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N-glycans and metastasis in galectin-3 transgenic mice

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ABSTRACT

Poly-N-acetyl-lactosamine (polyLacNAc) on N-glycans facilitate lung specific metastasis of melanoma cells by serving as high affinity ligands for galectin-3, expressed in highest amounts in the lungs, on almost all its tissue compartments including on the surface of vascular endothelium. PolyLacNAc not only aids in initial arrest on the organ endothelium but in all the events of extravasation. Inhibition of polyLacNAc synthesis, or competitive inhibition of its interaction with galectin-3 all inhibited these processes and experimental metastasis. Transgenic galectin-3 mice, viz., gal-$3^{+/+}$ (wild type), gal-$3^{+/-}$ (hemizygous) and gal-$3^{-/-}$ (null) have been used to prove that galectin-3/polyLacNAc interactions are indeed critical for lung specific metastasis.

Gal-$3^{+/-}$ mice which showed <50% expression of galectin-3 on the lungs also showed proportionate decrease in the number of B16F10 melanoma metastatic colonies affirming that galectin-3 and polyLacNAc interactions are indeed key determinants of lung metastasis. However, surprisingly, the number and size of metastatic colonies in gal-$3^{-/-}$ mice was very similar as that seen in gal-$3^{+/+}$ mice. The levels of lactose binding lectins on the lungs and the transcripts of other galectins (galectin-1, -8 and -9) which are expressed on lungs and have similar sugar binding specificities as galectins-3, remain unchanged in gal-$3^{+/+}$ and gal-$3^{-/-}$ mice. Further, inhibition of N-glycosylation with Swainsonine (SW) which drastically reduces metastasis of B16F10 cells in gal-$3^{+/+}$ mice, did not affect lung metastasis when assessed in gal-$3^{-/-}$ mice. Together, these results rule out the possibility of some other galectin taking over the function of galectin-3 in gal-$3^{-/-}$ mice. Chimeric mice generated to assess if absence of any effect on metastasis is due to compromised tumor immunity by replacing bone marrow of gal-$3^{-/-}$ mice with that from gal-$3^{+/+}$ mice, also failed to impact melanoma metastasis. As galectin-3 regulates several immune functions including maturation of different immune cells, compromised tumor immunity could be the major determinant of melanoma metastasis in gal-$3^{-/-}$ mice and warrants thorough investigation.

1. Introduction

Metastasis is the major cause of deaths in cancer patients, yet the underlying molecular mechanisms are poorly understood, possibly, because of complexity and multistep nature of the disease [1]. To successfully metastasize the cancer cells must complete a sequential series of events which include separation from the primary tumor, invasion through surrounding tissues and basement membranes, entry and survival in the circulation and arrest in a distant target organ vasculature, extravasation and adaptation to the new organ growth environment [1–3].

Tumors often metastasize regionally to organs in the anatomic vicinity like those receiving the afferent blood vessel from the primary or the draining lymph nodes. As these organs receive maximum number of cells, some of these cells may get mechanically trapped in the fine vasculature and get adapted to the new organ environment and give rise to metastatic colonies. Colon cancers metastasizing to liver and several tumors colonizing lymph nodes are classical examples of this pattern of metastasis proposed by Ewing Ref. [2]. However, many cancers bypass several

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organs in their blood flow path and metastasize to very specific distant organ sites. The 'seed and Soil' hypothesis was proposed by Dr. Stephen Paget in 1889 to explain the phenomenon of organ specificity of metastasis based on the autopsy studies of >700 breast cancer patients. He compared the organ microenvironment with soil and tumor cells with seeds and explained that like seeds cancer cells although get dispersed to all the organs, but survive and give rise to metastatic colonies only in the organs that can support their growth [2,4]. Apart from the organ microenvironment, adhesive interactions, chemokines and their receptors have also been shown to be the key participants in organ specific metastasis [2,5,6].

