Provisional chapter

Emerging GM3 Regulated Biomarkers in Malignant Melanoma

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Additional information is available at the end of the chapter

1. Introduction

Gangliosides, GSLs, are expressed in the outer leaflet of the plasma membrane of animal cells and involved in a variety of functions, including serving as antigens, receptors for bacterial toxins, mediators of cell adhesion, and mediators and modulators of signal transduction. Moreover, the accumulated lines of evidence have shown that gangliosides play pivotal roles in cancer metastasis. As the first and simplest member in the metabolic series of the ganglioside family, GM3 is a metabolic precursor of more complex natural gangliosides, which in turn determines their contents and biological functions in cells and tissues. GM3 has been demonstrated to be involved in regulation of various processes including cell proliferation, differentiation, apoptosis, embryogenesis and oncogenesis, etc. However, it is difficult to understand the defined functional concepts of GM3 in cancer metastasis because GM3 indirectly exert their effects via regulating target genes.

Target genes indicated in cellular transformation and tumor progression have been divided into two categories: proto-oncogenes and tumor suppressor genes. In cancer, it can thus be speculated that an altered balance of tumor suppressor genes towards proto-oncogenes may contribute to tumor transformation. Alterations in proto-oncogenes and tumor suppressor genes are largely dependent on point mutation, amplification or translocation. Consequently, the normal control mechanisms that constrain the expression of genes are undermined, and thus the oncogene is continually expressed, resulting in tumor transformation [1]. Similarly, genetic transformation has been linked to melanoma genesis and progression. These genes encompass many signaling pathways, including the RTK, PI3-K, Rb, p53, Wnt, and NF-κB pathways [2]. Further evidence implicated the downstream oncogenes of these pathways in melanoma ranges from Ras, B-Raf, Kit,

Mitf, Cyclin D1, CDK4 to HDM2 [1], [3]. Although genetic discoveries related to melanoma transformation have been accelerated greatly in recent years, the involvement of GM3 in melanoma metastasis via these proto-oncogenes or tumor suppressor genes has not yet been clearly established.

The present chapter was aimed to give insights into the mechanisms that GM3 regulates melanoma metastasis via its target genes. To keep the discussion focused, we will discuss the relationship between GM3 contents and their abilities to regulate proto-oncogenes or tumor suppressor genes, which in turn mediate melanoma metastasis. To screen GM3 target genes, we obtained cells (CSSH-1) that overexpressed B4galt6 cDNA and cells (CAH-3) that suppressed its expression, which in turn result in GM3 modulation [4], [5]. In the CSSH-1 cells, GM3 contents were doubled, but in the CAH-3 cells, GM3 expression was halved compared with vector transfectant control, SM-1 and CM-1, respectively [4], [5]. To further confirm the roles of GM3 in melanoma cells, St3gal5 silenced cells were established by transfecting B16 cells with St3gal5 siRNA and it was found that the introduction of St3gal5 siRNA to B16 cells resulted in GM3 depletion as compared with the scrambled siRNA transfectant control [4], [5]. Moreover, we would elucidate the mechanism that GM3 regulate melanoma metastasis via the genes, such as Ly-GDI, TNF-α, MMP-9, MMP-2, Caveolin-1 and Plaur, etc. and the functions of these genes on the phenotypes of melanoma cells, such as invasive proliferation, adhesion, migration and invasion would be finally addressed based on our and others' publications (Table 1) [6].

2. Materials and methods

2.1. Cell lines and culture

Murine melanoma B16 cells were kindly provided by Dr. Kiyoshi Furukawa of Nagaoka University of Technology, Japan. The cells were maintained in medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen Corporation, NY, USA) supplemented with 10% fetal bovine serum (TBD; Tianjin Hao Yang Biological Company, Tianjin, China), 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified (37°C, 5% CO2 and 95% air) incubator (Sanyo, Toyota, Japan). The cells were usually grown in a 60-mm culture dish (BD Falcon, CA, USA) and passaged once they reached 75% confluence. To observe the effects of pharmacological inhibitors on genes expression, cells were treated with pharmacological inhibitors for 24 h before analyzing genes expression by RT-PCR.

2.2. Chemicals and antibodies

LY294002 and LY303511 were purchased from Sigma-Aldrich (St. Louis, MD, USA). The Rneasy mini kit to extract total RNA was obtained from Qiagen (Hilden, Germany). The RT-PCR kit was from Takara Biotechnology Corporation (Dalian, China). All other reagents were from Invitrogen (Carlsbad, CA, USA), unless otherwise specified.

Regulation Manner	Gene Name	CSSH-1/SM-1	CAH-3/CM-1	B11/B16	Biological Functions
Positive	Caveolin-1	1.378	0.321	0.146	(1)[7], (4)[7], (5)[7]
	Ly-GDI	2.156	0.423	0.387	(5)[8]
	PKN-1	1.658	0.626	0.495	(4)[9]
	E-cadherein	1.875	0.695	0.721	(1)[10], (3)[11], (5)[12], (6)[13]
	Gelsolin	1.841	0.543	0.502	(4)[14]
	MMP-9	1.915	0.174	0.282	(4)[15], (5)[16]
	MMP-2	1.532	0.534	0.472	(4)[17], (5)[18]
	Apaf1	1.350	0.608	0.509	(2)[19]
	Rho B	2.247	0.427	0.318	(5)[20], (6)[20], (8)[20]
	Midkine	1.403	0.518	0.417	(1)[21]
	Lymphotoxin α	2.245	0.475	0.497	(6)[22]
	Tnf α	2.188	0.349	0.292	(4)[23], (5)[23]
	Plau	1.453	0.397	0.750	(5)[24], (6)[24]
	Plaur	2.209	0.543	0.720	(2)[25]
Negative	Integrin β5	0.783	1.465	1.754	(1)[26, 27], (2)[28], (3)[29], (4)[30]
	Vimentin	0.111	1.984	2.089	(7)[31]
	TGF-β1	0.571	2.124	3.309	(1)[32], (4)[33], (5)[33]
	TGFBR 2	0.716	1.453	1.903	(1)[34]
	N-Cam	0.282	2.901	2.223	(3)[35]
	Src	0.639	1.347	1.925	(1)[36], (3)[36], (4)[37], (5)[36]

(1) Invasive Proliferation; (2) Apoptosis; (3) Adhesion; (4) Motility; (5) Invasion; (6) Metastasis; (7) Tumor marker; (8) Transformation

Table 1. GM3 involved in regulating cancer related genes in melanoma.

