TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Galectin-3 Inhibits Cancer Metastasis by Negatively Regulating Integrin β 3 Expression

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Accepted for publication
December 17, 2018.

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Galectin-3 (Gal-3; gene *LGALS3*) is a member of the β -galactose-binding lectin family. Previous studies showed that Gal-3 is expressed in several tissues across species and functions as a regulator of cell proliferation, apoptosis, adhesion, and migration, thus affecting many aspects of events, such as angiogenesis and tumorigenesis. Although several reports have suggested that the level of Gal-3 expression correlates positively with tumor progression, herein we show that highly metastatic mouse melanoma B16/BL6 cells express less Gal-3 than B16 cells with a lower metastatic potential. It was found that overexpression of Gal-3 in melanoma cells in fact suppresses metastasis. In contrast, knocking out Gal-3 expression in cancer cells promoted cell aggregation mediated through interactions with platelets and fibrinogen *in vitro* and increased the number of metastatic foci *in vivo*. Thus, reduced Gal-3 expression results in the up-regulation of β 3 integrin expression, and this contributes to metastatic potential. These findings indicate that changes of Gal-3 expression in cancer cells during tumor progression influence the characteristics of metastatic cells. (*Am J Pathol* 2019, 189: 900–910; <https://doi.org/10.1016/j.ajpath.2018.12.005>)

It is widely accepted that cancer cells acquire malignant characteristics in the tumor microenvironment by genetic and epigenetic modification during tumorigenesis.^{1,2} Cell proliferation is one hallmark of tumorigenesis; spontaneous cell-autonomous growth of the tumor is caused by constitutive activation of several growth factor receptors, such as epidermal growth factor receptor, by mutation and alteration of cell signaling.^{3,4} As well as cell proliferation, tumor cells acquire malignant behaviors by developing invasive potential and high mobility to infiltrate neighboring organs and tissues or to metastasize remotely.

One well-known mechanism resulting in the promotion of cell invasion is the epithelial-mesenchymal transition. The master regulator controlling this system is transforming growth factor- β (TGF- β).⁵ This cytokine causes cell morphology to change to a mesenchymal phenotype via induction of transcription factors, such as snail and twist, which result in cell mobilization. It has been reported that such highly mobile cancer cells that acquire the ability to migrate are followed by macrophages that gravitate toward vascular regions and transmigrate into the intraluminal cavity of blood

vessels through the vascular wall.⁶ After invasion into the peripheral blood, cancer cells must protect themselves against apoptosis caused by sheer stress in the blood stream and against attack by immune cells during the process of locating and colonizing an adequate metastatic niche. It has been reported that cancer cells aggregate together with platelets for protection against harmful factors in the peripheral blood.⁷ It has not been elucidated whether the TGF- β -mediated signal pathway is influenced by the binding of platelets to cancer cells.

Herein, we sought factors regulating cancer cell metastasis by focusing on differences in gene expression between parental B16 melanoma cells and cells from the more highly metastatic B16/BL6 subline. Of many candidate genes,

Supported in part by Japan Agency for Medical Research and Development grants 18cm0106508h0002 and 18gm5010002s1102, the Japan Society for the Promotion of Science grants-in-aid for scientific research (A) 16H02470 (N.T.).

Y.H. and W.J. contributed equally to this work.

Disclosures: None declared.

galactose-binding lectin-3 (Galectin-3; Gal-3) was studied. The expression of Gal-3 is reduced in B16/BL6 cells; however, its function in metastatic processes remains unknown.

Gal-3 is a member of a lectin family with conserved carbohydrate-recognition domains that bind β -galactosidase.⁸ Fourteen different well-conserved Gals (Gal-1 to Gal-14) have been identified and classified into three subtypes, as follows: i) the prototype group, ii) the tandem repeat group, and iii) the chimera group.⁹ The prototype group contains one carbohydrate-recognition domain, whereas the tandem repeat group consists of two carbohydrate-recognition domains that are separated by a linker sequence. Gal-3 is the only chimera-type galectin in vertebrates, and it contains one carbohydrate-recognition domain connected to a long N-terminal proline- and glycine-rich domain.¹⁰ Gal-3 has been reported to act mainly as an intracellular molecule influencing cell apoptosis, the cell cycle, and RNA editing^{11,12}; however, it can also act as a secreted molecule regulating cell adhesion by mediating interactions with adhesion molecules.

Herein, we investigated how Gal-3 affects cancer cell metastasis by experimentally overexpressing or silencing its expression in cancer cells. To define whether cancer cell autonomous Gal-3, extrinsic Gal-3, or both are involved in cancer cell metastasis and how Gal-3 deficiency affects the survival of tumor-bearing mice, cancer cell metastasis was studied using Gal-3-deficient mice. It was also analyzed whether Gal-3 expression is associated with platelet adhesion to enhance metastasis and how Gal-3 expression is regulated on stimulation with TGF- β .