Tumor cells show several cell surface modifications associated with the metastatic phenotype. One of the frequently observed modifications is the altered expression of β1, 6 branched N-oligosaccharides on cell surface glycoproteins. These oligosaccharides have been strongly associated with the invasive and metastatic phenotype of tumor cells [7,8]. The expression of these types of oligosaccharides on human tumors correlated with the disease progression [9–12]. Majority of the cancer cell lines expressing these oligosaccharides metastasize either to lungs or to liver. The E-Selectin expressed on the liver acts as a receptor for β1,6 branched N-oligosaccharides terminally substituted with Lewis antigen on tumor cell surface and facilitate liver metastasis [13–15]. Previously we showed that poly-N-acetyl lactosamine (polyLacNAc) substituted β1,6 branched N-oligosaccharides expressed on B16F10 cells promote lung specific metastasis via galectin-3 which is expressed in highest amounts on the lungs [16,17]. Galectin-3/PolyLacNAc pair not only facilitates initial arrest but also participates in all the subsequent steps of extravasation and organ colonization. Inhibition of polyLacNAc synthesis or competitive inhibition of their interaction has been shown to inhibit all these processes and metastasis. Galectin-3 transgenic mice used to confirm their participation in the metastatic process has thrown some very intriguing but interesting results, further pointing towards the complexity of the entire process.

2. Materials and methods

2.1. All the reagents used in this study are described in supplementary method 1

2.1.1. Cell culture and experimental metastasis assay

B16F10 murine melanoma cell line was obtained from National Centre for Cell Sciences, Pune, India. Melanoma cells were routinely cultured in Dulbecco's Modified Eagle's medium (DMEM), with or without glycosylation inhibitor SW (2 μg/ml) and metastasis assays were performed as described in Ref. [17].

2.1.2. Genotyping of galectin-3 transgenic mice by PCR

Propagation of transgenic mice is described in supplementary method 2. Galectin-3 status of the littermates was determined by PCR using genomic DNA obtained from the tails of the 3–4 week mice. PCR was performed using 1 μg genomic DNA and primers: PRIMER 1 (a primer annealing to the neo cassette region) 5′GGCTGACCGCTTCCTCGTGCTTTACGG3′, PRIMER 2 (a primer annealing to the non -disrupted region of intron 4 of intact galectin-3 gene) 5′GTAGGTGAGAGTCACAAGCTG GAGGCC3′ and PRIMER 3 (a common downstream primer annealing to EXON 5) 5′CACTCTCAAAGGGGAAGGCTGACTGTC3'. PCR was carried out and the product was analyzed on 2% agarose gel by electrophoresis followed by ethidium bromide staining. Gal-$3^{+/+}$ mice give an amplicon of 450 bp, gal-$3^{+/-}$ mice give 2 amplicons of 450 bp and 300 bp while gal-$3^{-/-}$ mice give an amplicon of 300 bp [18].

2.1.3. Preparation of total cell lysates and Western blotting

Preparation of total cell lysates and analysis of Western blotted proteins was done exactly as described in Ref. [16].

2.1.4. Detection of galectin-3 expression in mouse lungs by western blotting

Lungs of 6–8 week old mice of gal-$3^{+/+}$, gal-$3^{+/-}$ and gal-$3^{-/-}$ group were excised and subsequently homogenized in 1× Laemmli's Sample buffer, Western blotted and probed with rat anti mouse galectin-3 antibody as a probe.

2.1.5. Flow cytometric analysis

For flow cytometry, melanoma cells were first fixed by overnight incubation with 1.5% gluteraldehyde in PBS (pH 7.4). Analysis of surface expression of polyLacNAc on melanoma cells was performed using biotinylated L-PHA exactly as described in Ref. [17].

2.1.6. Real time PCR

Total RNA from the lungs of gal-$3^{+/+}$, gal-$3^{+/-}$ and gal-$3^{-/-}$ transgenic mice was extracted using TRIzol reagent and the cDNA synthesized and used as a template for real time PCR. The primer pair sequences of galectin-1, galectin-8, galectin-9 and RPL4 were obtained from Ref. [19]. Real time PCR was performed as described in Ref. [20].

