Mechanisms Involved in Regulation of Processes Critical for Cancer Metastasis by β1,6 branched N-oligosaccharides

By

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Akhil Kumar Agarwal entitled "Mechanisms Involved in Regulation of Processes Critical for Cancer Metastasis by β1,6 branched N-oligosaccharides" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution/University.

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List of Publications arising from the thesis

Journal

1. "Regulation of melanoma metastasis to lungs by cell surface Lysosome Associated Membrane Protein-1 (LAMP1) via galectin-3", **Akhil Kumar Agarwal,** Rajiv P. Gude and Rajiv D. Kalraiya, Biochemical and Biophysical Research Communications, **2014**, Vol. 449, Issue 3, pages 332-337.
2. "Glycosylation regulates the expression of Lysosome Associated Membrane Protein-1 (LAMP1) on the cell surface", **Akhil Kumar Agarwal** and Rajiv D. Kalraiya, Journal of Bioscience and Technology, **2014**, Vol. 5, Issue 3, pages 556-563.
3. "Role of tumor cell surface Lysosome Associated Membrane Protein-1 (LAMP1) and its associated carbohydrates in lung metastasis", **Akhil Kumar Agarwal**, Nithya Srinivasan, Rashmi Godbole, Srikanth Budnar, Rajiv P. Gude and Rajiv D. Kalraiya, under revision, Journal of Cancer Research and Clinical Oncology, **2014**.

Conferences (Abstracts Published)

1. "Role of Cell Surface Lysosome Associated Membrane Protein-1 (LAMP1) in Metastasis", **Akhil Kumar Agarwal**, Rashmi Godbole, Nithya Srinivasan and Rajiv D. Kalraiya, Glycobiology, **2012**, Vol 22, page 1600.
2. "Role of cell surface Lysosome Associated Membrane Protein-1 (LAMP1) and its associated sugars in organ specific metastasis", **Akhil Kumar Agarwal**, Rashmi Godbole and Rajiv D. Kalraiya, Journal of Cancer Research and Therapeutics, **2012**, Vol 8, Supplement 1, page S44.

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Presentations at Conferences

1. **Oral presentation** in National Conference on "**Glycobiology of Cancer; Lectins as tools and Targets**", Karnatak University, Dharwad, 7-9 November, **2013**.
2. **Oral presentation** in **8th National Research Scholars Meet in Life Sciences**, Advanced Centre for Treatment, Research & Education in Cancer (ACTREC), Kharghar, Navi Mumbai, 21-22 December, **2012.**
3. **Poster presentation** in National Conference on "**Glycobiology of Cancer; Lectins as tools and Targets**", Karnatak University, Dharwad, 7-9 November, **2013**.
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Akhil Kumar Agarwal

Dedicated to

My Beloved Family and Friends

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Ph. D. PROGRAMME

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SYNOPSIS

Introduction:

Cancer is one of the major causes of death in humans. Metastasis, the major cause of mortality seen in cancer patients, is a complex multi-step process. Tumor cells often get trapped in the fine vasculature of the first organ encountered. However, many tumors metastasize to very specific distant organ sites [1]. Tumor cells show several membrane modifications associated with metastasis. Altered expression of β1,6 branched N-oligosaccharides on cell surface glycoproteins is one such consistently observed modification. Its expression on several human cancers and many invasive and metastatic human and murine tumor cell lines has been shown to correlate with their invasive and malignant potential [2]. Further, its association with organ-specific metastasis is outlined by the fact that a majority of cell lines carrying these oligosaccharides metastasize specifically to either the liver or to the lungs [3, 4]. These oligosaccharides may mediate organ specific metastasis in two ways. Firstly, β1,6 branch serves as the preferred site for further substitutions like Lewis antigens, poly-N-acetyl-lactosamine (PolyLacNAc), sialic acids and others which may serve as ligands for several endogenous lectins such as selectins, galectins, siglecs and as yet unidentified endogenous lectins [2, 5]. Secondly, the multi-antennary highly substituted bulky carbohydrate structures formed as a result of β1,6 branching may alter the structural and functional properties of proteins which carry them, thus possibly aiding organ-specific metastasis. Some of the proteins important from metastasis point of view which are known to carry these oligosaccharides include cell adhesion molecules such as cadherins, integrins, CD44, growth factor receptors such as EGFR, matrix components like laminin and others like Lysosome Associated Membrane Proteins (LAMPs) [2].

LAMPs (mainly LAMP1 & LAMP2), unlike the other cell surface proteins listed above, belong to the class of highly glycosylated (17-20 N-glycan sites) lysosomal membrane proteins which decorate the luminal side of lysosomes. They are thought to protect

themselves and the lysosomal membranes from intracellular proteolysis due to the presence of highly branched oligosaccharides on them [6]. However, they are also expressed on surface of several metastatic tumor cells such as human melanoma, colon carcinoma, fibrosarcoma and myelomonocytic leukemia cells [7]. Cell surface expression of LAMP1 (but not LAMP2) has been shown to correlate with metastatic potential of murine melanoma cell line [8]. In addition, increased surface expression of LAMP1 has also been observed on normal cells involved in migratory and/or invasive functions such as activated cytotoxic T-lymphocytes, natural killer cells, macrophages as well as embryonic cells [9-12]. However, the mechanism by which cell surface LAMP1 may mediate these functions is largely unknown.

Purified LAMP1 has been shown to bind to some of the Extra Cellular Matrix (ECM) and Basement Membrane (BM) components as well as RGD peptides [13]. LAMP1 has also been shown to be present on unique cell surface domains involved in cell locomotion such as membrane ruffles and microspikes (filopodia) [14]. Further, its accumulation at the edges and extensions of A2058 human metastasizing melanoma cells [7] hints towards its potential role in tumor cell adhesion, spreading and motility possibly by serving as additional receptors for ECM and BM components. Besides, LAMP1 has also been found to be a major carrier of polyLacNAc substituted β1,6 branched N-glycans [3, 8]. An increase in β1,6 branching observed in fibroblasts, metastatic cell line SP1 and macrophage-melanoma fusion hybrids appeared to be associated with increased surface expression of LAMP1 [11, 15]. LAMP1 on cell surface has been shown to provide ligands in the form of sialyl-Le^x to E-selectin [16] and in the form of polyLacNAc to galectin-3 [7, 8].

Using low (B16F1) and high (B16F10) metastatic variants of lung colonizing B16 murine melanoma cells, it was previously shown that surface translocation of LAMP1, but not

LAMP2, correlated with the metastatic potential of these cells [8]. LAMP1 being highly glycosylated, its surface translocation provides high density of easily accessible high affinity ligands (polyLacNAc) for galectin-3. Galectin-3 has been shown to be expressed in highest amounts on the lungs and constitutively on the surface of lung vascular endothelium [8]. Further, blocking cell surface LAMP1 in high metastatic B16F10 cells using antibodies to LAMP1 resulted in significantly reducing their metastasis to lungs. From the above studies, several questions arise.

Key Questions:

- **Does cell surface LAMP1 participate in any of the cellular processes important for metastasis?**
- **What is the contribution of carbohydrates on LAMP1 in these processes and hence lung metastasis?**
- **Would knockdown of LAMP1 in high metastatic cells affect their cellular properties important for metastasis?**

Objectives:

The following objectives were proposed to answer these questions:

1. **To investigate the effect of translocating LAMP1 to cell surface in low metastatic cells, on different cellular properties important for lung metastasis.**
2. **To investigate the contribution of β1,6 branched N-oligosaccharides on LAMP1 in different cellular properties of metastatic cells.**
3. **To investigate the effect of downregulation of LAMP1 on the cellular properties of high metastatic murine melanoma cells.**

Methodology:

Cell culture and experimental metastasis assay

Melanoma cells were routinely cultured in DMEM containing 10% FBS, L-glutamine and antibiotics. For inhibition of N-glycosylation, cells were grown in presence of swainsonine (2 μg/ml) for 48 h. For metastasis assays, cells were injected intravenously in inbred strains of female C57BL/6 mice which were sacrificed after 21 days and melanoma colonies on their lungs were counted.

Overexpression of LAMP1 on surface of B16F1 cells

LAMP1 was amplified from total cDNA. After removing K18-YFP from pLV-K18-YFP-IRES-Puro, it was either self-ligated to obtain vector control or ligated to the amplified LAMP1 to generate wild-type LAMP1 (wtLAMP1) vector. This vector was further used for site-directed mutagenesis of its tyrosine^{386} to alanine to get mutant LAMP1 (mutLAMP1) vector. The vector control plasmids and the mutLAMP1 plasmids were co-transfected with helper plasmids in HEK293FT cells. Infectious viruses generated contained either control vector or mutLAMP1 vector. These were transduced in B16F1 cells. One vector control clone (VC) and two mutant LAMP1 clones (C1 & C11) were selected using puromycin and maintained as separate stocks.

Flow cytometric analysis

For flow cytometry of LAMP1, melanoma cells were incubated with anti-LAMP1 antibody followed by treatment with anti-rat FITC conjugate. The cells were fixed in paraformaldehyde before being acquired on FACS Calibur. For determination of lectin binding, paraformaldehyde fixed melanoma cells were stained either directly with FITC labelled lectin or with biotinylated lectins followed by extravidin-FITC. Untreated cells or cells treated with extra-avidin-FITC alone served as control. Fluorescent cells were acquired on FACS Calibur and analyzed using Cell Quest software.

Immunofluorescence staining

Paraformaldehyde fixed melanoma cells grown on coverslips were washed, blocked with BSA and incubated with LAMP1 antibody, followed by washes with PBS. Cells were further incubated with anti-rat FITC followed by washes with PBS. Nuclei were stained with DAPI and coverslips were mounted on slides using vectashield. Images were acquired using a confocal microscope.

Expression and purification of recombinant human galectin-3

Expression of recombinant human (rh) galectin-3 was induced in *Escherichia coli* BL-21 containing pET3C plasmid using IPTG. The protein was purified from the bacterial cell lysates using a Lactose-Sepharose affinity column and stored after vacuum drying.

Cell spreading assays

Melanoma cells were seeded on galectin-3, fibronectin and matrigel coated coverslips in serum free medium and incubated for 45 minutes at 37^0C in CO_2 incubator. Bound cells were paraformaldehyde fixed and permeabilized with Triton X-100. Phalloidin FITC was used for F-actin staining and DAPI for staining nuclei. The images of stained cells were acquired using a confocal microscope.