2.3. RNA extraction and RT-PCR

RNA extraction and analysis of amplified DNA were described in our previous work [4], [5], [9], [38-40]. The primers used in this study were designed with primer 3 software and syntehsized by Shanghai Genebase Biotechnology Corporation (Shanghai, China). Primer sequences used for the PCR in this study were as follows: Plau, Sense 5′-GCCCACAGA CCTGATGCTAT and Antisense 5′-TAGAGCCTTCTGGCCACACT; Plaur, Sense 5′-AGGTGGTGACAA-GAGGCTGT and Antisense 5′-AGCTCTGGTCCAAAGAGGTG; gelsolin, Sense 5′-TCCAA-CAAGGTGCCAGTGGA and Antisense 5′- CAGCACAAAGGCATCGTTGG; Caveolin-1, Sense 5′-CTACAAGCCCAACAACAAGGC and Antisense 5′- AGGAAGCTCTTGATG-CACGGT; Ly-GDI and Eef sequences are provided in our previous publications [5], [40]. The mRNA levels of the genes under consideration, using Eef mRNA as an internal control, were determined by RT-PCR semiquantitatively, as described previously [4, 5, 9, 38-40]. Candidate mRNA values are expressed as a ratio of candicate mRNA to Eef mRNA and are usually expressed as unity for control experiments.

2.4. siRNA and cDNA constructs

Target sequences were designed and synthesized as previously described [4, 5, 9, 38-40]. Effective siRNA sequence targeting Ly-GDI can be found in our previous publications [5, 40]. For ectopic expression of Caveolin-1 or Ly-GDI, total RNA was isolated from mouse FBJ-S1 cells. After the first-strand cDNA was synthesized, a Caveolin-1 or Ly-GDI transcript was amplified using the following sense and antisense primers: Caveolin-1, Sense 5′-GCTAG-CATGTCTGGGGGCAAATACGT and Antisense 5′-GGATCCTCA-TATCTCTTTCTGCGTGC; Ly-GDI, 5′- GCTAGCATGACGGAGAAGGATGCACAGCCA and Antisense 5′- GGATCCTCATTCTGTCCAATCCTTCT. The coding sequence of Caveolin-1 or Ly-GDI was inserted between NheI and BamHI digestion sites for sense expression in a pITES-puro3 expression vector with puromycin resistance (Clonetech, USA). The plasmids were prepared and confirmed by sequencing analysis.

2.5. Transfection

In RNA interference experiments, B16 cells were transfected as previously described [4, 5, 9, 38-40]. In brief, B16 cells were transfected with Ly-GDI siRNA for 3 days and the stably transfected cells were further selected by G418 treatment. In control experiments, cells were transfected with scramble siRNA and also selected with G418. For ectopic expression experiments, B16 cells were transfected with Caveolin-1 or Ly-GDI cDNA contructs before analysing the mRNA expression of corresponding genes after 3 days. Control cells were transfected with empty vector.

3. Involvement of GM3 targeted genes in mediating melanoma metastasis

Accumulating evidence using thin-layer chromatography techniques has shown that the gangliosides GM3 and GD3 are predominantly expressed in melanoma cells and are present in relatively higher concentrations than the more complicated gangliosides GT1, GD1 and GM1 in adult brain tissue [41]. In line with this report, GM3 has been detected in both human and murine melanoma cells by MAb M2590 [42], [43]. Although GM3 has been identified several decades ago, the progress on the mechanism of GM3 in mediating melanoma metastasis is slow. Prior works have shown that GM3 facilitates melanoma B16 cells to metastasize in C57BL/6 mice [44], [45], but the mechanism remains unknown. In view of the complicated steps of melanoma metastasis and multiple biological functions of GM3, it is necessary to address the targets molecules through which GM3 exerts its functions on melanoma metastasis.

3.1. Ly-GDI

Ly-GDI, a Rho GTPase dissociation inhibitor beta, is also known as RhoGDI2, Arhgdib or D4-GDI. It belongs to a family of RhoGDIs including RhoGDI1 and RhoGDI3. The family is named for its ability to inhibit the dissociation of bound GDP from its partner Rho GTPase, which reg-

ulates interactions with regulatory guanine nucleotide exchange factors, GAP, and the effector targets [46]. Among GDIs, Ly-GDI differs substantially at the structural level from the other two GDIs and is regarded as an invasive and metastatic suppressor gene in human bladder cancer cells [47], [48]. In line with these findings, Ota *et al.* [49] also reported that truncated Ly-GDI promotes metastasis of mouse colon cancer. Along with these prior works, our recent data demonstrate that Ly-GDI expression was positively regulated by GM3, which in turn suppresses anchorage-independent growth in mouse melanoma B16 cells [5]. As anchorage-independent growth has been previously regarded as one of the most important oncogenic properties of tumor cells, it is important to describe the signal transduction pathway between GM3 and Ly-GDI, leading to suppress melanoma invasive proliferation in soft agar medium.

