxin (Fig. 2B). NFAT1 expression in DX3 is evident, however, its expression is low, likely due to the cells lacking galectin-3, and this results in no autotaxin expression (Fig. 2A and B). Two NFAT1 binding sites are located within 300 bp from the transcription initiation site of autotaxin at approximately positions 290 and 209, respectively (Fig. 2C). It is likely that galectin-3 drives the expression of NFAT1, which binds to the autotaxin promoter. Therefore, we investigated the status of NFAT1 expression after silencing galectin-3. NFAT1 protein expression was significantly reduced by 3.8- and 8.5-fold in both WM2664 and A375SM cell lines, respectively, \( P < 0.05 \) (Fig. 2D). To determine whether NFAT1 directly affects autotaxin expression at the transcriptional level, ChIP targeting the NFAT1 transcription factor identified that NFAT1 binds to the autotaxin promoter, and silencing galectin-3 decreased the amount of NFAT1 binding in both cell lines (Fig. 2E).

To further establish autotaxin regulation by NFAT1, the autotaxin promoter was cloned in a PGL3 vector with mutations at either single or both NFAT1 binding sites. Luciferase activity after mutating either NFAT1 binding site was decreased by approximately 50% in both melanoma cell lines (Fig. 2F). This is comparable to the luciferase activity of the autotaxin promoter after silencing galectin-3. However, mutating both binding sites did not have an additive effect, concluding that both binding sites are equally required for NFAT1 transcriptional activity.

Silencing NFAT1 decreases autotaxin protein expression

To corroborate our initial studies that NFAT1 is responsible for autotaxin expression, we transiently silenced NFAT1 with siRNA in WM2664 and A375SM parental cell lines. We achieved approximately 90% silencing of NFAT1 in these cells. This resulted in decreased expression of autotaxin within the supernatant (Fig. 3A). Furthermore, enforced expression of NFAT1 in SB-2 melanoma cells (nonmetastatic, NFAT1 negative) induces the expression of autotaxin by approximately 6-fold (Fig. 3B) and significantly enhanced their invasive potential in Matrigel-coated filters (Fig. 3C). A second band is seen underneath autotaxin, and could be one of the shorter isoforms 2 or 3. However, the larger band appears to be isoform...
Interestingly, the smaller isoform is only seen in the SB-2 melanoma cells and not in the more metastatic cell lines. In addition, stable NFAT1 silencing with shRNA in A375SM cells also diminishes autotaxin expression at the mRNA and protein level (Supplementary Fig. S3 and Fig. 3D), which results in reduced FS-3 fluorescence (Fig. 3E).

Rescue of galectin-3 reverts NFAT1 and autotaxin expression

Introducing small hairpin RNA has been reported to have nonspecific off-target effects. Therefore, galectin-3 was rescued in both melanoma cell lines to determine whether this can reexpress NFAT1 and autotaxin expression. Indeed, after rescuing galectin-3 in both WM2664 and A375SM, both autotaxin and NFAT1 expression were reverted and rescued (Fig. 4). This confirms that galectin-3 is indeed responsible for regulating NFAT1 and autotaxin protein expression.

Reexpression of autotaxin partially rescues tumor growth and angiogenesis in vivo

To correlate whether galectin-3 regulates melanoma progression in part by modulating autotaxin expression and activity, we stably reexpressed autotaxin in galectin-3 silenced melanoma cells and injected them subcutaneously in nude mice (Supplementary Fig. S4). Reexpression of autotaxin partially rescues tumor growth as compared with galectin-3 shRNA melanoma cells transduced with an empty vector ($P < 0.05$; Fig. 5A). Furthermore, silencing galectin-3 increases TUNEL positive cells within the tumor, indicating there is more cell death. Reexpression of autotaxin decreases the number of TUNEL positive cells ($P < 0.05$), thus, indicating that autotaxin contributes to cell survival in vivo (Fig. 5B and C). Mice injected with A375SM melanoma cells expressing the NT shRNA vector grew at a faster rate than those with autotaxin reexpression (Fig. 5A). This suggests that galectin-3 regulates the expression of other genes and signaling pathways along with autotaxin to
enhance tumor growth and cell survival as shown in our previous publication (16).