Materials and Methods

Cell Lines and Reagents

B16 (mouse melanoma),¹³ MMAc (human melanoma; RIKEN, Tsukuba, Japan), and A2058 (human melanoma; JCRB, Osaka, Japan) cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (100 U/mL; Life Technologies, Tokyo, Japan). The B16/BL6 cell line (mouse melanoma)¹⁴ was maintained in RPMI 1640 medium (Sigma) with 10% FBS and 1% penicillin/streptomycin. The G-361 cell line (human melanoma; RIKEN) was maintained in McCoy5A (Sigma) with 10% FBS and 1% penicillin/streptomycin. Finally, the MeWo cell line (human melanoma; JCRB) was maintained in minimal essential medium (Sigma) with 10% FBS, 1% nonessential amino acids (Gibco, Grand Island, NY), and 1% penicillin/streptomycin.

Recombinant Gal-3 and TGF- β 1 (R&D Systems, Minneapolis, MN), TGF- β receptor 1 inhibitor (LY364947; Merck Millipore, Darmstadt, Germany), and fibroblast growth factor 2 and epidermal growth factor (EGF) (Kurabo, Osaka, Japan) were used to stimulate the cells. For this, cells were starved in FBS-free medium for 4 to 8 hours before adding reagents in 24-

well plates. Mouse Gal-3 siRNA and negative control siRNA were purchased from Sigma.

Plasmid Construction and Transfection

Full-length Gal-3 cDNA was isolated from intestinal total RNA using PCR-based cloning methods, as described previously.¹⁵ Primers used for cloning were as follows: sense, 5'-GGAATTCGGATGGCAGACAGCTTTTCGCT-3'; and antisense, 5'-GGAATTCTTAGATCATGGCGTGGTTAGC GCTG-3'. The PCR product was subcloned into thymine adenine (TA)-cloning vector pT7-Blue (Takara, Tokyo, Japan) and sequenced. An EcoRI/EcoRI fragment containing the full-length mouse Gal-3 cDNA was inserted into the EcoRI site of the pIRES2-EGFP vector (Clontech, Mountain View, CA). The human Gal-3 coding region was amplified from human umbilical vein endothelial cell cDNA using the primers 5'-AGCCAACGAGCGGAAAAT-3' (sense) and 5'-TGACT-TACAAGAGGGATATTGATGA -3' (antisense), ligated to the pTA vector according to the Target clone protocol (Toyobo, Osaka, Japan), and subcloned as an XhoI-BamHI fragment into the pIRES2-EGFP vector. B16 or B16/BL6 cells were transfected with pCMV-mGal3-IRES2-EGFP containing mouse Gal-3, or with mock vector as a control, and A2058 cells were transfected with pCMV-hGal3-IRES2-EGFP containing human Gal-3, and also using mock vector as a control. All cells were stably transfected using the Lipofectamine 2000 system (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and selected by antibiotic resistance to G418 (Geneticin; Gibco).

To establish Gal-3 knockout cells using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated proteins) genome editing system, single guide RNAs were designed using the CRISPRdirect Web application (<https://crispr.dbcls.jp>) for the mGal-3 first exon using pX330-U6-Chimeric_BB-CBh-hSpCas9 (plasmid number 42230; Addgene, Watertown, MA).¹⁶ The gene-specific single guide RNA sequences were 5'-CACCGGCCCTTATGGTGTCCCCGC-3' (sense) and 5'-AAACGCGGGGACACCATAAGGGCC-3' (antisense), and oligos were ligated into the BbsI site of pX330. The constructed targeting vector was transfected into the B16 cell line. Clones were selected, and elimination of Gal-3 expression at the mRNA and protein level was confirmed through real-time quantitative RT-PCR (RT-qPCR) and Western blot analysis, respectively (as described below).

Tumor Growth and Metastasis *in Vivo*

All animal studies were approved by the Osaka University Animal Care and Use Committee. C57BL/6 female mice (for experiments using B16 and B16/BL6) and KSN female nude mice (for experiments using A2058), aged 7 to 8 weeks, were purchased from Japan SLC (Shizuoka, Japan). Gal-3 knockout (Gal3^{-/-}) mice were generated as previously described.¹⁷ MMTV-PyMT mice were purchased from the Jackson

Laboratory (Bar Harbor, ME). Male MMTV-PyMT mice on the C57BL/6 background were mated with Gal3^{-/-} females. Male Gal3^{+/-}-PyMT offspring were mated with Gal3^{-/-} females to finally generate MMTV-PyMT;Gal3^{-/-} mice. Genotyping was performed following standardized protocols, as described on the website of the Jackson Laboratory (Supplemental Figure S1A). For the tumor cell metastasis model, 1×10^5 tumor cells in 200 μ L phosphate-buffered saline were injected into the lateral tail vein of mice and lung metastases were analyzed 3 weeks later. Lungs were counted for surface metastatic foci, and fixed lungs were serially sectioned for staining with hematoxylin and eosin. Bright-field images were obtained by conventional microscopy (DM5500

B; Leica, Wetzlar, Germany) or digital camera (DFC500; Leica). For the s.c. model, 1×10^6 tumor cells in 100 μ L phosphate-buffered saline were injected into the shaved flank of the animals. Tumor volumes were measured and calculated as follows: length $\times$ width $\times$ width $\times$ 0.52.