As a first step, we evaluated the effect of endogenous GM3 on Ly-GDI mRNA expression. Our data reveal that Ly-GDI mRNA expression is always proportional to endogenous GM3 contents (Table. 1), which suggests possible roles of GM3 in Ly-GDI regulation. To further confirm the notion that GM3 is responsible for Ly-GDI regulation, GM3 was exogenously added in the culture medium of GM3-depleted cells, such as B11 clone and CAH-3 cells, as well as B16 parental cells. Our data reveal that exogenous GM3 significantly bound to biological membranes, which resulted in upregulation of Ly-GDI expression in three cell lines [5]. Reciprocally, GM3 depletion was carried out by incubating cells with D-PDMP, which in turn suppresses Ly-GDI expression. Collectively, our data demonstrated that GM3 contents play pivotal role in regulating Ly-GDI expression [5].

To keep the discussion focused, we next aimed to characterize the signaling pathway of GM3 in regulating Ly-GDI expression. In light of the possible role of PI3-K pathway in GM3 signaling cascade [4], [40], we examined the effects of PI3-K inhibitor, LY294002, on Ly-GDI mRNA expression in the absence or presence of GM3. Treatment of B16 and CSSH-1 cells with LY294002 not only suppressed Ly-GDI mRNA expression, but also reversed the effects of GM3 on increasing Ly-GDI mRNA expression. Similarly, this pharmacological intervention was also effective in suppressing Akt phosphorylation at Ser 473 and Thr 308 without altering total Akt levels. To further exclude the possible non-specific effects of PI3-K inhibitors, we transfected B16 cells with Akt1 or Akt2 siRNA. Our results revealed that Akt1 or Akt2 knock down decreases Ly-GDI expression at mRNA level and the effects of suppression are more profound in Akt1/2 doubly silenced cells. It is noteworthy that GM3 are not able to upregulate Ly-GDI expression in Akt1/2 double knock down cells. Collectively, our results demonstrate that GM3 regulate Ly-GDI expression via PI3-K/Akt pathway [5].

Although we had found that PI3-K/Akt pathway plays pivotal role in regulating Ly-GDI expression, the question is easily raised whether GM3 directly activates PI3-K pathway or not. In view of the important role of Pdpk1 and mTOR complexes in PI3-K signaling cascade [50], we determined their expression in B11 cells. Our data revealed that GM3 knock down concurrently reduces Pdpk1 and Raptor expression, whereas induces Rictor expression. Taking the advantage of siRNA technique, we further found that Pdpk1 and Raptor, but not Rictor knock down abolished GM3 effects on Ly-GDI induction via blocking Akt phosphorylation at Thr 308 [5]. From these observations, we demonstrate that GM3 induces Ly-GDI expression via PI3-K, Pdpk1, Akt^{Thr308} and mTOR/Raptor pathway in melanoma B16 cells (Fig.

1). More importantly, the experiments were also carried out to determine if Ly-GDI is the key molecule in mediating melanoma B16 cells anchorage-independent growth. Our data demonstrate that Ly-GDI knock down significantly increased the proliferating ability of B16 cells in soft agar or serum free medium [5]. It is noteworthy that expression of GM3 is gradually increased during the progression of melanoma malignancy. For instance, GM3 was not detected in normal or naevi skin, but 60% of primary and 75% of metastatic melanoma expressed GM3 [51]. Our data along with previously published results [51] suggest that relatively lower levels of GM3 and Ly-GDI at the early stage of melanoma facilitate melanoma cells to undergo invasive proliferation in vigorous environment. These observations also provide insights into the molecular basis of GM3 on augmenting melanoma invasive proliferation at the early stage of pathology.