Previous studies have shown that galectin-3 can act as a proangiogenic molecule (15, 29). This same phenomenon occurs with autotaxin and LPA during development and cancer (30, 31). This could partially be due to decreased autotaxin expression. Indeed, silencing galectin-3 decreases the number of CD31 positive staining within the tumor, indicating there are fewer blood vessels (Fig. 5C). Reexpression of autotaxin increases the amount of CD31 staining in tumors obtained from Fig. 5A on day 27 suggesting that galectin-3 can regulate autotaxin expression and enhance angiogenesis in vivo (Fig. 5C and Supplementary Fig. S5). The increased number of blood vessels could be a contributing factor in which we see reduced TUNEL positive cells, and it is likely that our Gal-3/EV has increased apoptosis because of the lack of oxygen and nutrients within the tumor microenvironment. Our immunohistochemistry staining confirmed that galectin-3 remained silenced on day 27 after tumor injections, and that autotaxin was rescued in these cells (Supplementary Fig. S3).

Galectin-3 contributes to the metastatic phenotype in part by regulating autotaxin expression

As autotaxin expression enhances migration and invasion in vitro (18, 32) and high levels of LPA or the overexpression of autotaxin and LPA receptors induce metastasis in vivo (26, 33), we sought to determine whether the downregulation of autotaxin after silencing galectin-3 contributes to a reduced metastatic phenotype. When compared with NT shRNA A375SM, significantly fewer Gal-3 shRNA/EV A375SM cells metastasized to the lung with a median of 68 versus 9 (P < 0.05). When we reexpress autotaxin, there are significantly more metastatic lesions as compared with the empty vector with a median of 29 and 9, respectively (P < 0.05; Fig. 6A and B). As in our subcutaneous model, we do not see a complete rescue of the metastatic potential after inducing autotaxin expression.

Figure 3. NFAT1 regulates autotaxin protein expression in melanoma cells. A, transient silencing of NFAT1 in WM2664 and A375SM melanoma cells reduces autotaxin expression by 3- and 2-fold, respectively. *, P < 0.05. B, overexpression of NFAT1 in SB-2 melanoma cells (nonmetastatic, NFAT1 negative) induces autotaxin expression. **, P < 0.05. C, overexpression of NFAT1 alone significantly increases the invasive phenotype of melanoma cells. ***, P < 0.001. D, NFAT1 silencing decreases NFAT1 protein levels in A375SM cells. This results in decreased levels of autotaxin protein expression within the supernatant. E, reduced levels of autotaxin results in decreased activity as observed in the autotaxin activity assay. A silver stain was conducted with equal volumes of supernatant used for the autotaxin activity assay as a loading control.
Galectin-3 most likely regulates other genes, and conducts other functions such as carbohydrate binding on cell surface glycoproteins during circulatory transport and extravasation into the lung parenchyma, or it can inhibit apoptosis to enhance cell survival. Taken together, our data show that the transcriptional regulation of autotaxin by galectin-3 contributes to melanoma growth and metastasis. However, autotaxin upregulation by galectin-3 is not the only mechanism in which galectin-3 contributes to metastasis, and galectin-3 enhances melanoma progression through additional mechanisms, such as the regulation of IL-8 and VE-cadherin as we previously showed (16).

It is also likely that NFAT1 regulates multiple genes that affect melanoma growth and metastasis. We therefore decided in the last set of experiments to intravenously and subcutaneously inject A375SM melanoma cells transduced with NT or NFAT1 shRNA. Silencing of NFAT1 also results in a significant inhibition of tumor growth and metastasis (Fig. 6C and D). Taken together, our data supports the notion that NFAT1 plays a major role in melanoma growth and metastasis.

Discussion

We have previously shown that increased galectin-3 expression is correlated with the progression of melanoma in human patients, and its expression increases in highly metastatic cell lines (10, 16). Earlier reports from our laboratory identified that silencing galectin-3 decreases VE-Cadherin and IL-8 expression along with reducing tumor growth and experimental lung metastasis of C8161-c9 melanoma cells (16). In this study, we sought to identify whether galectin-3 expression can regulate previously unknown genes that are vital for melanoma growth and metastasis. By using cDNA microarray profiling after silencing galectin-3 we identified autotaxin as a novel downstream target.