2.1.7. Generation of chimeric mice

Chimeric mice were generated exactly as described by Mace et al. [21]. Briefly, the bone marrow in irradiated gal-$3^{-/-}$ mice was replaced, in group 1 with that from gal-$3^{-/-}$ mice which served as control, while in group 2 with that from gal-$3^{+/+}$ mice. Chimerism was confirmed by genotyping of these mice using tail and blood genomic DNA by PCR as described by Hsu et al. [18].

2.1.8. Statistical analysis

All data are represented as mean ± SE unless stated. Student's t-test was employed for calculating significance across different groups. (p value < 0.05 was considered significant).

3. Results

3.1. Characterization of gal-$3^{+/+}$, gal-$3^{+/-}$ and gal-$3^{-/-}$ transgenic mice

Galectin-3 transgenic mice were characterized using tail genomic DNA as the template for PCR. The amplicon size for gal-$3^{+/+}$ and gal-$3^{-/-}$ mice was 450 bp and 300 bp, respectively. However, the two amplicons 450 bp and 300 bp were obtained for gal-$3^{+/-}$ mice (Fig. 1A). These results indicate that the mice were successfully propagated and maintained.

3.2. Comparison of the levels of expression of galectin-3 in galectin-3 transgenic mice

Tissue extracts of lungs of transgenic mice was analyzed by Western blotting for the presence of galectin-3. As expected, the gal-$3^{+/+}$ mice show the expression of galectin-3 while there is absence of galectin-3 in gal-$3^{-/-}$ mice. Since the gal-$3^{+/-}$ mice have one mutated allele, show >50% reduction in the expression of galectin-3 as compared to that of gal-$3^{+/+}$ mice (Fig. 1B).

3.3. Expression of galectin-3 in lungs dictates the extent of metastasis of melanoma cells

Experimental metastasis assay using B16F10 cells in gal-$3^{+/+}$ and gal-$3^{+/-}$ mice showed that the levels of galectin-3 expression