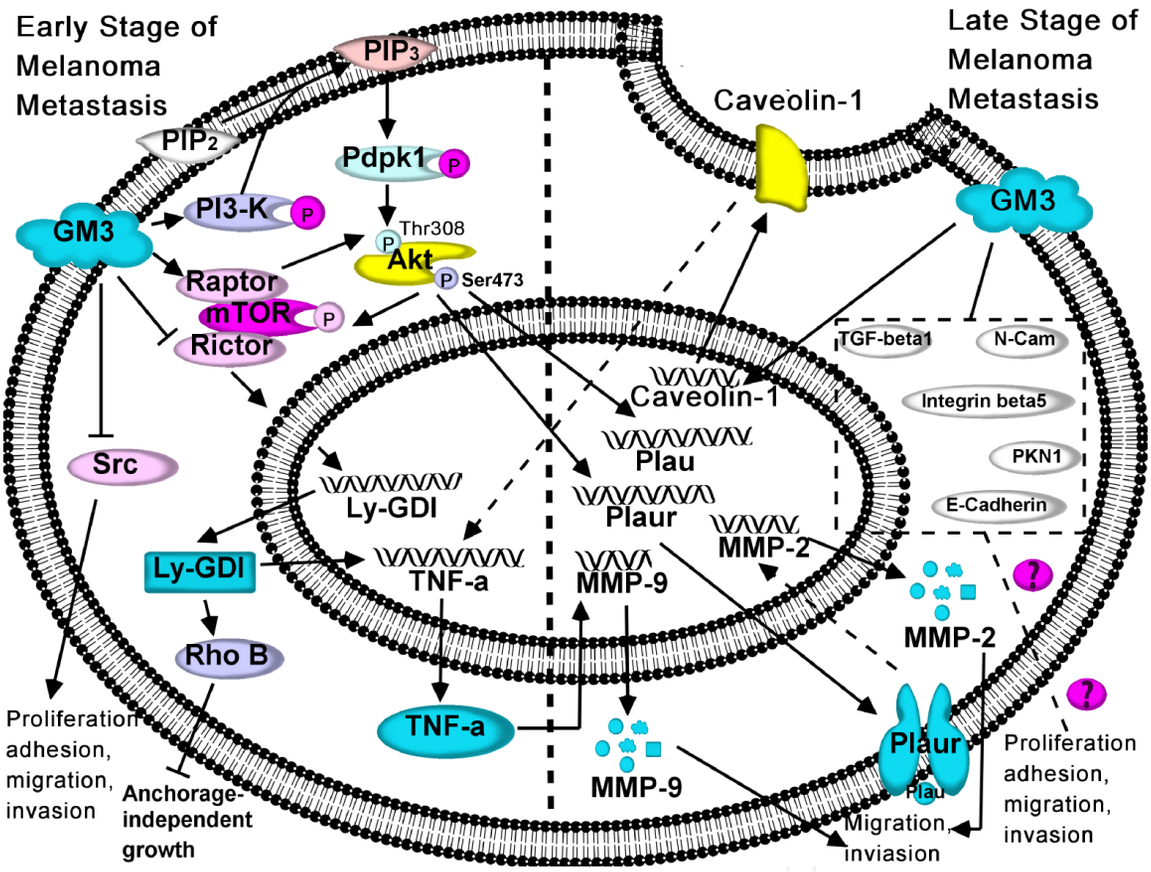

Figure 1. Proposed cascade of GM3 signaling events in murine melanoma B16 cells metastasis. In melanoma B16 cells, GM3 is gradually increased during the progression of melanoma malignancy and facilitates melanoma cells to metastasize. We here found that GM3 signals are transduced via PI3-K, Pdpk1, Akt and the mTOR/Raptor pathway, leading to the expression of Ly-GDI and RhoB, which in turn suppresses mouse melanoma cell proliferation in soft agar or serum deprived medium. In addition, GM3 is able to suppress melanoma cell proliferation, adhesion, migration and invasion by inhibiting Src mRNA expression. It is noted that the above mechanisms will result in cell invasive proliferation, migration and invasion because of relatively lower level of GM3 at early stage of melanoma progression. In contrast, higher level of GM3 at late stage of melanoma metastasis will trigger cell migration and invasion via MMP-2, MMP-9 and Caveolin-1 etc, although the signaling transduction pathway is the same as the above discussion. These *in vitro* observations not only decipher the codes of GM3 in regulating melanoma metastasis *in vivo*, but also help find new therapeutic strategies to treat melanoma disorders.

3.2. TNF-α

TNF-α is a multifunctional cytokine, which is synthesized as a 26kDa (233 amino acids) membrane-bound propeptide (pro-TNF-α) and is secreted upon cleavage by TNF-α converting enzyme [52]. Studies on the effects of TNF-α using experimental models of invasion and metastasis have shown that it can often act as a tumor promoting factor [53]. More specifically for melanoma, TNF-α has been reported to upregulate the expression of integrin subunits, which in turn enhance the interaction of human melanoma cells with ECM substrates [54], [55]. The more recent study from this laboratory showed that TNF-α induces integrin expression, cell attachment and invasion via fibronectin in human melanoma cells [55]. In light of these prior works, our recent data demonstrate that TNF-α located downstream of Ly-GDI to mediate melanoma metastasis (Fig. 4) [4], [39], [40]. To verify that TNF-α follows the same GM3 signaling cascade as Ly-GDI, we carried out the same experiments as above Ly-GDI. In line with GM3 regulating Ly-GDI signaling pathway [5], our results revealed that GM3 regulates TNF-α mRNA and protein expression via PI3-K, Akt and mTOR pathway [4], [40], suggesting that TNF-α is indeed the downstream target of Ly-GDI through which mediate biological functions of GM3 and Ly-GDI in melanoma metastasis. Once TNF-α was found to be a link in the chain of GM3 signaling, we focused on its biological effects on melanoma metastasis. The results demonstrate that TNF-α is able to enhance melanoma migration via inducing MMP-9 expression and activity [39], which will be further discussed in "MMP-9 and MMP-2" part (Fig. 1). On the other hand, Venessa *et al.* [56] proposed that TNF-α protect melanoma cells from apoptosis by BRAF inhibition. In contrast, Andrea *et al.* [57] reported that TNF-α exhibited antiproliferative effects on four melanoma cell lines. In line with this report [57], accumulating evidence demonstrates that TNF-α suppresses melanoma metastasis in patients or murine models [58]. Although TNF-α shows its effects on suppressing melanoma metastasis, we still could not negate its ability to induce melanoma migration and invasion. Since the debating is still going on and the multiple biological functions of TNF-α, we are not able to arbitrarily judge that TNF-α induces or reduces melanoma metastasis until now. It would be finally decided by the mechanism that TNF-α predominantly exerts.

3.3. MMP-9 and MMP-2

As above discussion, TNF-α is able to enhance melanoma migration via inducing MMP-9 expression and activity [39], we next aimed to elucidate its mechanism. In order to determine the role of MMP-9 in cell migration, we examined the effects of an MMP-9 agonist and antagonist on cell migration, as stimulation of MMP-9 expression by TNF-α has been reported in several studies [59], [60]. We therefore used TNF-α as a positive control against the MMP-9 inhibitor GM6001. RT-PCR results demonstrate that TNF-α markedly induces MMP-9 expression and activity, which is reversed by GM6001 treatment [39]. Furthermore, cell migration tested by transwell experiments showed that the numbers of cells migrating were consistent with MMP-9 expression [39]. This finding is in accordance with previously published data showing that TNF-α increases human melanoma cell invasion and migration *in vitro*, whereas they could not further extend their investigation to MMP-9 [23]. Bianchini *et al.* [59] proceed to find that invasive phenotype of murine melanoma cells stimulated with

TNF-α was characterized by an enhanced MMP-9 expression. More importantly, we extended their investigations to GM3 and further reveal that GM3 also regulate MMP-9 expression and activity via PI3-K, Akt, Ly-GDI and TNF-α pathway [39]. Therefore, our findings [39] along with the prior works [23], [59] demonstrate that TNF-α induces melanoma migration and invasion via augmenting MMP-9 expression.