Wound healing assays

Melanoma cells were cultured on 6 well plates precoated with galectin-3, fibronectin or matrigel, blocked with BSA and grown for 24 h. The cells were treated with mitomycin C for inhibiting cell proliferation. A wound was made on the monolayer and cells were maintained in serum free medium. Wound closure of cells was measured for 16-20 h by time lapse video imaging.

Preparation of total cell lysates, protein estimation, SDS-PAGE and Western blotting

Total cell lysates were prepared by lysing the melanoma cells in 20 mM Tris Chloride buffer containing 1% NP-40, 0.5% sodium deoxycholate, 150 mM sodium chloride and 1 mM each

of magnesium chloride and calcium chloride and protease inhibitor cocktail followed by sonication and centrifugation. Proteins were resolved on 10% SDS-PAGE and blotted on PVDF membrane. Blots were probed either with biotinylated lectins (L-PHA for β1,6 branched N-oligosaccharides and LEA for polyLacNAc) or antibodies against LAMP1 and β-actin (loading control).

Immunoprecipitation of LAMP1

Precleared total cell lysates of melanoma cells were incubated with anti-LAMP1 antibody followed by addition of protein G sepharose beads. After overnight incubation at 4°C, the beads were pelleted and washed with lysis buffer. The bound proteins were eluted by boiling the beads in 1X Laemmli sample buffer, separated on SDS-PAGE and Western blotted for LAMP1, LPHA and LEA.

Designing and cloning of short hairpin RNA (shRNA) constructs for downregulating LAMP1

Two shRNAs against LAMP1 were designed. ShRNA cassettes were PCR amplified using specific primers. PCR products were digested with XhoI and EcoRI, gel purified and ligated into XhoI and EcoRI linearized, inducible lentiviral vector pTRIPz. The transduction of LAMP1 shRNAs along with a non-targeting shRNA (NT) in B16F10 cells was done exactly as was done for mutant LAMP1 construct described above. The cells stably selected and sorted for NT and LAMP1 shRNAs were treated with doxycycline for 96 h for the expression of shRNAs.

Results:

Objective I: To investigate the effect of translocating LAMP1 to cell surface in low metastatic cells, on different cellular properties important for lung metastasis.

Surface expression of polyLacNAc substituted β1,6 branched N-oligosaccharides and LAMP1 correlates with metastatic potential of melanoma cells.

To confirm the correlation of expression of polyLacNAc substituted β1,6 branched N-oligosaccharides and cell surface LAMP1 with metastasis, the melanoma cell lines were evaluated for their expression. It was conclusively shown that the expression of β1,6 branched N-oligosaccharides and polyLacNAc indeed correlates with metastatic potential. Moreover, although the total levels of LAMP1 remained unaltered, its expression on the cell surface correlated with their metastatic potential. To explore its role, LAMP1 was overexpressed on the surface of low metastatic B16F1cells.

Expression of mutant LAMP1 (Tyr^{386} to Ala^{386}) in B16F1 cells results in elevated expression of LAMP1 on the surface.

Mutation in $tyrosine^{386}$ of the cytoplasmic tail of LAMP1 directs it to cell surface instead of lysosomes. Such a mutant LAMP1 was generated and stable expression of this mutant LAMP1 by lentiviral infection of B16F1 cells resulted in significantly higher surface expression of LAMP1 in both the clones (C1 & C11) as compared to either uninfected (F1) or those infected with vector control virus (VC) and even B16F10 cells. The impact of increased surface expression of LAMP1 on the cellular properties, important from the point of view of metastasis, was explored.

Increased expression of LAMP1 on the surface of B16F1 cells results in significantly increased spreading and motility on fibronectin and matrigel.

Purified LAMP1 has an affinity for ECM and BM components [13]. LAMP1 overexpressed on the surface is possibly used as an alternate receptor for these components. The clones C1 and C11 indeed showed significantly higher spreading on both fibronectin (ECM component) and matrigel (reconstituted BM) as compared to VC. They also showed much higher motility on these substrates as measured by wound healing assays. This strongly indicated that the

increased surface LAMP1 may alter the cellular properties which might eventually be important for metastasis.

Increased expression of LAMP1 on the surface of B16F1 cells had no effect on their galectin-3 mediated spreading and motility.

LAMP1 is a known ligand for galectin-3. Surprisingly, the increased expression of LAMP1 on the cell surface had no effect on spreading of C1 & C11 clones on galectin-3 as compared to VC and was very similar to that seen on uncoated coverslips. Besides, motility of C1 & C11 clones was also almost similar to VC in presence of either BSA or immobilized galectin-3. The lack of any effect is possibly because of low levels of polyLacNAc substitutions, the major galectin-3 ligands.

Increased expression of LAMP1 on the surface increases neither galectin-3 binding to B16F1 cells nor their metastatic potential.

In spite of >20 fold increase in expression of LAMP1 on the surface of clones C1 and C11 as compared to even B16F10 cells, it did not result in any gain of their metastatic potential as compared to the parent B16F1 cells. The analysis of levels of cell surface β1,6 branched N-oligosaccharides and polyLacNAc revealed a marginal increase in their levels in C1 & C11 clones as compared to VC. However, the binding of galectin-3 to these cells remained largely unaltered. It was possibly because of low levels of polyLacNAc substituted β1,6 branched N-oligosaccharides on each LAMP1 molecule overexpressed on cell surface. Immunoprecipitation experiment indeed showed that β1,6 branched N-oligosaccharides and polyLacNAc on LAMP1 from VC and C1 cells were comparable which were much lower as compared to that present on LAMP1 from F10 cells. This suggests that carbohydrates on LAMP1 may play a crucial role in lung metastasis.

Objective II: To investigate the contribution of β1,6 branched N-oligosaccharides on LAMP1 in different cellular properties of metastatic cells.

Treatment of B16F1 and B16F10 cells with swainsonine results in decreased glycosylation and consequently decreased surface expression of LAMP1.

To determine whether polyLacNAc substituted β1,6 branched N-glycans have any role in surface translocation of LAMP1 per se, B16F1 and B16F10 cells were treated with swainsonine (SW), an inhibitor of complex-type N-glycans. The treatment decreased the levels of both β1,6 branched N-glycans as well as polyLacNAc in these cells. Surprisingly, the treatment resulted in decrease in cell surface expression of LAMP1 as well. Moreover, the total LAMP1 levels (membrane+cytosolic) remained unaltered indicating that inhibition of glycosylation had no effect on its stability.

The surface expression of mutant LAMP1 or the spreading of cells expressing them on fibronectin and matrigel remains unaffected by glycosylation inhibition.

The mutant LAMP1 expressed in B16F1 cells largely remains on the cell surface. These cells were treated with SW to investigate the effect of glycosylation inhibition on mutant LAMP1 surface expression and on spreading of cells on fibronectin and matrigel. Results indicated that swainsonine treatment neither had any effect on surface expression of mutant LAMP1 on these cells nor on their spreading on fibronectin and matrigel. This clearly indicated that the increased expression of LAMP1 (in B16F1 cells expressing mutant LAMP1) as well as spreading of these cells on ECM and BM components was independent of glycosylation.

Since increased expression of LAMP1 on the surface of B16F1 cells did not enhance their metastatic potential, it was futile to see the effect on the same after inhibition of glycosylation.

Objective III: To investigate the effect of downregulation of LAMP1 on the cellular properties of high metastatic murine melanoma cells.

Expression of LAMP1 shRNAs in B16F10 cells results in decreased overall as well as surface levels of LAMP1.

To further establish the role of carbohydrates on LAMP1 in lung metastasis of B16 melanoma cells, two different shRNAs specific for LAMP1 (Sh1 and Sh2) along with a non-targeting shRNA (NT) were cloned in an inducible lentiviral vector, pTRIPz. The lentiviral particles generated were transduced in B16F10 cells. After stable selection of these clones, it was found that on induction of shRNA expression by doxycycline treatment, there was significant reduction in total LAMP1 as well as cell surface LAMP1 in Sh1 and Sh2 but not in NT clone which showed similar levels to that of untransduced B16F10 cells. Moreover, Sh1 clone showed greater downregulation compared to Sh2.

Downregulation of LAMP1 in B16F10 cells does not significantly alter their spreading and motility on fibronectin and matrigel.

LAMP1 is known to bind to ECM and BM components. However, both the LAMP1 shRNA clones did not show any significant decrease in spreading on fibronectin and matrigel as compared to NT clone. They also did not show any significant alterations in motility on these components indicating that the decreased surface LAMP1 did not alter the properties of cells on fibronectin and matrigel.

Downregulation of LAMP1 in B16F10 cells results in decreased galectin-3 binding and significantly decreased spreading and motility on galectin-3.

Since LAMP1 is a major carrier of polyLacNAc and is known to interact with galectin-3 through its polyLacNAc, effect of LAMP1 downregulation on galectin-3 binding and galectin-3 mediated properties was investigated. Both the LAMP1 shRNA clones indeed showed decreased galectin-3 binding and significantly decreased spreading and motility on

galectin-3 as compared to NT clone. The results strongly indicated that the decreased surface LAMP1 may significantly alter the properties of cells on galectin-3 which might eventually also affect lung metastasis.

Downregulation of LAMP1 in B16F10 cells results in significantly decreased lung metastasis of these cells.

To investigate if modulation of the galectin-3 mediated properties has any effect on lung colonization, experimental metastasis assay was performed. The LAMP1 downregulated clones showed significantly decreased lung metastasis as compared to untransduced or NT transduced B16F10 cells in a doxycycline inducible manner. This conclusively established the role of LAMP1 and its association with galectin-3 through its polyLacNAc in mediating lung metastasis.

These results would be discussed at length in thesis.

Summary and Conclusions:

The present study demonstrates that although increasing surface expression of LAMP1 aids in mediating interactions with the ECM and BM components, it has no influence on melanoma metastasis to the lungs unless it carries high density of ligands (polyLacNAc) for galectin-3. This was conclusively proven when downregulating surface LAMP1 in high metastatic B16F10 cells significantly reduced their spreading and motility on galectin-3 as well as their lung metastatic ability.