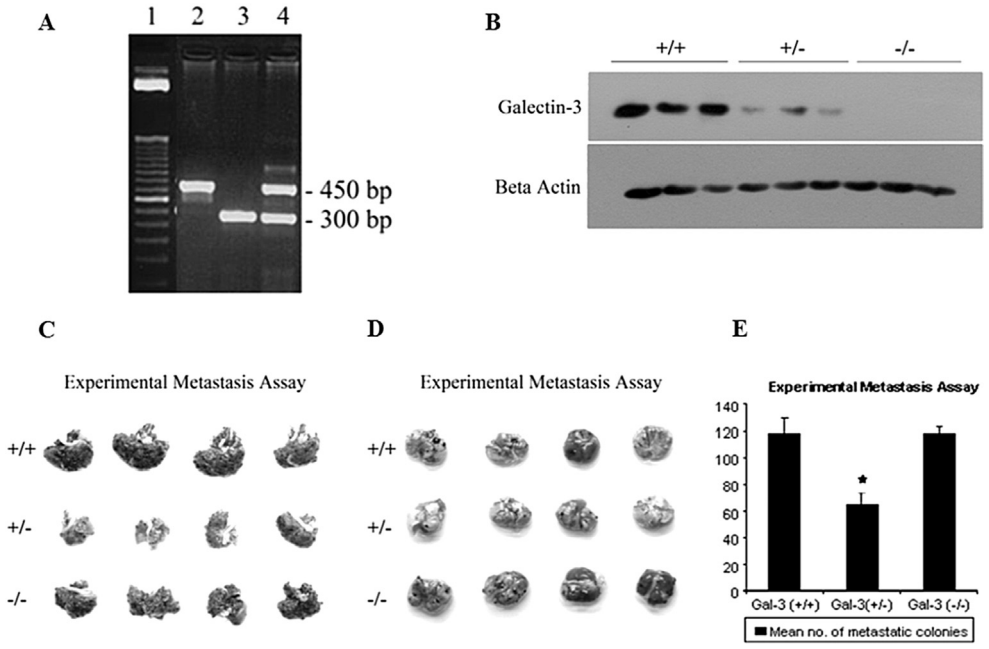

Fig. 1. Expression of galectin-3 in the lungs dictates the metastatic potential of B16F10 cells. (A) Assessment of galectin-3 status of mouse using PCR and utilizing genomic DNA as template and primers specific for galectin-3. Lane 1 represents 50 bp DNA ladder. Lane 2–4 represent PCR products using DNA from gal-$3^{+/+}$, gal-$3^{-/-}$ and gal-$3^{+/-}$ mice respectively. (B) Western blotting of 100 μg of lung lysates from gal-$3^{+/+}$, gal-$3^{+/-}$ and gal-$3^{-/-}$ mice using anti-galectin-3 antibody. Beta actin served as loading control. (C and D) Comparison of melanoma colonies in lungs of gal-$3^{+/+}$ (lane1), gal-$3^{+/-}$ (lane 2) and gal-$3^{-/-}$ (lane 3) mice, after injection with 0.18 million (C) and 0.075 million (D) B16F10 cells under experimental metastasis assay conditions. (E) Graphical representation of mean values of number of lung colonies of the three groups of mice under experimental metastasis assay conditions. Significance obtained by performing student's T-test is denoted by *, p value <0.05.

indeed dictates the metastatic outcome. Gal-$3^{+/-}$ mice which express significantly lower levels of galectin-3 in the lungs as compared to gal-$3^{+/+}$ mice, also showed corresponding decrease in lung metastasis. However, the gal-$3^{-/-}$ mice which show no galectin-3 on the lungs surprisingly showed same level of lung metastatic colonies as gal-$3^{+/+}$ mice. This pattern was always consistent even when tested by injecting different cell numbers (Fig. 1C and D).

Galectin-3 being a member of a large family of lectins the possibility of some other galectins taking over the role of galectin-3 was investigated.

3.4. Comparison of the expression of transcripts of galectin-1, -8 and -9 and lactose binding lectins in the lungs of transgenic mice

Galectin-1, -8 and -9 have similar carbohydrate binding specificities as galectin-3 and have also been shown to be expressed on the lungs [22]. In the absence of galectin-3 it is possible that one of these members takes over its function and thus may be overexpressed on the lungs. However, analysis of the transcript levels of these galectins showed insignificant difference between gal-$3^{+/+}$, gal-$3^{+/-}$ and gal-$3^{-/-}$ mice as assessed by real time PCR (Table 1).

Further, comparison of the total lactose binding lectins from the lungs of gal-$3^{+/+}$ and gal-$3^{-/-}$ mice were very similar except for the presence of galectin-3 in the fraction from lungs of gal-$3^{+/+}$ mice (Data not shown). These results appear to indicate that no other member of galectin family takes over the function of galectin-3 in facilitating metastasis. This raised the possibility that the metastasis in these mice may be due to some other mechanism and may not involve lectin-carbohydrate interaction. In such a scenario inhibition of N-oligosaccharides may not inhibit metastasis.

Table 1
Transcript levels of different galectin members in lungs of galectin-3 transgenic mice as compared to that in gal-$3^{+/+}$ mice.