In addition to MMP-9, it is noteworthy that MMP-2 is also induced by endogenous GM3 (Table. 1). Although we are still not figure out the mechanism that GM3 positively regulates MMP-2 expression in melanoma B16 cells, MMP-2 has been implicated to be associated with progression of the melanoma [61]. In more detail, although all skin and nodal metastasis were negative for MMP-2, higher MMP-2 concentrations were observed in patients with metastatic disease (stage IV) than in those with primary melanoma (stage I) or in controls [61]. In addition, Liu *et al.* [62] recently published research showing that d-GM3, a derivative of ganglioside GM3, stimulates melanoma cell migration and invasion by increasing the expression and activation of MMP-2. To keep the discussion focused, it should be also noted that expression of GM3 is gradually increased during the progression of melanoma malignancy [51]. Once GM3 was markedly induced at the late stage of melanoma, MMP-9 and MMP-2 would be activated by GM3, which in turn mediating migration and invasion in melanoma progression. Therefore, multiple biological functions and stage dependent expression manner of GM3 make it possible to induce invasive proliferation of melanoma at early stage and stimulate melanoma cell migration and invasion at late stage of melanoma progression (Fig.1).

3.4. Plau and Plaur

During the course of MMP-9 and MMP-2 investigations, experimental evidence had led to the recognition of Plau and Plaur [59], [62]. Indeed, Plau and Plaur are highly expressed in advanced stages of primary and metastatic melanoma progression [63]. In addition, Bianchini *et al.* [59] proposed that costimulation with TNF-α and IFNγ enhancing murine melanoma B16 cells metastasis via inducing Plaur mRNA and protein expression. Moreover, Liu *et al.* [62] extended the above findings to exogenous d-GM3 treatment, the results suggested that d-GM3 promotes melanoma cell migration and invasion through Plaur signaling dependent pathway, which in turn mediating melanoma cell migration and invasion via MMP-2. Along with these prior works [59], [62], our data revealed that endogenous GM3 is able to positively regulate Plau and Plaur mRNA expression in mouse melanoma B16 cells (Table. 1). Although the mechanism that GM3 regulates Plau and Plaur mRNA expression is still going on in our lab, we already found that PI3-K/Akt pathway plays key roles in regulating Plau and Plaur mRNA expression in B16 cells (Fig. 2). To elucidate the biological functions of Plau and Plaur, Bianchini *et al.* [59] already found that Plaur monoclonal antibody treatment caused a significant reduction of invasiveness in TNF-α/IFNγ stimulated melanoma cells. In addition, targeting Plaur has practical implications in treating cancer. For example, 1) downregulating Plaur expression leads to dormancy of cancer cells [64]; 2) administrating Plaur antagonists markedly inhibits metastatic ability, including that of melanomas [65]; 3)deficiency of plasminogen/Plau in mice slows tumor growth and progression [65]; and 4) inhibiting Plau and/or its binding with Plaur prevents metastasis in animal models [66]. Collectively, the additional role of Plau/

Plaur signaling in mediating GM3 function [67] makes Plau/Plaur system as an attractive target for cancer therapy.