Cell Lysis, SDS-PAGE, and Western Blot Analysis

Cell extracts were prepared by lysis with radio-immunoprecipitation assay buffer for 10 minutes on ice, followed by centrifugation at $17,800 \times g$ for 15 minutes at 4°C. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked with 5%

skimmed milk in 0.1% tris-buffered saline and Tween 20. Then, they were incubated with antibodies against Galectin-3/ MAC-2 (Cedarlane, Burlington, ON, Canada), integrin $\beta 3$ (ITG B3; number 13166; Cell Signaling Technology, Danvers, MA), or glyceraldehyde-3-phosphate dehydrogenase (Millipore, Temecula, CA), and then developed with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Antibody binding was visualized using electrochemiluminescence reagents (GE Healthcare, Milwaukee, WI).

RT-qPCR

Methods for RT-qPCR were previously described.¹⁸ Briefly, total RNA was extracted from cells using RNeasy-plus mini kits (Qiagen, Hilden, Germany) and reverse transcribed using the PrimeScript RT reagent kit (Takara), according to the manufacturer's protocol. Real-time PCR analysis was performed using Platinum SYBR Green qPCR SuperMix-UDC (Invitrogen) and an Mx3000p QPCR System (Agilent, Santa Clara, CA). Primers used in these studies were as follows: mouse Gal-3, 5'-TGCCCTATGACCTGCCCTT-3' (sense) and 5'-TCCTGCTTCGTGTTACACACAA-3' (antisense); mouse *GAPDH*, 5'-TGGCAAAGTGGAGATTGTTGCC-3' (sense) and 5'-AAGATGGTGTATGGGCTTCCCG-3' (antisense); mouse integrin αv , 5'-TCCGACGAGCACTGTTTCTC-3' (sense) and 5'-CATCCCGAAGATAGGCGACC-3' (antisense); mouse integrin $\alpha 6$, 5'-ATATCAAGCTCCCTCACGCG-3' (sense) and 5'-GCAAGAACAGCCAGGAG

GAT-3' (antisense); mouse integrin $\beta 1$, 5'-AACTTGTTGGT-CAGCAACGC-3' (sense) and 5'-AGCCAATCAGCGATC-CACAA-3' (antisense); mouse integrin $\beta 3$, 5'-TGACATCGAGCAGGTGAAAG-3' (sense) and 5'-GAG-TAGCAAGGCCAATGAGC-3' (antisense); mouse integrin $\beta 5$, 5'-CTGCACTTGCTGGTGTTCAC-3' (sense) and 5'-TTCAGGTGACACTGGCCATC-3' (antisense); human Gal-3, 5'-GGCCACTGATTGTGCCTTAT-3' (sense) and 5'-TCTTTCTTCCCTTCCCCAGT-3' (antisense); and human integrin $\beta 3$, 5'-GACAAGGGCTCTGGAGACAG-3' (sense) and 5'-ACTGGTGAGCTTTCGCATCT-3' (antisense). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase using the comparative threshold cycle method.

Platelet Isolation and Cell Aggregation Assay

Blood was drawn by cardiac puncture of the left ventricle into anticoagulant after anesthesia. To obtain a pure platelet fraction, density gradient centrifugation was performed using OptiPrep (iodixanol; Axis-Shield, Oslo, Norway), with a 1.063-g/mL density barrier produced by mixing 5 volumes of OptiPrep with 22 volumes of diluent [0.85% (w/v) NaCl; 20 mmol/L HEPES-NaOH, pH 7.4; and 1 mmol/L EDTA], and then centrifuging at $350 \times g$ for 15 minutes at 20°C. Cells were cultured on flat-bottom, ultra-low-attachment multiple-well plates (Corning, New York, NY) incubated on a shaker (WKN-2240; WakenBtech, Kyoto, Japan) for 24 hours.