Galectin-3 status in mice	Galectin-1	Galectin-8	Galectin-9
Gal-$3^{+/-}$ mice	1.5 fold increase	1.16 fold increase	1.05 fold increase
Gal-$3^{-/-}$ mice	1.244 fold increase	1.18 fold increase	1.04 fold increase

3.5. Inhibition of N-oligosaccharides on B16F10 melanoma cells does not impact metastasis when assayed in gal-$3^{-/-}$ mice

Inhibition of addition of polyLacNAc either by inhibiting synthesis of N-oligosaccharides using SW or using shRNA for enzymes that synthesize polyLacNAc, results in the loss of metastasis of B16F10 cells in gal-$3^{+/+}$ type C57BL/6 mice. However, inhibition of N-oligosaccharides using SW had no effect on experimental metastasis when assessed in gal-$3^{-/-}$ mice (Fig. 2A). The inhibition of expression of β1,6 branched N-oligosaccharides in SW treated B16F10 cells was confirmed using lectin blotting and flow cytometry using L-PHA as the probe (Fig. 2B and C). These results suggest that in absence of galectin-3 in mice, even carbohydrates on cancer cells become redundant for cancer cell metastasis. Galectin-3 is an important protein that is present in majority of the cells of immune system and performs a variety of regulatory functions. Complete absence of galecetin-3 possibly compromises the anti-tumor immunity and thus increased lung metastasis in gal-$3^{-/-}$ mice.

3.6. Generation of chimeric mice by replacing bone marrow of gal-$3^{-/-}$ mice with that of gal-$3^{+/+}$ mice does not impact metastasis of B16F10 cells

To test if absence of galectin-3 in some way alters the tumor immunity and thus metastatic outcome of cells in gal-$3^{-/-}$ mice,

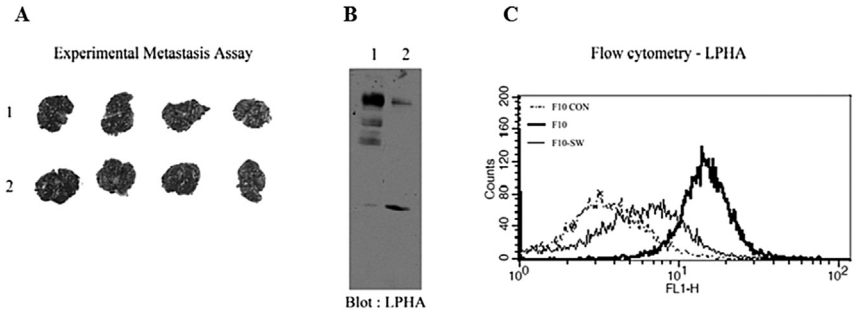

Fig. 2. Inhibition of N-oligosaccharides has no effect on the metastatic potential of melanoma cells in gal-3$^{-/-}$ mice. (A) Comparison of melanoma colonies in lungs of gal-3$^{-/-}$ mice after injecting 0.15 million (1) untreated and (2) Swainsonine treated B16F10 cells, under experimental metastasis assay conditions. The inhibition of N-oligosaccharides by SW was confirmed by (B) Western blotting and (C) flow cytometry using biotin labeled L-PHA. B16F10 cells treated only with avidin FITC served as control (B16F10 CON).

chimeric mice were generated. Replacement of bone marrow of gal-3$^{-/-}$ mice with that from gal-3$^{+/+}$ mice was confirmed by analyzing the tail and blood genomic DNA as a template for PCR. The bands at 300 bp in both the groups obtained with the tail genomic DNA confirmed their gal-3$^{-/-}$ phenotype (Fig. 3A). Further, the appearance of only 300 bp band in group 1 and bands of both 300 and 450 bp obtained with blood genomic DNA in group 2 confirms gal-3$^{-/-}$ phenotype in group 1 and successful chimerism in group 2 (Fig. 3B). However, generation of chimeric mice had no effect on the metastatic outcome of B16F10 cells in the lungs (Fig. 3C).

4. Discussion

Previous work in the lab very clearly demonstrated the role of high affinity ligands in the form of polyLacNAc on N-glycans on melanoma cells in mediating lung metastasis. By demonstrating the presence of galectin-3 in highest amounts on lungs and its constitutive expression on vascular endothelium and by using dominant negative and competitive inhibitors, galectin-3 was shown to be the potential polyLacNAc receptor that mediates metastasis on the lungs [16,20]. To prove that the galectin-3/polyLacNAc interaction is indeed indispensable for lung metastasis galectin-3 transgenic mice were used.