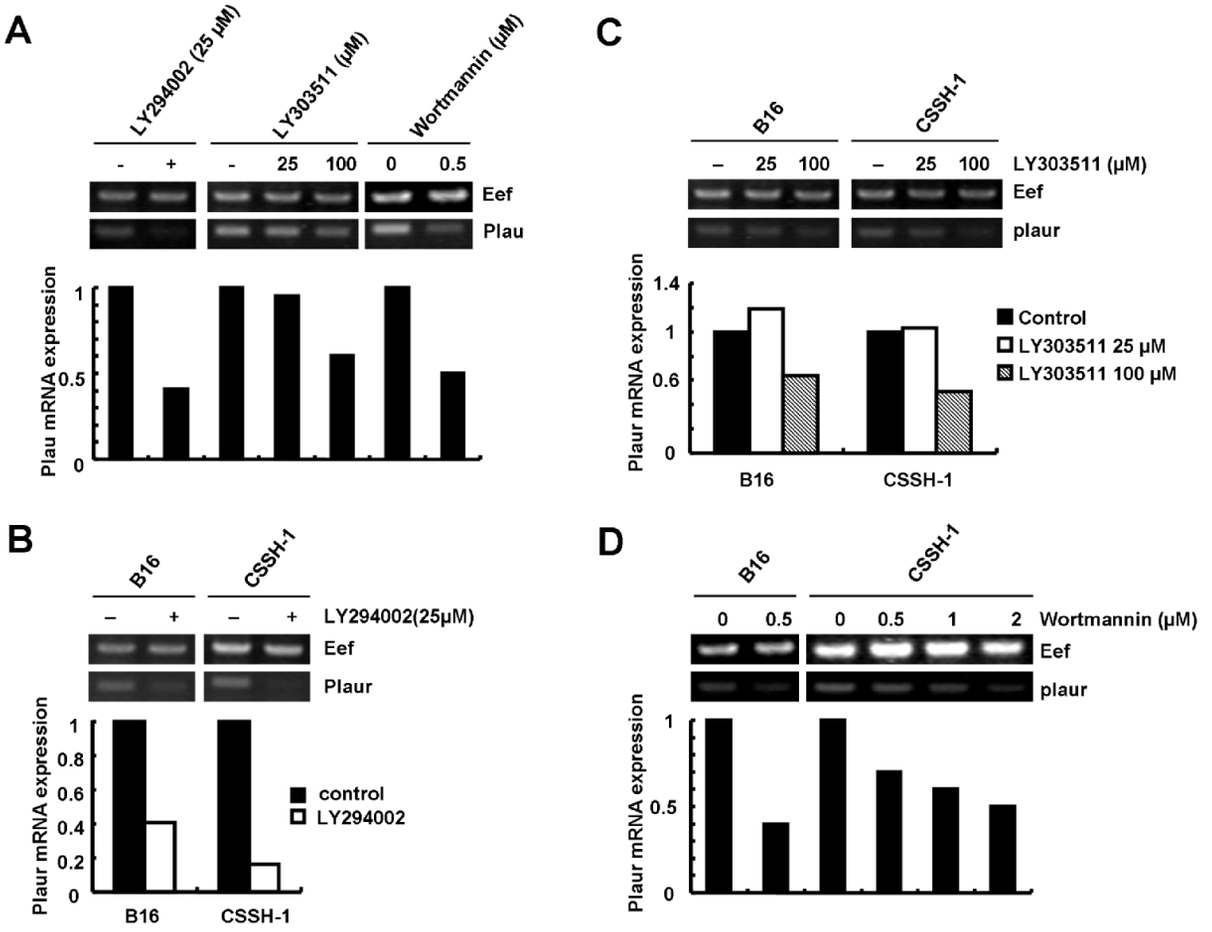

Figure 2. Involvement of PI3-K signaling pathway in Plau and Plaur synthesis in mouse melanoma B16 cells. B16 (A-D) or CSSH-1 (B-D) cells were incubated with either LY294002, LY303511 or Wortmannin for the indicated concentrations. Plau (A) and Plaur (B-D) mRNA expressions were determined by RT-PCR after 24h incubation. Eef served as internal control. The intensity of bands was analyzed relative to Eef for each treatment using the Bio-Profile Bio ID gel image system at 312nm.

3.5. Gelsolin

Gelsolin is a representative actin-regulatory protein with an 82kDa mass and is present in most vertebrate tissues. Gelsolin controls the length of actin polymers *in vitro* by a variety of mechanisms [68], [69]. Since actin filament reorganization is important for cell shape and motility, gelsolin has crucial roles in the control of these cellular functions [70], [71]. Accumulating evidence has shown that gelsolin expression is frequently downregulated in several types of human cancers, such as gastric, bladder, colon and lung [72]- [75]. In addition, ectopic expression of wild-type gelsolin resulted in suppression of tumorigenicity of both bladder and colon carcinoma cell lines [73], [76]. However, a new 85kDa truncated gelsolin (GSNp85), co-expressed with wild-type gelsolin, was frequently expressed in vertical growth phase mela-

nomas (Clark level II-IV) and metastatic growth phase melanomas [77]. Moreover, Litwin *et al.* [78] recently found that downregulation of gelsolin expression in melanoma A375 cells significantly reduce their migratory potential. These disparate observations might be attributed to different species of cancer cells. Consistent with the prior works [77], [78], we newly found that endogenous GM3 has ability to regulate gelsolin mRNA expression (Table. 1). In addition, PI3-K/Akt signaling pathway was found to exert critical role in mediating gelsolin mRNA induction (Fig. 3A). Moreover, gelsolin is found to be downstream targets of Ly-GDI in melanoma B16 cells by the experiments of Ly-GDI knock down (Fig. 3B). This observation not only demonstrates the critical role of Ly-GDI, but also provides new insights in melanoma metastasis (Fig. 1). More importantly, it is also indicating that alterations in the expression level of gelsolin and its subcellular distribution may be directly responsible for determining migration capacity of melanoma.

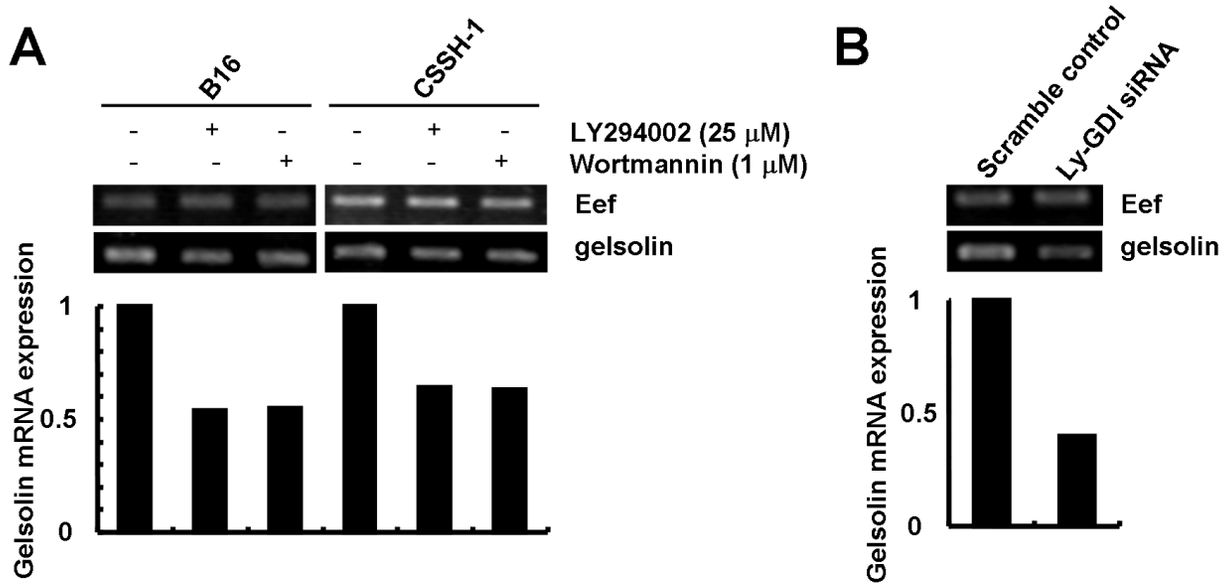

Figure 3. Involvement of PI3-K signaling pathway in gelsolin synthesis in mouse melanoma B16 cells. B16 (A, left panel) or CSSH-1 (A, right panel) cells were incubated with either LY294002 (25 μM) or Wortmannin (1 μM) for 24 h. In selected experiments, B16 cells were transfected with Ly-GDI siRNA and further established Ly-GDI knocking down cell lines by G418 selection (B). Gelsolin mRNA expression was determined by RT-PCR after 24h incubation. Eef served as internal control. The intensity of bands was analyzed relative to Eef for each treatment using the Bio-Profile Bio ID gel image system at 312nm.