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ABBREVIATIONS

AVP : Avidin HRPO

BLAST : Basic Local Alignment Search Tool

BM : Basement Membrane

BSA : Bovine Serum Albumin

cDNA : Complementary DNA

C/N ratio : Cytolasmic to nuclear area ratio

DAPI : Diamidino-2-phenylindole dihydrochloride

DEPC : Diethyl Pyrocarbonate

DMEM : Dulbecco's Modified Eagle's Medium

DMSO : Dimethyl Sulphoxide

ECL : Enhanced Chemiluminiscent

EDTA : Ethylene Diamine Tetra Acetate

ECM : Extracellular matrix

ER : Endoplasmic Reticulum

FITC : Fluorescein isothiocynate

FACS : Fluorescent Activated Cell Sorter

FBS : Fetal Bovine Serum

FN : Fibronectin

Gal3 : Galectin-3

GnT-V : N-acetylglucosaminyltransferase-V

HRPO : Horse Radish Peroxidase

kDa : Kilo Dalton

LAMPs : Lysosome Associated Membrane Proteins

LAMP1 : Lysosome Associated Membrane Protein-1

LAMP2 : Lysosome Associated Membrane Protein-2

LB : Luria Bertani

LEA : *Lycopersicon esculentum* Agglutinin

LPHA : Leucoagglutinin Phytohemagglutinin

Mat : Matrigel

MMP : Matrix Metalloproteinase

MutLAMP1 : Mutant LAMP1

NP-40 : Nonidet P-40

NT : Non-targeting shRNA

PAGE : Polyacrylamide Gel Electrophoresis

PBS : Phosphate Buffered Saline

PIPES : Piperazine-N,N′-bis (2-ethanesulfonic acid)

PMSF : Phenyl Methyl Sulfonyl Fluoride

PCR : Polymerase Chain Reaction

PolyLacNAc : Poly-N-acetyllactosamine

PVDF : Poly Vinylene DiFlouride

SA : Sialic Acids

SDS : Sodium Dodecyl Sulphate

shRNA : short hairpin RNA

SW : Swainsonine

TRITC : Tetramethyl Rhodamine Isothiocynate

TEMED : N, N, N', N',-Tetramethylethylenediamine

TTBS : Tween- Tris Buffered Saline

Un : Uncoated

wtLAMP1 : Wild-type LAMP1

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CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Cancer

Cancer is one of the most dreaded diseases and is the second major cause of mortality seen worldwide after heart diseases. Latest world cancer statistics shows that an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012 alone (GLOBOCAN 2012, IARC). There are more than 100 distinct types and subtypes of cancers. Cancer is known to arise due to uncontrolled proliferation of normal cells within the body due to defects in regulatory circuits that govern normal growth, signalling and homeostasis. A large body of evidence indicates that tumorigenesis in humans is a multistep process. For a normal cell to become cancerous, it must acquire six important characteristics, viz., sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [1]. Progress in the last decade has added two additional emerging hallmarks to this list—reprogramming of energy metabolism and evading immune destruction [2]. To understand the etiology of cancer, deciphering the cause and consequence of acquiring each of the eight hallmarks of cancer becomes a necessity.

1. **Sustained proliferative signalling:** The most essential trait of cancer cells is their sustained ability to proliferate. Cancer cells deregulate the production and release of growth-promoting signals that control the cell growth and division cycle, thereby disrupting the homeostasis of cell number and maintenance of normal tissue architecture and function. Cancer cells can acquire sustained proliferative signalling by either producing growth factor ligands themselves or send signals to tumor-associated stromal cells to release growth factors [3]. Alternatively, they can enhance receptor signalling by

elevating the levels of receptor proteins at the cell surface. For example, receptors like Epidermal Growth Factor Receptor (EGFR) and HER2 are found to be overexpressed in cancers such as breast, gastric and oesophageal carcinomas [4]. Cancer cells can also enhance receptor signalling by structurally altering the signaling molecules to facilitate ligand-independent signalling. For instance, about 40% of human melanomas contain activating mutations affecting the structure of the B-Raf protein, resulting in constitutive signaling through the Raf to mitogen activated protein (MAP)-kinase pathway [5].

2. **Evading growth suppressors:** For sustained proliferation, cancer cells also need to overcome the anti-proliferative signals that act as major intracellular barriers to cellular proliferation. Tumor cells evade anti-proliferative signals by downregulating the receptors through which these signals transmit. For instance, tumor cells attenuate Transforming Growth Factor-β (TGF-β) signaling, an important anti-proliferative signal, by downregulating TGF-β receptors or expressing mutant or dysfunctional receptors [6, 7].

3. **Resisting cell death:** Cancer cells, for successful proliferation, also must avoid cell death mechanisms. Apoptosis or programmed cell death is one of the major hurdles to development of cancer. Evidences indicate the attenuation of apoptosis in tumors that show resistance to therapy [8]. Apoptosis is mainly regulated by B-cell lymphoma 2 (Bcl-2) family of proteins which has both pro- and anti-apoptotic members. Tumor cells evade apoptosis by either downregulating the expression of death receptor proteins such as CD95 which shows reduced expression in neuroblastoma and lymphoma, or by overexpression of proteins which are inhibitors of apoptosis such as survivin which is found to be overexpressed in several cancers [9].

4. **Enabling replicative immortality:** Cancer cells require unlimited replicative potential for their growth. Normal cells are able to pass through only a limited number of successive cell-growth-and-division cycles mainly due to a loss of 50-100 bp telomeric DNA from ends of each chromosome after every cycle of cell division. Cancer cells overcome this problem of replicative senescence by overexpressing the enzyme, telomerase which prevents the loss of telomeric DNA by maintaining the ends of chromosomes [10].

5. **Inducing angiogenesis:** In normal cells, the process of angiogenesis is regulated by the balance between angiogenesis inducers and their countervailing inhibitors. However, in tumor cells, the balance is shifted towards angiogenic inducers [11]. At the primary site, tumors do not grow beyond the size of 2 mm in diameter due to lack of nutrients and oxygen. Hypoxic condition within the tumor induces the expression of hypoxia inducible factor (HIF) which, in turn, initiates the process of angiogenesis by regulating the expression of molecules like Vascular Endothelial Growth Factor (VEGF) which are involved in endothelial cell proliferation [12].

6. **Activating invasion and metastasis:** Epithelial-Mesenchymal Transition (EMT) has been widely implicated as a means by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis and to disseminate to distant organs i.e., metastasize. Cancer cells that attain these properties of invasion and metastasis are called malignant and it is the major cause of mortality in cancer patients. More than 90% of the patients die because of the invasive and metastatic cancers rather than the primary tumors. Although important, this is the least understood aspect of tumor biology because of its inherent complexity and multi-step nature [13].

7. **Reprogramming of energy metabolism:** Cancer cells not only show deregulated control of proliferation, but a corresponding adjustment of energy metabolism to stimulate cell growth and division. Under aerobic conditions, normal cells first undergo glycolysis and then Kreb's cycle and under anaerobic conditions, they mainly undergo glycolysis. However, cancer cells have been found to reprogram their metabolism in such a way that even under aerobic conditions, they chiefly undergo glycolysis leading to a state termed "aerobic glycolysis" (also known as Warburg effect) [14]. They also show increased expression of glucose transporters such as GLUT1 which facilitate the import of glucose into cytoplasm [15]. The glycolytic pathway intermediates are utilized by the tumor cells for the generation of nucleosides and amino acids which, in turn, help in biosynthesis of macromolecules and organelles required for assembling new cancer cells [16].

8. **Evading immune destruction:** Immune system plays a very important role in resisting or eradicating formation and progression of incipient cancers. Its role in tumor prevention is substantiated by striking increase of cancers in immunodeficient individuals [17]. Cancer cells evade immune system by disabling components of immune system. For example, they inactivate infiltrating Cytotoxic T Lymphocytes (CTLs) and Natural Killer (NK) cells by secreting TGF-β or other immuno-suppressive factors [18]. Besides, they can also recruit inflammatory cells that are immuno-suppressive such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) which are known to suppress the actions of cytotoxic lymphocytes [19, 20].

All these hallmark properties are mostly acquired either directly or indirectly through changes in the genomes of cancer cells. Alterations in two classes of genes, namely oncogenes and

tumor suppressor genes have been shown to be the major underlying factors which help tumor cells acquire these characteristics [21]. Ras has been found to be one of the most predominant oncogene involved in tumor progression. In about 25% of human tumors, Ras proteins are present in structurally altered forms that enable them to release a flux of mitogenic signals into cells, without ongoing stimulation by their normal upstream regulators. In human colon carcinomas, about half of the tumors bear mutant *ras* oncogenes [22]. On the other hand, p53 is the most common tumor suppressor gene that has been found to be mutated in most of the cancers. Mutation in p53, a mediator of apoptosis, results in impairment of apoptosis elicitation and culminates in uncontrolled growth of cells [23].

The growth of a tumor cell is termed "benign" if the rapidly growing cells remain confined to the primary site of origin. These tumors can easily be treated by surgery. However, when tumor cells acquire unique characteristics by virtue of which they invade the surrounding tissues and spread to near and distant sites, they are termed "malignant" and the process of their dissemination from one site to another is termed "metastasis" [24].

1.2 Metastasis

Metastasis, the spread of tumor cells from the primary tumor site to other non-contiguous sites, is the major cause of cancer-related deaths [25]. Approximately, 90% of deaths arising due to cancer can be ascribed to the process of metastasis. It is a complex, multi-step process involving detachment from the primary site, invading the surrounding normal tissues, intravasating into the blood vessels, surviving in circulation and finally homing into a secondary site (**Illustration 1**) [13]. Improved understanding of the mechanisms involved in each step of metastasis is warranted to unravel novel drug targets and prognostic markers.