Figure 2 Gal-3 deficiency in tumor cells increases metastatic ability. **A:** Real-time quantitative RT-PCR (RT-qPCR) of Gal-3 mRNA expression in B16 cells and Gal-3 knockdown B16 clones (B16/Gal3KO), as indicated. **B:** Western blot analysis of lysates from control B16 and B16/Gal3KO cells with anti-Gal-3 antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. **C:** RT-qPCR of mRNA expression of galectins 1 through 4, 6 through 9, and 12 in B16 and B16/Gal3KO cells. **D:** *In vitro* growth of B16 and B16/Gal3KO cells, as indicated. **E:** Quantitative evaluation of *in vivo* tumor growth. B16 or B16/Gal3KO cells were injected subcutaneously. **F:** **Top row:** Gross appearance of lung with metastatic foci 3 weeks after injection of melanoma cells, as indicated. **Bottom row:** Sections of lung tissues, as above, were stained with hematoxylin and eosin. **Arrows** indicate tumor metastasis nodules. **G:** Quantitative evaluation of the number of lung metastases counted in the lung shown in **F**. Data are expressed as means $\pm$ SD (**C–E**). $n = 3$ (**D**); $n = 4$ (**E**); $n = 5$ (**G**). * $P < 0.05$, ** $P < 0.01$ versus B16/Gal3KO. Scale bars: 5 mm (**F**, **top row**); 200 μ m (**F**, **bottom row**). ND, not detected.

Figure 3 Intrinsic Gal-3 produced by tumor cells is responsible for cancer metastasis. **A:** Scheme for the experimental metastasis model. **B:** Quantification of the number of metastatic nodes counted after injection of B16 [wild type (WT)] or Gal-3-knockout B16 melanoma (KO) cells into Gal3^{+/+} mice or Gal3^{-/-} mice, respectively. **C:** Sections of lung with metastatic foci 3 weeks after injection. Sections were stained with hematoxylin and eosin. **Arrowheads** indicate tumor metastasis nodules. **D:** Western blot analysis of Gal-3 in culture media or cell lysate of B16 or B16/BL6 cells. Data are expressed as means $\pm$ SD (**B**). $n = 4$ (**B**). $^{**}P < 0.01$. Scale bars = 100 μ m (**C**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Flow Cytometry

To assess the expression of integrins, cells were harvested using versene solution (Gibco) and resuspended in 4% FBS/phosphate-buffered saline. Cells stained with fluorescent-labeled anti-mouse antibodies specific for CD29 (BD Bioscience, San Jose, CA), CD41, and CD61 (eBioscience, San Diego, CA) were analyzed with a FACSCalibur (BD Bioscience).

RNA-Sequencing Analysis

Total RNA of B16 and B16/BL6 cells were extracted from cells using RNeasy-plus mini kits. Library preparation was performed using a TruSeq stranded mRNA sample preparation kit (Illumina, San Diego, CA), according to the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 2500 platform in a 75-base single-end mode. Illumina Casava software version 1.8.2 was used for base calling. Sequenced reads were mapped to the mouse reference genome sequences (mm10) using TopHat version 2.0.13 in combination with Bowtie2 version 2.2.3 and SAMtools version 0.1.19 (Johns Hopkins University, Center for Computational Biology, Baltimore, MD; <https://ccb.jhu.edu/software.shtml>). The fragments per kilobase of exon per million mapped fragments were calculated using Cufflinks version 2.2.1 (University of Washington, Seattle, WA; <http://cole-trapnell-lab.github.io/cufflinks>). The data were

uploaded to the Gene Expression Omnibus repository (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE122789).

Statistical Analysis

All data are presented as the means $\pm$ SD. For statistical analysis, the Statcel 4 software package (OMS; Higashikurume, Tokyo, Japan) was used, with analysis of variance performed on all data, followed by the Tukey-Kramer multiple comparison test. When only two groups were compared, *t*-test was used, except for the survival rate data, for which the log-rank test was used. The level of statistical significance was taken as $P < 0.05$.

Results

Gal-3 Expression Alters the Potential for Tumor Metastasis

Gal-3 expression levels were first evaluated on different cancer cells to determine how it influences metastasis. Most cancer cells express Gal-3, as previously reported^{19,20} (Supplemental Figure S2A). However, the highly metastatic mouse melanoma variant B16/BL6 had lower expression than poorly metastatic B16 cells, both at the mRNA and protein levels (Figure 1A). In fact, Gal-3 expression was significantly higher in B16 cells, according to comprehensive analysis by RNA sequencing (Supplemental Figure S2B). To test the hypothesis that Gal-3