3.6. Caveolin-1

Caveolin-1 is a 22-24 KDa protein originally identified as a structural component of caveolae, specialized invagination of the plasma membrane. These caveolae represent compartments in which key signaling transduction molecules are concentrated to provide an efficient system for cellular cross talk. However, relatively little information is available concerning the role of Caveolin-1 in melanomas. Early studies demonstrate that increased Caveolin-1 expression was associated with enhanced malignancy in a non-cutaneous, retinal melanoma [79]. An even

more recent study identified exosomes in the plasma of melanoma patients with high levels of Caveolin-1.In this particular case, exosomes are associated with malignant tumor progression as a small vesicle secreted by both normal and tumoral cells [80]. These prior works demonstrate that Caveolin-1 are relevant to function in melanoma metastasis.

Additional studies support the notion that presence of Caveolin-1 helps melanoma metastasis. Felicetti et al. proposed that Caveolin-1 expression is associated with increased metastatic potential in different human melanoma cell lines. Specifically, Caveolin-1 expression increased cell proliferation, anchorage-independent growth, migration and invasion in WM983A melanoma cell line. Alternatively, Caveolin-1 down-regulation in metastatic Caveolin-1 overexpressing melanomas reduces their proliferation, as well as their tumorigenicity [7]. Consistent with prior works [7], we further found that Caveolin-1 was regulated by endogenous GM3 (Table. 1). More importantly, our recent data revealed that Caveolin-1 is able to regulate TNF-α (Fig. 4), which in turn mediates melanoma migration or invasion through MMP-9 as discussed above [39]. Our data along with previous reports [7] further implicated the important role of GM3-enriched membrane subdomain, especially Caveolae, in melanoma metastasis.

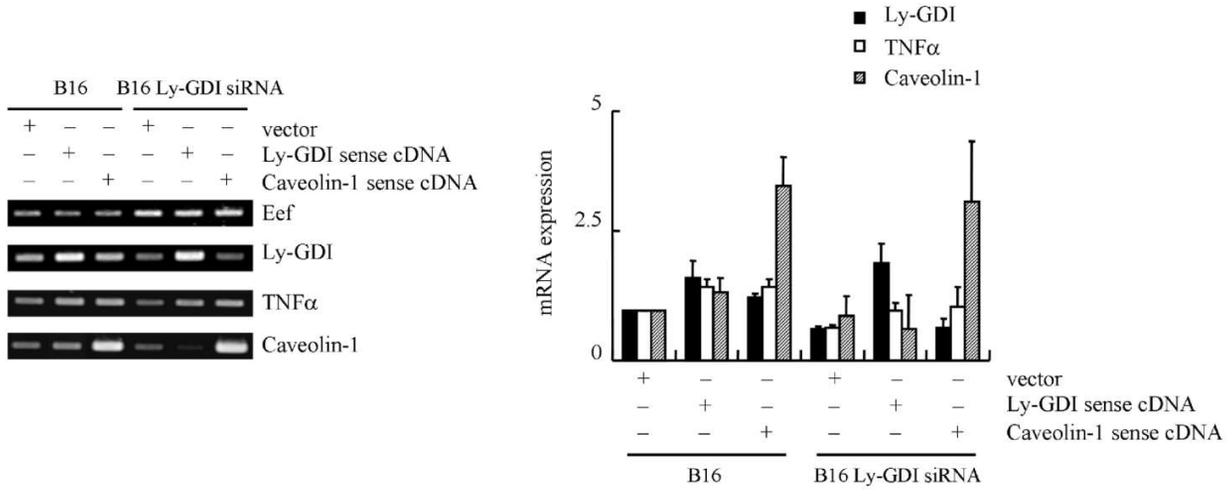

Figure 4. Ly-GDI overexpression induces TNF-α mRNA expression in mouse melanoma B16 cells. B16 or Ly-GDI knocking down cells were transfected with Ly-GDI or Caveolin-1 cDNA construct. Ly-GDI, TNF-α and Caveolin-1 mRNA expressions were determined by RT-PCR after 3 days transfection. Eef served as internal control. The intensity of bands was analyzed relative to Eef for each treatment using the Bio-Profile Bio ID gel image system at 312nm.

3.7. Src

Over the past few decades, studies of Src and the SFKs have given new insights into the role of these proteins in regulating cell adhesion, invasiveness and motility in cancer cells and in tumor vasculature, rather than directly influencing cell proliferation [81]. Src expression and activity are increased in melanoma cell lines and in melanoma tumors *in vivo* [82]. Src activity was detected in the following cell lines: LOX, IMVI, MALME-3M, SK-MEL-2, SK-MEL-5, SK-MEL-28, M19-MEL, UACC-62 and UACC-257. The highest activity was that in the M14 melanoma cell lines.