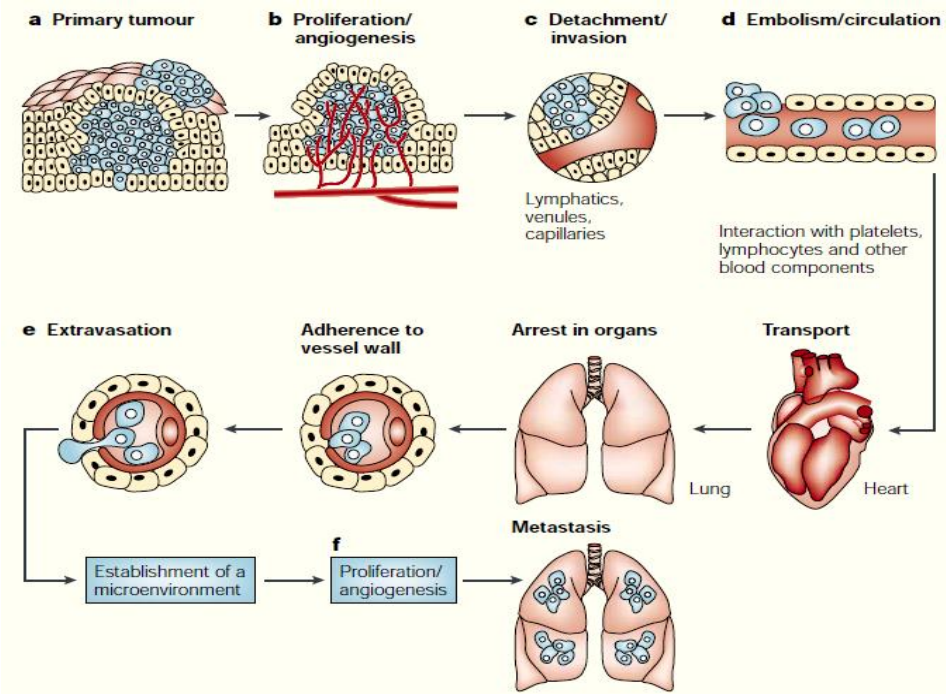

Illustration 1: The Metastatic Cascade – adapted and modified from [26].

1.2.1 Detachment of cells from the primary tumor

Cell-cell and cell-Extracellular Matrix (ECM) interactions play a pivotal role in maintaining the normal cellular architecture and in turn, the efficient functioning of a tissue. Alterations in these interactions are essential for detachment of the cells from their primary tumor tissue. A large body of evidence suggests that such alterations occur due to a phenomenon known as Epithelial-Mesenchymal Transition (EMT). It involves several mutations to transform an epithelial cell to attain a mesenchymal morphology resulting in acquisition of properties such as enhanced migration and invasiveness [27, 28]. Although the molecular mechanisms underlying EMT remain elusive, alterations in levels of several cell-adhesion molecules including cadherins, the hyaluronate receptor - CD44, immunoglobulin-like cell-adhesion molecules (Ig-CAMs) and integrins have been shown to promote EMT. These alterations help in modulation of both homotypic cell-cell interactions (e.g., by reduced expression of E-

cadherins) as well as heterotypic cell-ECM interactions (e.g., by upregulation of N-cadherins and α6β4 integrins) [29, 30].

1.2.2 Degradation and invasion of surrounding normal tissues

After successful detachment from the primary site, cancer cells need to create space for their movement. Cells of the epithelia, endothelia and mesothelia are normally separated from the surrounding interstitial stroma by 50-100 nm thin structure called Basement Membrane (BM). Tumor cells degrade the surrounding ECM and Basement Membrane (BM) by the action of several hydrolytic enzymes or proteases. There are several classes of proteases which can be secreted by the tumor cells to degrade ECM/BM. It includes Plasminogen activators - urokinase plasminogen activator (uPA) and plasmin, Adamalysin-related membrane proteinases that contain disintegrin and metalloproteinase domains (ADAMs), glycosidases, lysosomal cysteine cathepsins and matrix metalloproteinase (MMP) family of secreted and membrane proteinases. The ECM/BM degradation by these proteases promotes metastatic spread of primary tumor cells as it results in release of growth factors which stimulate growth of new blood vessels by angiogenesis [31].

1.2.3 Tumor cell motility

Metastasis essentially involves the movement of cells from one site to another. Cell motility is thus the next and one of the most important steps in metastasis. It is a dynamic multistep process which involves leading edge protrusion, turnover of focal adhesions, and generation of tractional forces, tail retraction and final detachment of the cell. The migrating cell is thus highly polarized with complex regulatory pathways that integrate the above processes. Directional motility can be provided by certain chemo-attractants such as degraded ECM components (collagen peptides), growth factors, and motility factors secreted by the tumor

cells. This type of motility is termed as **chemotaxis**. Motility provided by adhesion to altered ECM/BM and mediated largely though integrins is termed **haptotaxis.** Binding of integrins to the immobilized ECM leads to their activation and localization at the leading edge of the cell. Thus, the integrin heterodimers constitute a fundamental requirement for cells to acquire the traction necessary for movement [32]. Intermediate or rather **optimum levels of adhesion** allow traction at the cell front while releasing contacts at the rear end, resulting in net forward movement of the cell.

1.2.4 Intravasation

The process by which the metastatic tumor cells penetrate the blood vessels to gain entry into circulation is called intravasation. Intravasation occurs as metastatic cells often secrete various degradative enzymes to disrupt the vascular BM which acts as a major barrier for the entry of cells into the circulation. It is also facilitated by the acquisition of angiogenic property by the tumor cells. **Angiogenesis** is the process by which the tumor cells induce the growth of blood vessels in their vicinity. New blood vessels in tumors evolve by sprouting outgrowths from pre-existing vessels or by de novo recruitment of rare, circulating vascular cell precursors. This leads to an increased expression of angiogenic promoters like Vascular Endothelial Growth Factors (VEGF) and Angiopoietins in comparison to the angiogenesis inhibitors belonging to the statin family [33-35]. The newly formed blood vessels around tumors however, are poorly formed as they lack BM and the endothelial lining is often discontinuous. This leads to leaky endothelium in intratumoral blood vessels as opposed to tight endothelial junctions in normal vessels. This leakiness further promotes intravasation into circulation [36].

1.2.5 Survival in circulation

After the intravasation of tumor cells into the blood stream, they are prone to events which threaten their survival such as the hemodynamic shear forces, toxicity induced by high levels of oxygen and immune-mediated killing. Tumor cells overcome shear by either travelling as clumps in the center of blood vessels or by forming clumps with platelets called emboli which are recognized as self, thereby evading the immune system and facilitating hematogenous arrest into microcirculation [37]. Interactions between cell surface carbohydrates in the form of Lewis antigens on tumor cells and P-selectins on platelets have been implicated in tumor cell embolization [38]. In the blood circulation, tumor cells are also prone to anoikis, a form of apoptosis induced in the absence of adhesion to substratum. Resistance to anoikis by increasing expression of tyrosine kinase receptor (TrkB) and its kinase activity, is another strategy adopted by tumor cells to survive in circulation [39].

1.2.6 Extravasation and organ homing

After strategically surviving the harsh environment in the circulation, tumor cells get arrested at a distant site and extravasate into the surrounding tissue. By utilizing the blood and lymphatic vasculature, the tumor cells may metastasize to organs in the anatomical vicinity of the primary site or may traverse to distant sites and colonize specific organs, which resulted into two different theories explaining organ metastasis.

1. **Mechanical/Anatomical mode of metastasis:** This theory of metastatic dissemination was put forth by **James Ewing** in 1928, according to which, preferential colonization of organs could be primarily attributed to the route of the blood and lymphatic flow from the primary site [40]. He suggested that maximum

number of circulating tumor cells become mechanically trapped in the vascular bed of first organ encountered. Liver metastasis of colon carcinoma could be explained on this basis as liver is the first organ encountered through portal circulation and receives maximum number of colon cancer cells via portal vein from colon that drains into liver. Similarly, prostate cancers are also believed to colonize vertebral bones through vertebral venous plexus of spine [41]. Though regional metastasis could easily be explained by Ewing's hypothesis, it was unable to explain the distant metastasis of specific tumors.

2. **Organ-specific metastasis:** Certain tumors are seen to metastasize to very specific distant organs. For example, choroidal melanomas bypass several organs and colonize the liver selectively; prostrate and thyroid cancers metastasize to bone; breast cancer metastasizes specifically to lung, liver, bone and brain [24, 42]. The pre-disposition or bias to spread towards certain organs is known as "organ specific metastasis". Several clinical and experimental studies have clearly demonstrated occurrence of **organ specific metastasis.** In 1889, Dr. Stephen Paget analyzed 735 autopsy records of women with fatal breast cancer and was struck by his observations. He observed discrepancy between the relative blood supply and frequency of metastasis in some organs, i.e., liver metastasis occurred at far more frequency than any other organ, such as spleen, which has the same exposure to cancer cells because of similar blood flow. Hence, he suggested that formation of secondary tumors is not a matter of 'chance'. Based on his observations, he put forth the **seed and soil hypothesis** which states that: **Certain tumor cells (the 'seed') have specific affinity for the milieu of certain organs (the fertile 'soil') and metastasis forms only when seed and soil are compatible** [43].

1.2.7 Evidences supporting organ-specific metastasis

Clinical evidence supporting organ specific metastasis came from experiment by Tarin et al in 1984. Ovarian cancer patients suffering from late-stage disease with malignant ascites were fitted with peritoneo-venous shunts to relieve pain. These shunts directed ascitic fluid containing millions of metastatic cells back into blood circulation. Yet, patients did not develop disseminated metastases, sometimes even after two years of continuous shunting. Furthermore, even when metastases were observed after autopsy, they were frequently indolent growths [44]. The concept of organ specific metastasis of tumor cells was further strengthened by the works of Hart, Nicolson and Isaiah Fidler who made significant contributions not only towards the occurrence but also towards the understanding of mechanisms of organ specific metastasis. They demonstrated that the intravenous injection of different cell lines in mice showed diverse and unique patterns of metastatic spread of each cell line. For example, RAW-117 cells showed predisposition towards the liver, while reticulum cell sarcoma and S91 Cloudman melanoma cells metastasized to the spleen and lung, respectively [42]. Similarly, Fidler convincingly proved the organ specific metastasis of melanoma cells to lungs, by injecting parabiotic pairs of mice having a common circulation. Even though only one mouse received the injection, tumor cells displayed specific adherence and metastasis to the lungs of both the mice [45]. He was also successful in the enrichment of highly metastatic cells by repeatedly allowing low metastatic cells to adhere to their target tissue [46]. Their studies thus convincingly proved that adhesive interactions occurring between molecules on the tumor cell surface and counter receptors on the target organ, and tissue microenvironment of the host are important determinants for organ specific metastasis.