Figure 4 Lack of Gal-3 also promotes metastasis in the spontaneous mammary tumor model. **A:** Survival of MMTV-PyMT;Gal3^{+/+} and MMTV-PyMT;Gal3^{-/-} mice. The P value is from log-rank testing. **B:** The number of metastatic foci in lung developing in MMTV-PyMT;Gal3^{+/+} and MMTV-PyMT;Gal3^{-/-} mice at 28 to 30 weeks of age. **C:** Hematoxylin and eosin staining of lung tissues from 28-week-old MMTV-PyMT;Gal3^{+/+} and MMTV-PyMT;Gal3^{-/-} mice. Arrowheads indicate metastatic foci in lung. **D:** Quantification of the number of metastatic foci was in five random fields of four independent lung sections. Data are expressed as means $\pm$ SD (**B** and **D**). $n = 7$ (**A**, MMTV-PyMT;Gal3^{+/+} and MMTV-PyMT;Gal3^{-/-} mice); $n = 4$ (**B**, MMTV-PyMT;Gal3^{+/+} and MMTV-PyMT;Gal3^{-/-} mice). $^{**}P < 0.01$. Scale bars = 100 μ m (**C**).

expression affects tumor metastasis, Gal-3 expression was enforced in B16/BL6 cells (Figure 1B). An *in vivo* experimental metastasis model in which B16/BL6 cells, B16/BL6-mock cells, or B16/BL6 cells in which Gal-3 expression was enforced were injected into the tail veins of recipient mice was used. After 3 weeks, the number of pulmonary metastatic foci was counted. B16/BL6 cells in which Gal-3 expression was enforced generated a lower number of metastatic foci than the parental B16/BL6 cells (Figure 1, C and D). A Gal-3 expression plasmid was transfected into B16 cells, causing them to highly overexpress this lectin (B16/Gal3 cells). Again, Gal-3 overexpression was confirmed by RT-qPCR and Western blot analysis (Figure 1E). When B16, B16-mock, or B16-Gal3 cells were injected, as described above, the latter showed significantly lower tumor metastatic ability than controls (Figure 1, F and G). These data suggest that Gal-3 expression in tumor cells suppresses tumor metastasis.

Gal-3 Deficiency in Tumor Cells Increases Metastatic Ability

To determine the effect of Gal-3 expression on tumor cells by loss of function, Gal-3-knockout B16 melanoma cells were generated using CRISPR/Cas9 genome editing technology by causing a deletion in Gal-3 exon 1. The lack of Gal-3 mRNA and protein expression in the knockout clones thus obtained was confirmed through RT-qPCR and Western blot analysis (Figure 2, A and B). Knockout Gal-3 expression did not affect other galectin family members at

the mRNA level (Figure 2C). It has been reported that Gal-3 expression is up-regulated during cancer progression at primary sites in several cancers.²¹ To test whether Gal-3 deficiency conveys a growth advantage in mouse melanoma, cell proliferation analyses were performed on B16 or Gal-3-knockout B16 melanoma cells. Gal-3 deficiency in B16 cells decreased cell growth potential *in vitro* and *in vivo* at the primary site (Figure 2, D and E). Furthermore, ectopic Gal-3 or inhibition by lactose did not affect cell proliferation (Supplemental Figure S3).

Next, B16 or Gal-3-knockout B16 melanoma cells were intravenously injected into the tail vein of wild-type mice and the number of metastatic foci was counted after 3 weeks. Gal-3 silencing in B16 melanoma greatly enhanced metastatic potential (Figure 2, F and G). These results indicate that Gal-3 expression in tumor cells suppresses tumor metastasis, but continuous Gal-3 expression induces cell and tumor growth at the primary site.

Lack of Gal-3 Expression in Tumor Cells Themselves Enhances Metastatic Colony Formation

To evaluate whether extrinsic Gal-3 may also affect tumor metastasis *in vivo*, Gal3^{-/-} mice were used (Figure 3A). Results suggested that extrinsic Gal-3 does not affect tumor cell metastasis in the lung (Figure 3, B and C). These results suggest that it is mostly Gal-3 expression in the tumor cells themselves that contributes to the formation of metastatic foci in distant organs.

It is known that Gal-3 has different functions as an intracellular or a secreted protein.^{22,23} Culture medium was concentrated from B16 or B16/BL6 cells, and intracellular or extracellular Gal-3 expression was assessed. The amount of cytoplasmic Gal-3 was found to be greater than secreted Gal-3, and the ratio of the two forms correlated with the level of expression of Gal-3 in the cells (Figure 3D). The possibility that secreted Gal-3 does have an influence on tumor metastasis cannot be excluded, but the results suggest the notion that metastatic potential is mostly regulated by intracellular Gal-3.

Lack of Gal-3 Also Enhances Metastasis in a Spontaneous Tumor Model in Mice

To investigate the role of Gal-3 in metastasis of tumors other than melanoma in the tumor cell inoculation model in mice, a spontaneous genetic mammary tumor model, MMTV-PyMT transgenic mice, was used. Knocking out Gal-3 did not affect the expression of the PyMT antigen (Supplemental Figure S1B). Survival of MMTV-PyMT transgenic mice on a Gal3^{-/-} background was significantly shorter than MMTV-PyMT mice on a wild-type background (Figure 4A). To quantify the effects of Gal-3 loss on lung metastasis, metastatic lesions were assessed in hematoxylin and eosin–stained sections of lung from mice at 28 to 30 weeks of age. The number and area of metastatic lesions were significantly greater in MMTV-PyMT;Gal3^{-/-} mice (Figure 4, B–D). These results indicate that lack of Gal-3 accelerates tumor metastasis in this spontaneous breast cancer model.