Src can activate STAT3, STAT5 and other downstream targets in melanoma [83]. The expression of STAT3 is highly expressed in both primary and metastatic melanoma in humans, although the expression level is variable [81]. In addition, STAT3 is activated in human melanoma, but not in melanocytic or in benign melanocytic neoplasms [84]. Moreover, blocking STAT3 signaling in mouse B16 melanoma cells resulted in the release of soluble factors capable of inducing apoptosis and cell-cycle arrest [85]. *In vivo*, inerfering with STAT3 signaling suppressed growth of syngeneic murine melanoma B16 tumor [86]. In addition to STAT3, it was recently found that STAT5 signaling in melanoma cell lines is mediated by the EGFR tyrosine kinase and by intracellular non-receptor tyrosine kinase, such as Src and JAK1. The expression of STAT5 is increased in melanoma metastasis compared with normal human melanocytes and STAT5 is activated in 62% of human melanoma metastasis. Alternatively, inhibiting STAT5 expression significantly reduced the expression of Bcl-2 and decreased cell viability and increased apoptosis in the melanoma cell lines. In view of the critical role of Src in melanoma invasive proliferation, we further found that Src was negatively regulated by endogenous GM3 (Table.1). This result along with previous reports [51], [83]- [86] indicated that GM3 regulates melanoma invasive proliferation via Src at the early stage of melanoma or at the stage of transformation, but not in advanced stage of melanoma (Fig. 1). Although we haven't found detailed mechanism that GM3 regulates Src mRNA expression, which in turn resulting in melanoma metastasis, it is still noteworthy to emphasize that Src mediated melanoma invasive proliferation might be the pivotal mechanism, which mediates melanoma transformation or onset of disease at the early stage when GM3 expression is still relative low. Therefore, it is also important to elucidate the biological function of Src when the expression is still low at the early stage of melanoma progression. These future discoveries will make us understand which mechanism predominantly contributed to the metastasis progression of melanoma at different stages or at different steps of cancer metastasis.

3.8. Rho B

Recent studies confirmed the role of the Rho proteins in cancer by showing their involvement in cell transforamtion, invasion, metastasis and angiogenesis. The major members of the Rho subfamily comprise the Rho A, Rho B and Rho C proteins. Rho B is quite different from Rho A and Rho C in many aspects, although it shares 87% homology [20]. For example, Rho B has a tumor suppressive role, including inhibiting cell proliferation and inducing apoptosis in several human cancer cells, and inhibiting tumor growth in murine model, in contrast, activation of Rho A promotes cell malignant transformation, proliferation, invasion and metastasis, like other small GTPases such as Ras, Rac1 and Cdc42 [87] Moreover, Rho B, unlike the constitutively expressed Rho A, is inducible by genotoxic stress, such as U.V. light, growth factors (TGF-β1) and chemotherapeutic drugs (cisplatin and 5-FU). In our experimental system, we found that mRNA expression of Rho B is suppressed by Ly-GDI knocking down [5]. This observation partially implied that Rho B located downstream of Ly-GDI to mediate its inhibitory effects on melanoma invasive proliferation and would also exerts its effects at early stage of melanoma progression (Fig. 1).

3.9. Other genes

During the course of our investigation in melanoma metastasis, we also found the involvement of some pro-oncogenes or tumor suppressor genes, such as TGF-β1, N-Cam, integrin β5, PKN1 or E-cadherin *et al.*, (Table. 1) in GM3 biological functions to mediate melanoma cells invasive proliferation, adhesion, migration and invasion, which finally contribute to melanoma transformation and progression. Although these steps of melanoma metastasis are not separate, our *in vitro* experiments have partially revealed the mechanism of melanoma metastasis at different stages. More importantly, deciphering the mechanism of GM3 in mediating melanoma metastasis will help find new therapeutic strategies to treat melanoma disorders.

4. Conclusion

Metastasis, the spread of malignant tumor cells from a primary site to distant sites, is the most life-threatening complication of cancer and a major problem of cancer treatment [88], [89]. The metastatic process consists of multiple steps: 1) invasive proliferation as benign tumor at the primary site 2) dissociation of tumor cell(s) from the primary site with a concomitant loss of cell-cell and cell-ECM adhesions; 3) tumor-cell adhesion to and subsequent local digestion of basement membrane; 4) retraction of endothelial cells and subsequent intravasation; 5) survival within the vasculature; 6) extravasation from vasculature at a distinct site and 7) growth in a "foreign" or ectopic organ environment [90], [91]. In view of these prior theories, our data summarized here reinforce the notion that GM3 potentially plays a dual role in melanoma development, as has been described in our previously published works[4-6,][39], [92]. At early stage of melanoma metastasis, lower level of GM3 induces melanoma invasive proliferation via Src, Rho B and Ly-GDI *et al.* Alternatively, higher levels of GM3 at the late stage of melanoma metastasis triggers cell migration and invasion via MMP-2, MMP-9 and Caveolin-1 etc. These *in vitro* observations not only decipher the codes of GM3 in regulating melanoma metastasis *in vivo*, but also help find new therapeutic strategies to treat melanoma disorders.

Nomenclature

GSLs, sialylated glycosphingolipids; RTK, receptor tyrosine kinase; PI3-K, phosphatidylinositol-3-kinase; Rb, retinoblastoma; PKN1, protein kinase 1; MMP-9, matrix matelloproteinase-9; MMP-2, matrix metalloproteinase-2; Apaf1, apoptotic protease activating factor 1; TNF-α, tumor necrosis factor-α; Plau, urokinase-type plasminogen activator; Plaur, urokinase-type plasminogen activator receptor; TGF-β1, transforming growth factor-β1; TGFBR2, transforming growth factor, beta receptor 2; RhoGDI, RhoGDP dissociation inhibitors; GAP, GTPase-activating proteins; Pdpk1, 3-phosphoinositide dependent protein kinase-1; mTOR, mammalian target of rapamycin; Raptor, regulatory associated protein of mTOR; ECM,

extracellular matrix; d-GM3, De-N-acetyl GM3; IFNγ, interferon γ; SFKs, Src-family kinases; STAT3, signal transducer and activator of transcription 3; STAT5, signal transducer and activator of transcription 5; JAK1, Janus kinase 1.

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