1.2.8 Factors influencing organ-specific metastasis

1.2.8.1 Adhesive interactions between tumor cell surface and target organ

Adhesive interactions of tumor cells with vascular endothelium, the sub-endothelial basement membrane, the parenchymal cells as well as the ECM of the target organ encountered during extravasation, has been found to profoundly affect the metastatic outcome. Involvement of organ specific endothelium in metastasis can be understood by the fact that vascular beds of different organs show marked heterogeneity in their molecular makeup and cell surface determinants [47, 48]. Moreover, metastatic cells have been repeatedly shown to adhere selectively to capillary endothelial cells from preferential secondary organs. The presence of specific 'homing' receptors on endothelial cells, which could either be constitutively expressed or induced in response to stimuli, have also been shown to influence metastasis. For example, molecules such as Lung Endothelial Cell Adhesion Molecule-1 (Lu-ECAM-1) and Dipeptidyl peptidase IV (DPP IV) are specifically expressed in venular and capillary endothelia of lungs respectively, and are reported to facilitate adhesion and metastasis specifically to lungs [48, 49]. Expression of adhesion molecules on the endothelium such as E-Selectin which recognizes sialyl Lewis X carbohydrate antigens or galectin-3 which recognizes T/Tn antigens and polyLacNAc expressed on the tumor cells, may also aid in organ-specific homing [50-52]. After initial rolling, arrest and spreading of tumor cells on endothelium, they cause the endothelium to retract, thus coming in contact with vascular BM after degrading which they finally encounter the organ parenchyma.

Besides endothelium, tumor cells can also bind to components of exposed sub-endothelial BM and organ parenchymal cells. For instance, HT1080 cells have been shown to get arrested in the pulmonary vasculature through interaction of their $\alpha 3\beta 1$ integrin with Laminin-5 in the exposed BM [53]. Similarly, interactions between Kupffer cells, which form

an integral part of the liver parenchyma, and colorectal carcinoma cells has been found to facilitate liver specific metastasis of these cells [54]. Besides, specific factors present in the ECM of the target organ may also determine organ specific metastasis. For example, asialoprotein found in the bone-ECM has been shown to facilitate adhesion of breast cancer cells to the bone [55].

1.2.8.2 Appropriate microenvironment in the target organ

A congenial microenvironment in the target organ, which supports growth and invasion of tumor cells, is also an important determinant for successful metastasis. Favourable 'soil' of a specific organ is often found to be enriched with growth factors which exclusively support propagation of specific types of tumor cells. For instance, Transforming Growth Factor alpha (TGF-α), a key mediator of liver regeneration, appears to be responsible for liver metastasis of colon cancer cells expressing elevated levels of functional receptors for Epidermal growth factor [56]. Furthermore, the invasive ability of human colon cancer cells has also been shown to be directly influenced by organ-specific fibroblasts present in colon and lung [57]. Similarly, mammary carcinoma cells display greater growth response to lung conditioned media as compared to other organs, which also correlates with their metastatic potential [42].

1.2.8.3 Chemokines and their receptors

Recent studies have shown that low molecular weight cytokines called **chemokines** and their interactions with their specific **receptors** could be a possible determinant of organ specific metastasis [58]. Typical sites of metastatic colonization of breast cancer such as bone marrow, lung, liver and lymph nodes constitutively express CXCL12, a chemokine whose receptors, CXCL4, are expressed on breast tumor cell surface [59]. Another study documents

melanoma cases that express CCR9 and shows that these tumor cells metastasize specifically to the small intestine where CCL25 is expressed [60].

Irrespective of whether organ specific colonization is due to adhesive interactions between the tumor cells and the target organ, the growth microenvironment within the organ, or chemokine gradients, **tumor cell surface molecules** play an important role in successful establishment of metastasis. Tumors indeed show marked alterations in the expression of several classes of cell surface molecules which aid them in negotiating different steps of metastasis.

1.2.9 Cell surface modifications associated with metastasis

Analysis of the molecules on tumor cell surfaces has revealed that they are indeed different from their normal counterparts, and play a crucial role in regulating the overall process of metastasis. Direct evidence for this came from the work of Poste and Nicholson who fused plasma membrane vesicles derived from highly metastatic cells to low metastatic cells. As a consequence, there was a marked increase in the metastatic properties of these vesicle-modified low metastatic cells [61]. Surface of metastatic tumor cells show alterations in expression of several membrane adhesive molecules involved in cell-cell and cell-ECM adhesion [62, 63]. For instance, reduced expression of E-cadherins on tumor cells aids detachment by weakening cell-cell adhesion. Similarly, reduced expression of Neural Cell Adhesion Molecule (NCAM), correlates with poor prognosis for several cancers due to defects in cell-ECM adhesion [64]. Altered expression of several integrins has also been found to correlate with metastasis. For instance, expression of the integrins $\alpha v\beta 3$, $\alpha 5\beta 1$, $\alpha 6\beta 4$, $\alpha 4\beta 1$ and $\alpha v\beta 6$ is correlated with disease progression and metastasis in various tumor types

such as melanoma, breast, prostrate, pancreatic and colon cancers [65]. Altered expression of growth factor receptors such as EGFR, VEGFR, etc. also serve as cardinal signs of metastasis [56].

Another important hallmark of metastatic tumors is altered cell **surface glycosylation**. Glycosylation is one of the most abundant post-translational modifications, and nearly 50% of all known proteins and more than 80% of the cell surface proteins in eukaryotes are glycosylated. Many of the cell surface proteins like the adhesive proteins and growth factor receptors are glycosylated, which further modulates the functioning of the receptors [66].

1.2.10 Aberrant glycosylation and metastasis

Aberrant glycosylation expressed in glycolipids, proteoglycans and glycoproteins in tumor cells has been implicated as an essential mechanism in defining the stage, direction, and fate of tumor progression. Glycosylation reactions are catalyzed by the action of glycosyltransferases, which add sugar chains to various complex carbohydrates such as **glycolipids, proteoglycans** and **glycoproteins**.

1.2.10.1 Glycolipids in cancer metastasis

Glycolipids consist of an oligosaccharide chain attached to a ceramide moiety by a glycosidic linkage. Glycolipids can be divided into seven major families based on their oligosaccharide structures which include the ganglio-, globo-, lacto-, neolacto-, isoglobo-, mollu-, and arthro-series [67]. Aberrant expression of glycolipids such as those belonging to lacto series, Lewis a (Le^a), Lewis b (Le^b) and Lewis x (Le^x) antigen have been reported in adenocarcinomas. Similarly, increased expression of gangliosides such as GD_3, GM_3 have been observed in

melanomas, while GD_2 have been shown to accumulate on neuroblastoma cells [68]. Glycolipids also form an integral part of membrane microdomains called **lipid rafts** which modulate transmembrane signaling related to growth, motility, invasion and metastasis. Raft associated receptors including integrins, protein kinase C, tyrosine kinase-linked growth factor receptors and G-protein-coupled receptors affecting protein kinase A, are directly modulated by gangliosides and sphingolipids [69].

1.2.10.2 Proteoglycans in cancer metastasis

Proteoglycans consist of a protein core to which one or more glycosaminoglycans (GAGs) are covalently attached. Depending on their glycosaminoglycan chains, proteoglycans can be categorized into five different types, viz., chondroitin suphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparan sulphate (HS) and hyaluronic acid (HA). Proteoglycans can be found both in the ECM as well as on the cell membrane. In the ECM, they form hydrated gels and thus provide stability to the ECM and also serve as a depot for several growth factors. On cell membrane, they act as co-receptors for integrins and proteases and participate in growth factor signaling. The expression of several proteoglycans has been found to be altered during cancer progression [70]. For example, expressions of haparan sulphate proteoglycans such as glypican-1 and syndecan-1 have been found to be elevated in cancers such as gliomas and breast cancer [71, 72].

1.2.10.3 Glycoproteins in cancer metastasis

Glycoproteins are a group of glycoconjugates which carry oligosaccharide chains covalently attached to proteins. Carbohydrates attached to proteins can be classified into three major groups: GPI- linked proteins, O-glycans, and N-glycans [67].

In **GPI linked proteins**, the carboxy terminal of the protein is attached to glycosylphosphatidylinositol (GPI) anchor, causing the protein to be bound to the non cytoplasmic surface of the membrane, solely by this anchor [67]. Proteases (MT-MMPs) and protease receptors (uPAR) which are directly implicated in cancer cell invasion belong to GPI anchored proteins.

O-linked glycoprotein biosynthesis occurs by the covalent addition of N-acetyl galactosamine (GalNAc) to the –OH group of serine or threonine residues. This is followed by sequential addition of other oligosaccharides directly onto the polypeptide chain, mediated by specific glycosyl transferases. Among the O-glycosylated proteins, mucins are major carriers of altered glycosylation. Overexpression of mucins such as MUC-1 or episialin has been widely reported in carcinomas. Mucins often mask the expression of antigenic peptides by MHC molecules altering host-immune response towards tumor [67].

Asparagine (Asn) or N-linked sugar chains are synthesized as a co-translational event in the endoplasmic reticulum (ER) and Golgi apparatus. The pathway involves sequential addition of oligosaccharides to the amide group ($-NH_2$) of the asparagine residue having a target sequence Asn-X-Ser/Thr, where X can be any amino acid residue except Pro or Asp.

Biosynthesis of N-glycans can be divided into four distinct phases [67], each associated with different compartments of the secretory pathway:

1. Transfer of fourteen sugars Glc3Man9GlcNAc2 (where Glc is glucose, Man is mannose and GlcNAc is N-acetylglucosamine) en bloc from a Dolichol linked, lipid like precursor donor to nascent Asn-residues of glycoproteins in the lumen of the rough ER;
2. Glycosidase-mediated trimming in the rough ER and Golgi;

3. Substitution by GlcNAc-Transferases (GnTs) in the median-Golgi; and
4. Elongation by terminal oligosaccharides in the trans-Golgi network to complete the glycan structures.