The three subpopulations of galectin-3 transgenic mice successfully genotyped and characterized provide an important tool to investigate the role of galectin-3 in metastatic process (Fig. 1A and B). The gal-3$^{+/-}$ mice in which the expression of galectin-3 was <50% as compared to gal-3$^{+/+}$, also showed correspondingly reduced lung metastasis, indicating that galectin-3 on the lungs is indeed an important determinant of metastatic outcome. Surprisingly, however, the gal-3$^{-/-}$ mice which lacked galectin-3 on the lungs showed almost similar extent of metastasis as the gal-3$^{+/+}$ mice (Fig. 1C and D).

Galectins are a family of β-galactoside binding lectins which often exhibit functional redundancy. Besides galectin-3, other members including galectin-1, -8 and -9 have been shown to be expressed on the lungs and have very similar oligosaccharide specificity [22]. However, analysis of the transcripts of these galectins (Table 1), or comparison of the lactose binding proteins (data not shown) in the lungs from gal-3$^{+/+}$ and gal-3$^{-/-}$ mice did not show any significant difference. Although, the results do not completely rule out the possibility of some other galectin taking over the function of galectin-3, it weakens this possibility. An alternative approach was devised to test this. While inhibition of N-oligosaccharides significantly inhibits metastasis of B16F10 cells in gal-3$^{+/+}$ mice, their inhibition should also inhibit metastasis in the gal-3$^{-/-}$ mice, in case other galectins take over galectin-3 function. However, SW treatment failed to have any effect on the metastatic properties of these cells in gal-3$^{-/-}$ mice (Fig. 2), pointing towards an alternate mechanism.

Almost a million tumor cells are believed to be in circulation in a patient diagnosed with cancer; however, only a few of them metastasize. Experimentally it has been shown that, although, > 95% of the B16F10 cells injected via tail vein can be recovered from the lungs of mice within 2 min of injection, majority of them are cleared by 24 h. Only about 2% of the injected cells remain in the lungs by 24 h [23]. Even of these 2% arrested cells, only those that are able to interact with the host organ environment are possibly able to survive and grow as 100-150 metastatic colonies. Interactions between molecules like polyLacNAc on cancer cells and galectin-3 on the organs like lungs would assume importance at this stage. However, host immune competence would play a key

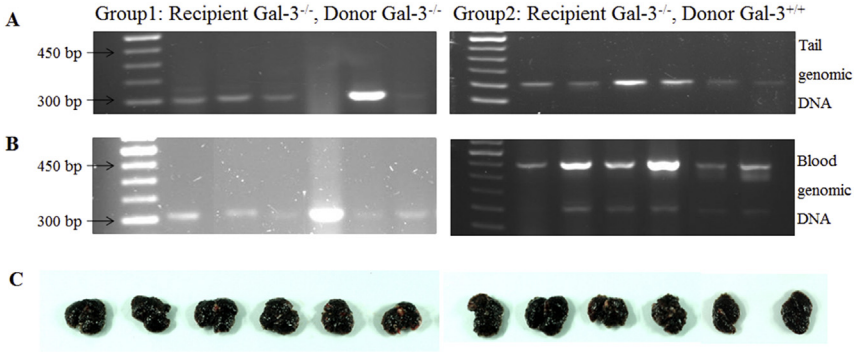

Fig. 3. Experimental metastasis assay using chimeric mice. (A) Agarose gel image showing PCR amplification band at 300 bp using tail genomic DNA. (B) Agarose gel image showing the PCR amplification band at 300 bp and 450 bp using blood genomic DNA as a template. (C) Represents the lung images taken after experimental metastasis assay.

role in clearing majority of the cells from the target organ. Agents which augment NK cell activity in mice have indeed been shown to be very efficient in inhibiting metastasis, which was ineffective in beige mice which lack NK cells or in mice where NK cells were depleted [23]. Are gal-3$^{-/-}$ mice not competent enough immunologically?