Gal-3 Alters Cancer Cell Aggregation through Integrin $\beta 3$ Expression

Tumor metastasis begins with detachment of tumor cells from the primary site and their invasion through blood/lymphatic vessels and progressive colonization of distant organs. Counteracting these processes, in the peripheral blood, immune cells and shear stress may damage the cancer cells. When these detach from the primary site, most of them are unable to seed a distal site.²⁴ For escape from the immune system, it is well-known that cancer cells form aggregates with large numbers of platelets^{7,25} and that this potential cell aggregation in the blood vessels facilitates the formation of metastatic colonies.²⁶ To elucidate the effect of Gal-3 on tumor metastasis during migration in the blood vessels, cell aggregation was investigated *in vitro*. Cancer cells were cultured with platelets and fibrinogen on ultra–low-attachment plates, and their aggregation was evaluated after 24 hours. The results showed that Gal-3–deficient B16 cells have a greater cell aggregation potential than parental B16 cells (Figure 5, A and B). To elucidate the mechanism responsible for the high aggregation potential of cancer cells and platelets, as affected by Gal-3 expression, adhesion molecules on the tumor cells were studied. Metastatic cancer cells often express integrin $\beta 3$ and adhere to platelets.^{27,28} Herein, integrin family expression was investigated and it was found that integrin $\beta 3$ was up-regulated in Gal-3–knockout B16 melanoma cells, whereas no significant

Figure 5 Gal-3 alters cancer cell aggregation through integrin $\beta 3$ expression. **A:** Appearance of cultured B16 or Gal-3–knockout B16 melanoma (B16/Gal3KO) cells with or without platelets (PLTs) and fibrinogen (FIB) after culturing for 24 hours. **B:** Quantification of diameter of cell aggregates, as observed in **A**. **C:** Quantitative RT-PCR of integrin αv (Itgav), $\alpha 6$ (Itga6), $\beta 1$ (Itgb1), $\beta 3$ (Itgb3), and $\beta 5$ (Itgb5) in B16 and B16/Gal3KO cells. Western blot analysis of integrin $\beta 3$ in B16 and B16/Gal3KO cells. **D:** Cell surface expression of integrins, as indicated in B16, B16/Gal3KO, and the negative control (gray area) by fluorescence-activated cell sorting analysis. **E:** Western blot analysis of integrin $\beta 3$ expression in B16 and B16/Gal3KO cells after stimulation with exogenous Gal-3 (8 μ g/mL) for 24 hours. **F:** Western blot analysis of Gal-3 siRNA–transfected B16 cells. siRNAs 1 and 2 indicate two different Gal-3–targeted siRNA oligonucleotides. Scrambled siRNA oligonucleotide was used as the negative control. Data are expressed as means $\pm$ SD (**B** and **C**). $n = 3$ (**B** and **C**). $*P < 0.05$, $**P < 0.01$. Scale bars = 200 μ m (**A**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

Figure 6 Gal-3 expression is reduced by transforming growth factor (TGF)- β 1. **A:** Real-time quantitative RT-PCR (RT-qPCR) of Gal-3 expression in B16 cells cultured with purified mouse plasma. Gal-3 expression seen 1 and 24 hours after stimulation. **B:** Cell morphology after stimulation with mouse plasma, as indicated in **A**. **C** and **D:** Quantitative RT-PCR of Gal-3 expression in B16 cells after stimulation with different doses of purified plasma (**C**) or serum (**D**) for 24 hours. **E:** Quantitative RT-PCR of Gal-3 expression in B16 cells after stimulation with 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), or 10 ng/mL TGF- β 1 for 24 hours. **F:** Western blot analysis of Gal-3 expression in B16 cells (lysate) after stimulation by TGF- β 1, as indicated, for 48 hours. **G:** Morphologic analysis of B16 cells after 10 ng/mL TGF- β 1 treatment for 24 hours. **H:** Quantitative RT-PCR of Gal-3 expression in B16 cells cultured with or without TGF- β receptor 2 inhibitor for 30 minutes and then stimulated with or without purified plasma for 24 hours. $n = 3$ (**A**, **C–E**, and **H**). * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μ m (**B** and **G**). DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

change was found in the expression of other integrin family members (Figure 5, C and D).