Our data represent a novel mechanism in which autotaxin is regulated by galectin-3. After silencing galectin-3 we observed reduced protein expression of the transcriptional regulator NFAT1. Indeed, the autotaxin promoter contains 2 NFAT1 binding sites within 300 bp upstream from the transcription initiation site. Here, we validate NFAT1 regulation of autotaxin by using dual luciferase promoter analysis, NFAT1 siRNA and NFAT1 overexpression methods. Furthermore, we add another layer to this mechanism by confirming that galectin-3 expression in metastatic melanoma cell lines regulates NFAT1 protein expression.

NFAT1 exists within the cytoplasm in a highly phosphorylated, inactive state. Dephosphorylation is regulated by the phosphatase activity of the calcium-regulated protein calcineurin, which induces nuclear localization and enhances transcriptional activity (34). NFAT1 kinases such as casein kinase 1 and GSK3β revert this process by phosphorylating and inactivating NFAT1 (34). However, conflicting data has been
reported on GSK3B. Yoeli-Lerner and colleagues reported that silencing GSK3B decreased the NFAT1 transcriptional activity. They also show that GSK3B shRNA decreased the protein expression of NFAT1 and its downstream target Cox-2 (35). In our system, we also observe decreased protein expression of NFAT1 after silencing galectin-3. Others have shown that galectin-3 can complex with and be phosphorylated by CK1 and GSK3B (7, 36). This implicates galectin-3, GSK3B, and NFAT1 within the WNT signaling pathway. In metastatic melanoma cells, galectin-3 expression could be required within this pathway to maintain NFAT1 protein expression.

Autotaxin is primarily secreted into the microenvironment where it conducts its LysolPLD function. We report that silencing galectin-3 decreases autotaxin expression within the supernatant of melanoma cell lines, thus, supporting the notion that decreased autotaxin levels in the supernatant reduce the amount of cleaved FS-3. However, our FS-3 assay is semi-quantitative because only FS-3 is used without the addition of LPC. FS-3 is then used as a substrate quickly and the rate of fluorescence is not linear. Furthermore, LPA can inhibit autotaxin in a negative feedback loop and NT shRNA cells produce more LPA within the media. This makes it difficult to directly correlate activity with expression in bio-logic samples. Nonetheless, this assay shows that the autotaxin activity levels correlate with the supernatant protein expression. LPA contributes to diverse physiologic processes by activating multiple LPA receptors and induces various downstream signaling pathways via coupling to an array of G-protein alpha subunits (37, 38). These molecular changes include protein kinase C (PKC) and NF-xB activation, RAP-MAPK signaling, activation of phosphatidylinositol 3-kinases (PI3K), and activation of GTPases such as RAC and RhoA (39–41). LPA production leads to physiologic processes such as cancer cell invasion, endothelial cell migration, and the upregulation of the chemokine IL-8 (16, 42–44).

Our laboratory has previously shown that silencing galectin-3 in melanoma cells significantly reduced tumor growth and metastasis of C8161 melanoma cells (16). This again is now shown with our A375SM melanoma cell line. We also observed a significant reduction in the number of blood vessels within the tumor after silencing galectin-3. Others have also reported that galectin-3 acts as a proangiogenic molecule in breast cancer models (29). It is likely that one mechanism by which galectin-3 induces angiogenesis is through the regulation of autotaxin. In zebrafish, autotaxin and its receptors are required for proper vascular development (45). Others have reported that autotaxin can enhance human umbilical vein endothelial cells (HUVEC) migration in the presence of LPC along with inducing angiogenesis (31, 43). Silencing autotaxin expression in HUVECs significantly decreases LPA receptors and VEGFR2 expression (43). It has also been shown that VEGF can increase autotaxin expression and HIF-1α activation by LPA (43, 46). This implies that autotaxin and VEGF signaling can positively regulate each other during the angiogenic process. When we rescue autotaxin in our galectin-3 shRNA melanoma cells, we see a significant increase in blood vessels within the tumor. In our model, it is likely that autotaxin expressed by the tumor cells enhances LPA production in the tumor microenvironment, which then acts upon endothelial cells to further increase the expression of LPA and VEGF receptors. This effect enhances endothelial cell migration and increases the number and size of tumor blood vessels. Recently, it has also been shown that autotaxin can regulate melanoma lymphangiogenesis by activating LPA receptors on lymphatic cells (47).
Galectin-3 Regulates NFAT1 via Autotaxin