At the end of the complex pathway in the ER and Golgi, three different types of N-oligosaccharides, namely, oligomannose, hybrid, or complex-type having a common pentasaccharide core (Man3GlcNAc2) may be formed. Formation of these structures depends on presence of remodeling enzymes, availability of oligosaccharide substrates and retention time of the protein in the ER and golgi. While the oligomannose structure (Man8GlcNAc2) is formed as an intermediate product before the trimming process, the hybrid structure is formed if a bisecting GlcNAc residue is added by GnT-III enzyme, before the removal of 2 Man residues. The complex-type N-glycans may be bi-, tri- or tetra-antennary and depending on the sequential action of N-acetyl glucosaminyl transferases (GnT I-V), each of the antennae of the complex N-glycans may be further linked with terminal GlcNAc residues, galactose (Gal), followed by the addition of sialic acid. Some chains may also terminate with repeats of Gal and GlcNAc, termed as poly-N-acetyllactosamine which may bear additional sugars like sialic acid and fucose. These numerous terminal substitutions on complex-type N-glycans provide tremendous heterogeneity to oligosaccharides [73].

While the bi-antennary structures are commonly seen on normal cells, the tri- and tetra-antennary complex-type N-glycans are characteristic of highly metastatic cells. N-acetyl glucosaminyltransferase-V (GnT-V) is the key enzyme involved in the formation of highly branched N-oligosaccharides. It is a trans Golgi enzyme encoded by the mannoside acetylglucosaminyltransferase 5 (Mgat5) gene, which adds GlcNAc to the mannose of the trimannosyl core in a β1,6 linkage [74]. The plant lectin-Leuco Phyto Heam agglutinin lectin

(LPHA) binds specifically to the β1,6 branch of tri- and tetra-antennary N-glycans, and is hence used as a probe for the analysis of the β1,6 branched N-glycans (**Illustration 2**) [75, 76]. The altered expression of β1,6 branched N-linked oligosaccharides is a prominent characteristic of the metastatic cells [77, 78].

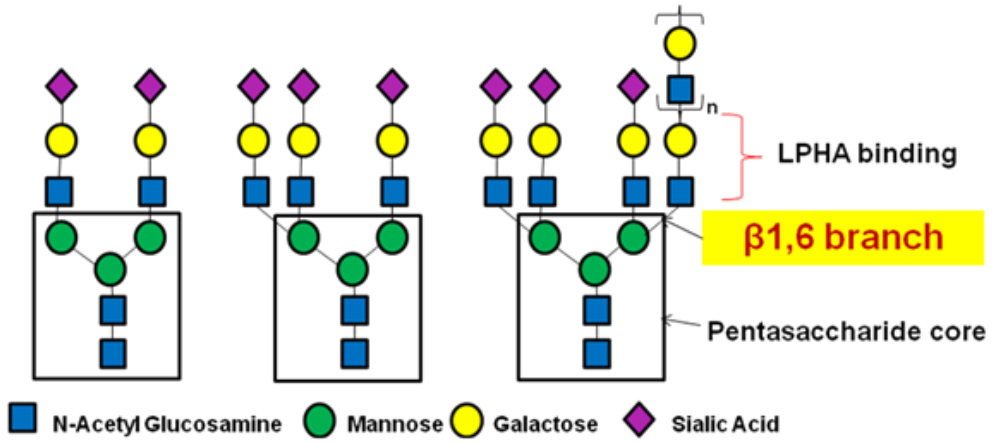

Illustration 2: Branching of N-linked oligosaccharides in normal and metastatic cells. Normal cells have a bi-antennary structure and invasive and metastatic cells show tri- or tetra-antennary structures, where addition of β1,6 branch is the rate-limiting step. β1,6 branch is specifically recognized by the lectin leuco-phytohaemagglutinin (LPHA).

1.3 β1,6 branched N-oligosaccharides and Cancer

The importance of increased expression of the β1,6 branch in metastatic progression has emerged from several clinical evidences seen in human tumors. For instance, increased expression of these oligosaccharides has been found to correlate with disease progression and poor prognosis in several metastatic human tumors such as liver, colorectal, breast, endometrium, melanoma and highly invasive gliomas as compared with normal and benign lesions, by LPHA immunohistochemical staining [79-84].

Clinical observations were also supported by enormous experimental evidence using various cell lines and animal models, which has firmly established the association of the β1,6 branch

with metastasis. Increased expression of the β1,6 branch has been found to correlate with metastatic potential of several human and murine tumor cell lines [77, 85-87]. Transfection of GnT-V cDNA (to increase β1,6 branch) into mouse mammary tumor cell lines significantly enhanced their metastatic potential [87]. Moreover, transformation of non-metastatic cells with known oncogenes such as T24 H-Ras, V-K-ras, or tyrosine kinase oncogene v-fps were found to induce increased GnT-V activity as well as metastatic potential [88]. Swainsonine, an inhibitor of complex-type N-glycan processing, was found to inhibit metastasis of highly metastatic B16F10 melanoma to lungs [86]. GnT-III uses the same substrates as GnT-V, but catalyzes the addition of a bisecting GlcNAc to core mannose preventing GnT-V from adding the β1,6 branch. Highly metastatic B16 melanoma cells when transfected with GnT-III gene, showed suppressed lung metastasis [89]**.** Further, glycosylation mutants of metastatic MDAY-D2 cell lines, deficient in GnT-V activity, showed loss of metastatic potential, although they retained their tumorigenic potential [77]. Recent studies using anti-sense and si-RNA to GnT-V have also confirmed that loss of β1,6 branch leads to a significant reduction in invasive and metastatic potential of tumor cells [90-92]. The significance of β1,6 branch in metastasis has also been proved using Mgat5 (gene coding for GnT-V enzyme) knock-out mice. Mammary tumor growth and metastases induced by the polyomavirus middle T oncogene was considerably less in Mgat5 (-/-) mice as compared to Mgat5 (+/+) counterparts [74].

All these evidences, therefore, unequivocally demonstrate that β1,6 branched N-oligosaccharides, the enzymatic product of GnT-V, plays a crucial role in facilitating metastatic progression. The increased expression of β1,6 branched N-glycan structures appears to modulate metastasis by participating in two major processes, viz., invasion and organ specific metastasis.

1.3.1 Association of β1,6 branched N-glycans with invasive phenotype

A large body of evidence indicates a strong association between expression of β1,6 branched N-oligosaccharides with invasive normal cells as well as malignant cancer cells that are highly invasive. They are often expressed by normal cells involved in invasive functions such as trophoblasts during embryogenesis and endothelial cells during angiogenesis [93, 94]. In addition, human tumors such as invasive glioma cells and invading edges of esophageal carcinoma exhibit significantly increased expression of these oligosaccharides [84, 95]. Although a clear association between β1,6 branching and invasive potential of cancer cells has been established, the underlying mechanisms still remain elusive. Among the emerging mechanisms, they appear to augment invasion by modulating matrix lysis, for instance, by transcriptional repression of TIMPs- the tissue inhibitors of matrix–metallo proteases [96]. These oligosaccharides have also been shown to modulate the adhesive and motile characteristics of the cells [90].

1.3.2 Association of β1,6 branched N-glycans with organ-specific metastasis

Another important finding is that majority of the metastatic cell lines expressing β1,6 branched N-oligosaccharides primarily metastasize either to the liver or to the lungs**.** For instance, the murine lympho-reticular cell line MDAY-D2 overexpressing β1,6 branched N-glycans preferentially metastasize to liver as compared to other organs [97]. In contrast, oncogenic Ras mediated increase in expression of β1,6 branched N-glycans on metastatic mouse mammary carcinoma cell line, SP-1, and immortalized cell lines NIH3T3 and Rat-1 fibroblasts leads to both, acquisition of metastatic phenotype and lung specific colonization [88]. Moreover, the B16 murine melanoma variants carrying β1,6 branched N-oligosaccharides also specifically metastasize to lungs [86].

1.3.3 Mechanisms by which β1,6 branched N-glycans mediate organ specific metastasis

The role of β1,6 branched N-linked oligosaccharides in organ-specific metastasis is speculated for two main reasons.

1.3.3.1 They could provide several novel ligands for the endogenous lectins

The β1,6 branch serves as the most preferred site for further substitutions, resulting in significant heterogeneity. The enzymes in the glycosylation pathway may add terminal sugars such as fucose, sialic acids, poly-N-acetyllactosamine (polyLacNAc), and others which may serve as ligands for endogenous lectins such as selectins, siglecs and galectins, respectively (**Illustration 3**) [98]. For instance, the addition of **fucose** in α1,3 and α1,4 linkages to the terminal GlcNAc residues of N-glycans leads to the formation of a related set of carbohydrate structures called as Lewis antigens. Lewis antigens, in turn, may facilitate adhesive interactions by binding to receptors of the selectin family [67]. Addition of sialic acids to fucosylated Lewis antigens leads to the formation of **Sialyl-Lewis antigens** on the tumor cells which could act as ligands for endogenous lectins such as E-selectin expressed on the vascular endothelium [99]. Lewis and Sialyl Lewis antigens are often over-expressed in human colorectal adenocarcinomas and have been shown to mediate attachment of colon tumor cells to selectins present in liver [100]. In addition, the terminal **sialic acids** may be recognized by the siglec family of endogenous lectins. It is speculated that the carcinoma cells having high levels of α2,3 and α2,6 linked sialic acids bind to vascular siglecs on target organ during metastasis [101]. Similarly, repeating units of N-acetylglucosamine and galactose termed as poly-N-acetyllactosamine (**polyLacNAc**) are also added preferentially on the β1,6 branch [102]**.** PolyLacNAc have been found to serve as high affinity ligands for the endogenous lectins called galectins [51, 103]. Tri- and tetra-antennary N-Glycans carrying polyLacNAc bind to galectins and form molecular lattices which oppose endocytosis of the

glycoproteins [104-106]. Moreover, polyLacNAc on melanoma cells have been shown to mediate attachment of these cells to galectin-3 present on lung vascular endothelium [51].