As described above, it is possible that Gal-3 is also secreted from cells and affects cell adhesion. However, on stimulation of B16 with soluble Gal-3, there was no change of β 3 integrin expression in B16 cells (Figure 5E). Also, even a high concentration of Gal-3 or addition of lactose to inhibit exogenous Gal-3 did not affect integrin β 3 expression (Supplemental Figure S4). Gal-3 expression was next silenced by siRNA, and it was found that its knockdown enhanced the expression of integrin β 3, as expected (Figure 5F). Thus, these results indicate that down-regulation of Gal-3 in tumor cells induces integrin β 3 expression, resulting in acceleration of tumor metastasis.

Gal-3 Expression Is Reduced by TGF- β 1 Stimulation

To determine how the change of Gal-3 expression in tumor cells is regulated during metastasis and which factors promote this in peripheral blood, B16 cells were stimulated with purified murine plasma. Gal-3 expression of stimulated cells dramatically decreased after 24 hours, and morphologic changes of B16 cells were also induced (Figure 6, A and B). These effects were caused by plasma in a dose-dependent manner; however, merely using serum from the mice did not alter the expression of Gal-3 (Figure 6, C and D). These results suggest that a molecule contained in peripheral blood stimulates tumor cells, resulting in attenuation of Gal-3 expression.

Previously, it was reported that TGF- β 1 from platelets promotes tumor metastasis and that TGF- β 1 stimulation

results in reduced Gal-3 expression in notochordal nucleus pulposus cells.²⁹ Herein, B16 tumor cells were treated with several cytokines, including TGF- β 1, and TGF- β 1 treatment was found to reduce Gal-3 expression by B16 cells (Figure 6E). At the protein level, high concentrations of TGF- β 1 reduced Gal-3 expression by B16 cells (Figure 6F) and induced morphologic changes to a scattered and spindle-shaped appearance, as in epithelial cancer cells (Figure 6G). The down-regulation of Gal-3 expression stimulated by plasma was abrogated using an inhibitor of the TGF- β receptor 2 (Figure 6H).

Tumor Cell Metastasis Is Affected by Gal-3 in Human Melanoma Cells

To determine the effect of Gal-3 expression on human melanoma metastasis, its expression was quantified on four human cell lines. This varied from line to line, but a negative correlation between Gal-3 and integrin β 3 was also observed in human melanoma cells (Figure 7A). Human melanoma line A2058 was selected for further study, and human Gal-3 was transduced into it to generate Gal-3–overexpressing cells (A2058/Gal3) (Figure 7B). Overexpression of Gal-3 caused slightly increased cell growth (Figure 7C). As observed in B16 melanoma cells, integrin β 3 expression in A2058/Gal3 cells was decreased relative to parental A2058 cells (Figure 7D). More important, when cells were injected into the tail veins of nude mice, overexpression of Gal-3 suppressed cancer cell metastasis in the lung, as was observed for B16 melanoma cells (Figure 7, E and F). In addition, integrin β 3 expression was silenced in

Figure 7 Tumor cell metastasis is affected by Gal-3 in human melanoma cells. **A:** Western blot analysis of integrin $\beta 3$ (Itgb3) and Gal-3 expression in human melanoma MeWo, A2058, G-361, and MMac cells. **B:** Quantification of Gal-3 mRNA expression levels in parental A2058 and Gal3-overexpressing A2058 cells (A2058/Gal3). **C:** *In vitro* cell growth. A2058 and A2058/Gal3 cells were cultured for 72 hours, after which cells were enumerated. **D:** Quantitative RT-PCR of integrin $\beta 3$ expression by A2058 and A2058/Gal3 cells. **E:** Gross appearance of the lung with metastatic foci 6 weeks after injection of A2058 or A2058/Gal3 cells. Hematoxylin and eosin staining of fixed lung sections, as above. Arrows indicate tumor metastatic nodules. **F:** Quantitative evaluation of the number of lung metastatic foci 6 weeks after injection of A2058 or A2058/Gal3 cells into KSN nude mice. Data are expressed as means $\pm$ SD (**B–D**). $n = 3$ (**C** and **F**). * $P < 0.05$, ** $P < 0.01$ versus A2058. Scale bars = 5 mm (**E**, top two rows); 100 μ m (**E**, bottom row). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

A2058 cells by siRNA and it was found that survival of mice injected with these A2058 cells in which integrin $\beta 3$ had been knocked down was increased relative to mice injected with parental A2058 cells (Supplemental Figure S5, A and B). The number of metastatic foci was counted in hematoxylin and eosin–stained lung tissue sections 6 weeks after injection of tumor cells, and it was found that silencing integrin $\beta 3$ in A2058 cells suppressed metastatic potential, as expected (Supplemental Figure S5C).