melanoma cells also express LPA receptors in the EDG (Supplementary Fig. S6A) and purinergic (Supplementary Fig. S6B) family. As reviewed by Jankowski, others have reported that autotaxin can induce melanoma cell motility by activating PI3Kα and induce the expression of urokinase-type plasminogen activator. Furthermore, LPA can enhance migration through PAK1 phosphorylation and downstream focal adhesion kinase (FAK) activation (48). These mechanisms result in increased melanoma motility. The production of LPA by autotaxin within the tumor microenvironment can potentially have a direct effect on tumor cells by activating signaling pathways that enhance tumor growth, inflammation, and cell survival. Reexpression of autotaxin partially rescues experimental lung metastasis after silencing galectin-3. Therefore, galectin-3 can regulate metastasis by multiple mechanisms directly, or indirectly through the regulation of autotaxin and the multiple genes and pathways that are deregulated by LPA.

To further establish a link between NFAT1 and melanoma growth and metastasis, we silenced NFAT1 in A375SM melanoma cells. We observed a decrease in tumor growth and the number of experimental lung metastasis after silencing NFAT1. As NFAT1 can potentially regulate multiple genes, further studies are needed to identify what genes NFAT1 regulates that affect tumor growth and metastasis. The genes identified after silencing galectin-3 then might be deregulated through the protein loss of NFAT1. Interestingly, others have reported that galectin-3 knockout mice show reduced B16-F10 metastasis to the lung. This is reportedly because of decreased ability of the tumor cells to bind to and extravasate into the lung parenchyma (49). In addition, it has also been shown that NFAT1−/− mice have a reduced number of B16-F10 metastatic lesions (50). Along with our data, this implicates that both galectin-3 and NFAT1 play major roles in modulating the tumor microenvironment to support melanoma growth and metastasis.

Taken together, our data provide novel mechanism by which galectin-3 contributes to melanoma growth and metastasis via the regulation of the NFAT1/ATX/LPA pathway. This is likely to be one of many ways in which galectin-3 can promote melanoma progression. Our data only scratches the surface, as it is still unclear how galectin-3 affects NFAT1 protein expression. Yet, this is the first report to assign a mechanistic role for NFAT1 in melanoma progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: R.R. Braeuer, T. Kamiya, D.J. McConkey, E. Shoshan, A. Raz, M. Bar-Eli


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.R. Braeuer, T. Kamiya, D.J. McConkey, E. Shoshan, R. Song, M. Bar-Eli

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.R. Braeuer, M. Zigler, W. Choi, D.J. McConkey, A. Raz, M. Bar-Eli

Writing, review, and/or revision of the manuscript: R.R. Braeuer, M. Bar-Eli

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Kamiya, L. Huang, A. Raz, M. Bar-Eli

Study supervision: M. Bar-Eli


Acknowledgments

The authors thank Dr. Didier Trono (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) for providing the lentiviral backbone in which galectin-3 shRNA was inserted.

Grant Support
This work was supported by the NIH Grant R01 CA67908, NIH Specialized Programs of Research Excellence in Skin Cancer Grant P50-CA093459 (M. Bar-Eli), and NIH R01CA46120 (A. Raz).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 21, 2012; revised September 7, 2012; accepted September 11, 2012; published OnlineFirst September 17, 2012.

References


Galectin-3 Contributes to Melanoma Growth and Metastasis via Regulation of NFAT1 and Autotaxin

Russell R. Braeuer, Maya Zigler, Takafumi Kamiya, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-2424

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/09/18/0008-5472.CAN-12-2424.DC1

Cited articles
This article cites 50 articles, 27 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/22/5757.full.html#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.