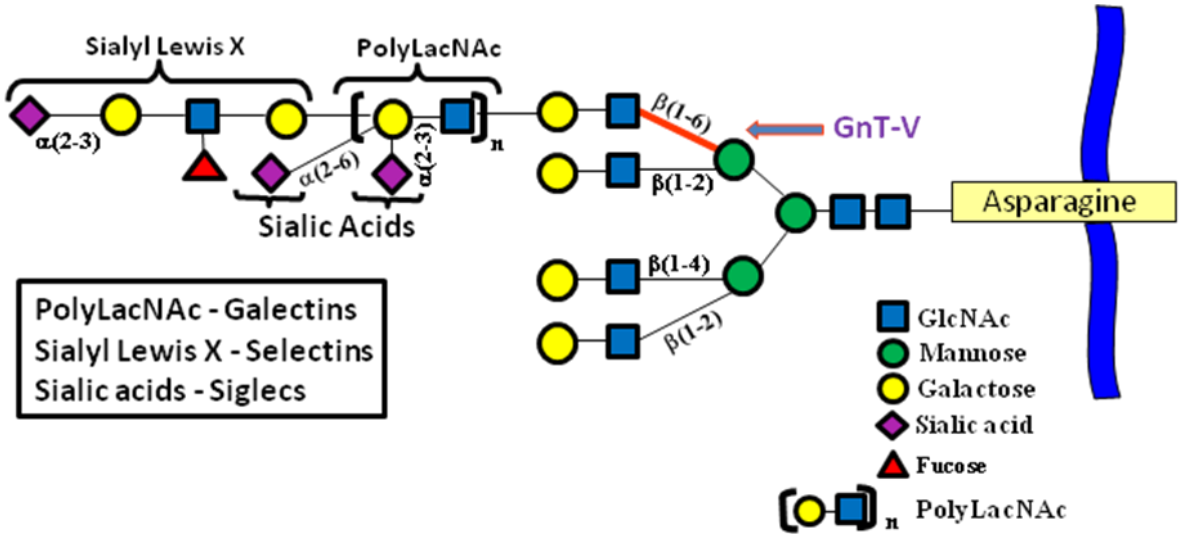

Illustration 3: Different substitutions on β1,6 branch recognized by different endogenous lectins. polyLacNAc substitutions on β1,6 branch are recognized by Galectins, sialyl Lewis antigens are recognized by Selectins and sialic acid substitutions are recognized by Siglecs.

1.3.3.2 They may alter the structural and functional characteristics of proteins that carry these oligosaccharides

Larger substitutions on these oligosaccharides may alter structural and functional characteristics of proteins that carry them. Several proteins which are important from metastasis point of view, for example cadherins, integrins, CD44, EGF-R (epidermal growth factor receptor), ECM components like laminin, proteolytic enzymes like matriptase and Lysosome associated membrane proteins (LAMPs) are known to carry these oligosaccharides [85, 98, 107-110]. It is possible that some of these modifications aid organ-specific metastasis by altering functional properties of these proteins required for cellular invasion and

metastasis. Reduced β1,6 branching of E-cadherin on melanoma cells by GnT-III transfection has been shown to suppress metastasis by increasing cell-cell adhesion [107]. Heterodimerization of the α5 and β1 subunits of integrins and their binding to fibronectin in ECM requires glycosylation and depends on the number of oligosaccharide branches and type of terminal substitutions [111, 112]. Surface expression of LAMP1 as well as the levels of polyLacNAc substituted β1,6 branched N-oligosaccharides on it have been found to correlate with the metastatic potential of B16 melanoma cells [51]. Moreover, high metastatic B16F10 cells treated with blocking antibodies to LAMP1 showed significantly reduced lung metastasis indicating involvement of LAMP1 in the metastatic process.

1.4 LAMP1 (Lysosome Associated Membrane Protein-1)

Lysosome Associated Membrane Protein-1 (LAMP1) (also known as CD107a, LAMPA, lgp120), as the name suggests, is an abundant, heavily N-glycosylated integral membrane protein that lines the lysosomes. It is encoded by the gene located on human chromosome 13q34 as a polypeptide containing 389 amino acids, corresponding to about 40 kDa [113]. LAMP1 has 17-20 potential N-glycosylation sites and six O-glycosylation sites [114-116]. The oligosaccharides present on LAMP1 are complex-type N-glycans containing mainly repeating structures of N-acetylglucosamine and galactose, commonly termed as poly-N-acetyllactosamine (polyLacNAc) [115, 117, 118]. LAMP1 is also a substrate for the enzyme GnT-V for addition of the β1,6 branch which is further preferentially substituted with polyLacNAc [77, 119]. The mature form of LAMP1 is approx. 120 kDa owing to the addition of N- and O-glycosylation structures. Thus, the carbohydrate structures contribute about 55-65% of total molecular weight of LAMP1.

A major portion of the protein containing all the glycosylation sites remains in the luminal side which is large and is separated by a proline rich hinge region into two disulphide containing domains, followed by a single transmembrane spanning segment and a short cytoplasmic domain having 11 amino acids containing a Gly-Tyr sequence required for transport to lysosomal membranes (**Illustration 4**) [120, 121]. LAMP1 along with another abundant glycoprotein, LAMP2 and others like LIMP1/CD63, LIMP2/LGP85 and minor transmembrane glycoproteins form a continuous glycocalyx on the luminal side of lysosomes. The complex N-glycans of these glycoproteins are resistant to the lysosomal hydrolases and thus protect the lysosomal membranes from autoproteolysis by lysosomal enzymes, thereby maintaining the structure and integrity of lysosomes [122].

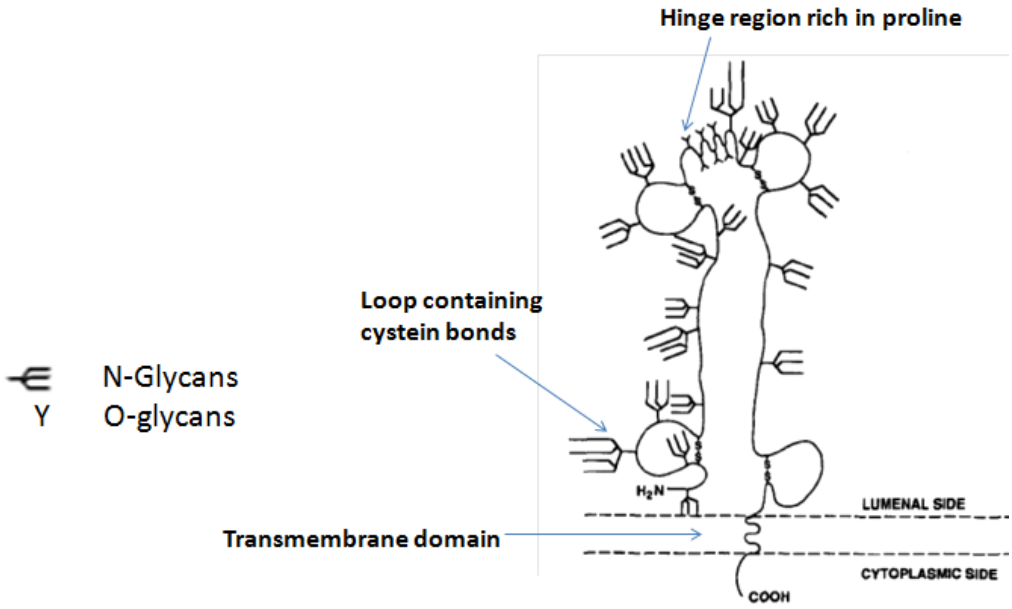

Illustration 4: LAMP1 structure: The intraluminal portion can be divided into two internally homologous domains separated by a region rich in proline residues. 4 half-cysteine residues are connected to each other, forming four disulfide loops in the intraluminal portion. The structure depicted is adapted and modified from [114].

Although LAMPs and other lysosomal membrane proteins are mainly localized in the limiting membranes of lysosomes and late endosomes, they are not static components. They are rather in a dynamic equilibrium between the lysosomes, endosomes and plasma membranes. Apart from maintaining membrane integrity, the lysosome membrane proteins are also involved in many other functions. For instance, the minor lysosomal proteins LIMP-2/LGP85 seems to have specific functions in maintaining endosomal transport and lysosomal biogenesis [123]. LAMP2 plays an important role in chaperon mediated autophagy. Its deficiency in humans leads to Danon disease - a fatal cardiomyopathy and myopathy. Furthermore, the absence of both LAMP1 and LAMP2 leads to embryonic lethality and autophagic vacuole accumulation in almost all embryonic tissues [124-126].

However, LAMP1 has not yet been attributed any specific, indispensable role on lysosomes as it was observed that LAMP1 deficient mice are viable and fertile with normal lysosomal properties and absence of any major histological and ultrastructural abnormalities of organs. LAMP2 appears to take over the function of LAMP1 in these cells suggesting that LAMP1 is dispensable for normal lysosomal functions [127]. However, besides being lysosomal membrane proteins, LAMP1 and LAMP2 are also found to get translocated to the cell surface in a number of circumstances. There are emerging evidences for LAMP1 being a highly functional glycoprotein in this setting.

1.4.1 Expression of LAMPs on the cell surface during lysosomal sorting

LAMPs form an integral part of the lysosomal membrane. Two different pathways, direct and indirect, have been proposed to participate in the biosynthetic transport of LAMPs to lysosomes. The direct pathway is a completely intracellular route that involves transport of newly synthesized LAMPs enclosed in Adaptar protein-2 (AP2) associated clathrin coated

vesicles (CCVs) and transported from the trans-Golgi network (TGN) to either early or late endosomes and then to lysosomes. Sorting of LAMPs to lysosomes via the indirect pathway occurs though plasma membrane, wherein glycoproteins are enclosed in Adaptar protein-1 (AP1) associated clathrin coated vesicles (CCVs) and transported to plasma membrane. At the plasma membrane, these glycoprotein molecules get enclosed in AP2 associated CCVs and are endocytosed to early endosome, followed by late endosomes and finally to the lysosomes. Hence at any point of time, a basal level of 0.5-1% of total LAMPs is expressed on the cell surface. In both the pathways, the transport from early to late endosomes is mediated by AP1 and AP2 associated CCVs [128-130].

Sorting of the LAMPs to lysosomes is due to the **tyrosine sorting motif GYXXØ** (where G is glycine, Y is tyrosine, X is any amino acid and Ø is any bulky amino acid) in their cytoplasmic tail. In mammalian cells, virtually all YXXØ signals mediate rapid internalization from the cell surface. The G, Y, and Ø residues as well as the exact placement of the motif relative to the transmembrane domain are critical for efficient biosynthetic targeting of the LAMPs to lysosomes [131]. If the tyrosine of the sorting motif is mutated to any other amino acid, it is observed that the levels of mutated LAMP1 increase on plasma membrane as they are not endocytosed effectively [130, 132].