Discussion

Herein, a negative correlation between Gal-3 expression and high metastatic ability of melanoma cells in both mouse and human was found. Survival of mice in which mammary cancer occurs spontaneously (MMTV-PyMT) was shorter when Gal-3 expression was abrogated (Gal3^{-/-}), and there was a greater number of metastatic foci in the lungs. Integrin

$\beta 3$ expression increased by the down-regulation of Gal-3 expression in cancer cells, resulting in greater platelet adhesion mediated by fibrinogen, and consequently, promotion of cancer cell metastasis. In these experiments, Gal-3 expression was down-regulated on stimulation with TGF- β in cancer cells, which was followed by integrin $\beta 3$ up-regulation facilitating platelet adhesion, as noted above. This contributes to protecting the cancer cells from apoptosis in the peripheral blood and encourages the formation of metastatic foci.

When tumor cell proliferation is compared with cell invasion, it is important to consider which is the crucial event for malignant progression of the tumor, which, in turn, is important for prognosis.^{30–34} A cancer stem cell model for mouse glioma suggests that increased cancer cell invasive behavior results in a poorer prognosis, whereas their proliferative capacity plays a subservient role.³⁵ In these experiments, in contrast to a previous report suggesting that Gal-3 induces malignant tumor growth associated with cancer cell

proliferation,³⁶ Gal-3 expression was found to inhibit malignant progression of the tumor by reducing metastasis. In addition to inhibiting metastasis, Gal-3 induced cancer cell proliferation *in vitro*. Cancer cell proliferation and cancer metastasis may be concurrently required for malignant progression of the tumor. However, overexpression or down-regulation of a single molecule (Gal-3) altered proliferation or metastatic ability of cancer cells differently. Therefore, it is possible that even a single molecule can affect several different processes of cancer cell biology in a context-dependent manner.

Gal-3 acts primarily as an intracellular protein, but it is also secreted from cells in an unusual manner because there is no signal sequence on its coding sequence.³⁷ Whether malignant tumor progression is more strongly facilitated by intracellular or extracellular Gal-3 has not been clearly determined thus far. In these experiments, extracellular Gal-3 did not alter the degree of metastasis of Gal-3-deficient cancer cells (Figure 3). Moreover, exogenous Gal-3 did not alter the expression of integrin $\beta 3$. Therefore, cancer cells acquire metastatic behavior via a cell-autonomous lack of Gal-3, as extracellular Gal-3 did not rescue the effect.

Herein, TGF- β suppressed the expression of Gal-3 in cancer cells and integrin $\beta 3$ expression negatively correlated with Gal-3 expression. To the best of our knowledge, there are a few reports showing a relationship between Gal-3 and integrin $\beta 3$. For example, Gal-3 in tumor cells correlates with integrin $\alpha v \beta 3$ to promote tumor progression and angiogenesis,^{38–40} and knockdown of Gal-3 in mesenchymal stromal cells increases integrin $\beta 3$ expression in the acute myeloid leukemia microenvironment.⁴¹ However, how integrin $\beta 3$ expression is controlled by Gal-3 is not well understood. Further precise analysis is required for clarifying the control of expression of integrin $\beta 3$ by Gal-3. Previous reports have been suggested that Gal-3 is shown to function as a transcription factor for other cancer-related genes.⁴² For example, Gal-3 induces cyclin D1 promoter activity in human breast epithelial cells and directly up-regulates the transcriptional activity of thyroid transcriptional factor 1. Gal-3 forms a complex at the activator protein 1 site on the mucin 2 promoter associated with tumor progression. Also, Gal-3 regulates specificity protein 1, a transcriptional factor targeting integrin $\beta 3$,⁴³ and melanoma cells abundantly express E26 transformation-specific sequence 1, a partner molecule of SP1.⁴⁴ We suggest that Gal-3 is directly or indirectly bound to the integrin $\beta 3$ promoter region and inhibits its expression. Knocking out Gal-3 did not affect the localization of integrin $\beta 3$ (Supplemental Figure S6).

The function of Gal-3 in cancer cell metastasis is conserved in humans. In fact, a lower level of Gal-3 expression in human melanoma was found in metastatic relative to primary lesions.⁴⁵ Because extracellular Gal-3 may not affect metastasis, induction of intracellular Gal-3 in cancer cells would be required to inhibit tumor metastasis in cancer therapy. Although cell invasive behavior

rather than cell proliferative ability results in a poorer prognosis, to understand how and when Gal-3 expression should be induced is critical because Gal-3 also induces cancer cell proliferation.

Acknowledgments

We thank Dr. Feng Zhang (Broad Institute of Harvard and Massachusetts Institute of Technology) for supplying pX330 plasmid; Drs. Daisuke Okuzaki and Daisuke Motooka (Research Institute for Microbial Diseases, Osaka University) for performing RNA-sequencing analysis; and Noriko Fujimoto, Maho Omiya, and Yoko Mori for technical assistance.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.12.005>.

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