Galectin-3 plays an important role in regulating different functions of innate and adaptive immune systems [24–27]. Almost all types of immune cells express galectin-3 [24]. As galectin-3 is present in the nucleus, cytoplasm and on the cells surface and as part of Extracellular matrix ((ECM), its function varies depending on its subcellular localization. Galectin-3 in the cytoplasm is anti-apoptotic while nuclear galectin-3 is pro-apoptotic whereas that on the cell surface or on ECM/Basement membrane (BM) performs very different functions [28–30]. As a result galectin-3 influences immune response in a variety of ways which are often conflicting.

Galectin-3 influences innate immunity by modulating adhesion and migration of monocytes, macrophages and dendritic cells. It promotes adhesion of neutrophils to laminin and regulates neutrophil traversing through BM at sites of inflammation. Galectin-3 also, regulates T-cell signaling, activation, cytokine secretion, apoptosis and regulatory T-cell proliferation. Galectin-3 deficiency results in increased frequency of immune suppressor cells such as CD4^{+}CD25^{+}FOXP3^{+}T$_{reg}$ cells [24,25,27]. In contrast, galectin-3 induces apoptosis in CD8^{+}T cells in mouse model of colorectal cancer [27]. Although, gal-3$^{-/-}$ mice are viable, they have a distinct phenotype in terms of their immune functions related to autoimmunity, and responses to allergy, inflammation and infectious diseases. However, a unified picture of galectin-3 mediated effects on host immunity and thus metastasis is yet to emerge clearly. It is very likely that the gal-3$^{-/-}$ mice are defective in their critical immune function which may positively or negatively influence metastatic outcome.

In such a scenario, analogous to the beige mice which lack NK cell activity, the gal-3$^{-/-}$ mice would also have much higher burden of mechanically arrested melanoma cells in the lungs. If they are not cleared efficiently, some of these possibly give rise to metastatic colonies, especially because the high metastatic B16F10 cells have been selected specifically for lung colonization by serial in vitro and in vivo passaging of B16F1 cells. Some of these would be able to adapt to the growth environment of the lungs even in absence of polyLacNAc and galectin-3. This would be very similar to what is proposed for anatomical/mechanical mode of metastatic spread. However, a recent report on experiments with B16F1 cells using gal-3$^{+/+}$ and gal-3$^{-/-}$ mice has suggested that the gal-3$^{-/-}$ mice may be more competent in terms of their anti-tumor immunity as compared to gal-3$^{+/+}$ mice via their enhanced NK-cell activity [31]. In contrast to these, our preliminary experiments with B16F1 cells in these mice did not give statistically significant variation in the number of metastatic colonies, mainly because the total number of metastatic colonies of B16F1 cells in the lungs as such, was very low.

Generating chimeric mice by replacing the bone marrow of gal-3$^{-/-}$ mice with that of gal-3$^{+/+}$ mice may possibly restore immunity and thus should inhibit metastasis. Although, chimeric mice could be successfully generated, metastasis of B16F10 cells in the lungs could not be inhibited in these mice (Fig. 3). Galectin-3 also appears to be required for maturation of several immune cells like the maturation of plasma cells into memory B cells and the selection of CD4^{+}T and CD8^{+}T cells in the thymus [32]. These results suggest that in the absence of galectin-3, even after replacing the bone marrow, the gal-3$^{-/-}$ mice may not achieve an effective anti-tumor immunity.

In conclusion, by employing gal-3$^{+/+}$ and gal-3$^{+/-}$ transgenic mice and lung homing high metastatic B16F10 cells, the present investigation very clearly demonstrates that specific interactions between molecules on the tumor cells and on the target organ indeed play an important role in facilitating organ specific metastasis. By utilizing gal-3$^{-/-}$ mice, it also demonstrates the importance of host immunity in controlling metastasis and the complex manner in which it is regulated. This warrants a thorough investigation.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.030.

Transparency document

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