1.4.2 Expression of LAMPs on the cell surface during the process of exocytosis

Plasma membrane wounds are repaired by a mechanism involving Ca^{2+}-regulated exocytosis. Elevation in intracellular Ca^{2+} triggers fusion of lysosomes with the plasma membrane. Ca^{2+} mediated exocytosis of lysosomes to wounded plasma membranes has been thus implicated as a mechanism of plasma membrane repair which also results in the expression of a pool of LAMP1 molecules on the cell surface. Moreover, lysosomal exocytosis and membrane

resealing were inhibited when antibodies against the cytosolic domain of LAMP1 were used, which specifically aggregated lysosomes [133]. This clearly emphasizes the role of surface LAMP1 in the process of exocytosis.

Oligosaccharides on LAMP1 also play a critical role in the process of exocytosis. For instance, LAMP1 present on the membrane of secretory lysosomes are a natural substrate for N-acetyl-α-neuraminidase (Neu1 or sialidase) which is responsible for controlling the sialic acid residues, thus determining the turnover of LAMP1 and lysosomal exocytosis. In the absence of Neu1, the LAMP1 was found to be hypersialylated which brought about an increase in expression of LAMP1 on the cell surface and also resulted in an increased lysosome exocytosis. Sialylation on LAMP1 is proposed to bring about a change in the cytoplasmic tail of LAMP1, altering its interaction with the transport machinery [134].

1.4.3 Expression of LAMPs on the surface of activated platelets and cells of the immune system

A regulated increased translocation of LAMP1 and LAMP2 from the lysosomal membrane to the plasma membrane has also been reported in thrombin activated platelets, thus indicating their role in adhesion, platelet aggregation and prothrombotic phenotype of these cells [135, 136]. Moreover, in cells of the innate immune system such as natural killer (NK) cells and cytotoxic T-lymphocytes, LAMP1 and LAMP2-containing granules fuse with the plasma membrane during degranulation and deliver the lytic components, and perhaps serve to protect the effector cells from self-lysis following the release of lytic enzymes like perforins and granzymes. Expression of LAMP1 has indeed been reported on surface of NK cells and cytotoxic T cells [137, 138]. Recently, increased surface expression of LAMP1 and not LAMP2 has been shown to protect NK cells from degranulation associated damage [139].

1.4.4 Expression of LAMPs on the surface of embryonic cells

LAMP1, also known as ESGp (Embryonal Surface Glycoprotein), is found to be expressed on the surface of embryonic cells after fertilization when they need to be more motile [140]. It has been implicated in cell–cell interactions, compaction of the embryo and its implantation into the uterus which are processes similar to those involved in metastasis [141, 142].

1.4.5 Expression of LAMPs and their carbohydrates on the surface of undifferentiated cells

Levels of carbohydrates specifically on LAMP1 (but not LAMP2) have been found to be greatly altered during differentiation of cells. For instance, there was a large decrease in the proportion of polyLacNAc associated with LAMP1 during differentiation of CaCo-2 cells [117]. Similarly, stage-specific embryonic antigen-1 (SSEA-1), a well-known carbohydrate antigenic epitope of undifferentiated cells, including neural stem cells (NSCs) is reported to be carried by LAMP1. The epitope contains the following sequence of Lewis X antigen: [Galβ1-4(Fucα1-3) GlcNAcβ-] and is a well characterized marker of undifferentiated cells. On differentiation of NSCs, SSEA-1 on LAMP1 was found to be completely ablated indicating that the expression of SSEA-1-positive LAMP1 is associated with the “stemness” of NSCs [143].

1.4.6 Expression of LAMPs and their carbohydrates on the surface of metastatic tumor cells

Several metastatic cells also show increased expression of LAMP1 on their cell surface and the expression correlates with their metastatic potential. For instance, it has been shown to be expressed on surface of human melanoma (A2058), human colon carcinoma (CaCo-2),

human fibrosarcoma (HT1080), human myelomonocytic leukemia (HL-60 and U937) and macrophage-melanoma fusion hybrid cells [144-146]. Its cell surface expression has been shown to correlate with metastatic potential of human colon carcinoma and murine melanoma cell lines [51, 85].

Highly metastatic lymphoid tumor cell line called MDAY-D2 was also found to express LAMP1 carrying the β1,6 branched N-linked oligosaccharides on cell surface. High metastatic colonic carcinoma cell lines were also found to express higher amounts of LAMP1 and LAMP2 on their surface as compared to low metastatic ones. LAMP1 on these cells showed higher levels of polyLacNAc terminating in sialyl Lewis x and sialyl Lewis a antigens on β1,6 branched N-glycans which are recognized by activation dependent vascular endothelial lectins called E-selectins. This helps in an increase in adhesion to vascular endothelia expressing E-selectin [85]. In addition, expression of β1,6 branched N-oligosaccharides substituted with poly-N-acetyllactosamine (PolyLacNAc) on LAMP1 of B16 melanoma cells has also been shown to correlate with their metastatic potential [51]. These evidences thus indicate a potential role of cell surface LAMP1 and its carbohydrates in the metastatic processes.

1.4.7 Involvement of LAMPs in cell motility and invasion

LAMPs have been found to be expressed in cellular processes involved in motility. Specific accumulation and localization of LAMP1 and LAMP2 was observed at the extensions and edges of the A2058 human metastasizing melanoma cell lines, suggesting that these molecules can serve as ligands for extracellular matrix components and facilitate cancer cell adhesion, spreading and locomotion during metastasis [144].

LAMP1 (expressed on the surface of human carcinoma cells), a prominent antigen recognized by BR96 antibody which binds to Lewis Y, was found to be present on unique

cell surface domains involved in cell locomotion such as microspikes (filopodia) and membrane ruffles [147]. Localization of LAMP1 on such actin rich structures suggests that LAMP1 can interact with other molecules which may regulate assembly of the cytoskeleton required for locomotion.

LAMP1 on highly metastatic mammary carcinoma cells is reported to bind to ezrin [148]. Ezrin (belonging to the ERM family of proteins) functions as a linker between the actin cortical cytoskeleton and various membrane-bound molecules [149, 150]. It mediates the interaction between actin and LAMP1 and is involved in vacuole acidification which aids local invasion of metastatic cells. These findings point towards a potential role of LAMP1 in actin remodeling and participation in membrane vesicle transport.

Ezrin has also been shown to bind directly to the N-terminal domain of the p125 focal adhesion kinase (FAK) and trigger FAK phosphorylation independently of cell matrix adhesion. Moreover, FAK phosphorylation at Tyr-397 was found to be independent of Src-kinase activity [151]. The phosphorylated Tyr-397 is a binding site for the PI3K adaptor protein, activating the PI3K/Akt signaling pathway which is shown to promote cancer cell invasion via increased motility and metalloproteinase production [152]. Moreover, ezrin itself undergoes phosphorylation at the Tyr-353 residue and transduces a survival signal through the PI3K/Akt pathway [153].

In addition, ezrin, in its active form, is shown to undergo direct Src-dependent phosphorylation of its Tyr-145 residue. This active residue then influences the Src-kinase activity which is required for focal adhesion kinase (FAK) activation which, in turn, activates myriad downstream signaling pathways [154]. Thus, through a complex series of events, ezrin can participate in cell adhesion, migration and invasion. The association between LAMP1 and ezrin thus indicates a plausible role of LAMP1 in mediating cell motility and invasion.

1.4.8 LAMP1 - A high affinity ligand for galectin-3

A key determinant in successful organ specific metastasis is the adhesive interactions between the molecules on the surface of tumor cells and the target organ. Galectin-3, an endogenous lectin expressed on the lungs, is a multifunctional molecule modulating processes of adhesion, invasion and metastasis [155]. LAMP1 molecules which were expressed on the surface of metastatic cells were identified as one of the major carriers of polyLacNAc, which is a high affinity ligand for galectin-3 [51]. LAMP1 being one of the major carriers of polyLacNAc, has been shown to serve as a ligand for galectin-3 in many tumor cells [51, 144, 156]. Therefore, it is possible that LAMP1 molecules may mediate the different steps involved in lung specific metastasis via galectin-3 binding.

1.5 Galectin-3

Galectin-3, a multifunctional protein, is a member of the family of β-galactoside binding lectins called galectins. Members of galectin family (15 members identified till date) share highly conserved Carbohydrate Recognition Domains (CRDs). They are classified into three subgroups based on their structural differences and the number of CRDs within their polypeptide chains. The **prototypical** galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15) contain one CRD, whereas the **tandem-repeat** galectins (galectin-4, -6, -8, -9 and -12) have two CRDs that are separated by a linker region in a single polypetide chain. Galectin-3 is the exclusive member of the **chimera-type** galectin subgroup and contains one CRD that is connected to an extended non-lectin N-terminal domain, which is rich in proline, glycine, and tyrosine residues [157, 158]. The COOH-terminal domain containing the CRD consists of 130 amino acids and is responsible for its binding to carbohydrate moieties. The CRD

contains a NWGR (Asp-Trp-Gly-Arg) motif which is essential for galectin-3 binding to β-galactosides [159]. In solution, galectin-3 largely occurs as a monomer. In the absence of its binding ligands, it can also form homodimer by self-association through its CRDs. However, in the presence of its carbohydrate binding ligands, galectin-3 can polymerize up to pentamers through its N-terminal domain. Multimerisation is a common feature of extracellular galectin-3, where it often cross-links its cell surface ligands to form lattice-like structures and triggers the initiation of cell surface molecule-associated cell signalling [160, 161].

Galectin-3 is found to be present intracellularly in the nucleus as well as the cytoplasm. Intracellular galectin-3 has been implicated in several processes such as cell growth and differentiation, pre-mRNA splicing, regulation of apoptosis, etc [162, 163]. For instance, in the nucleus, galectin-3 promotes pre-mRNA splicing and participates in spliceosome assembly via complexes with nuclear protein Gemin4 [163]. In the cytoplasm, galectin-3 can bind to Bcl-2 and inhibit cell apoptosis. NWGR motif present in galectin-3 is also present in the Bcl-2 family members of apoptosis regulators and is responsible for the anti-apoptotic activity of galectin-3 [164]. Cytoplasmic galectin-3 can also interact with the activated GTP-bound K-Ras and affect Ras mediated Akt signalling [165]. Like all other galectins, galectin-3 lacks a classical signal sequence and membrane-anchoring domain and appears to be synthesized on free polysomes, and is also secreted out of the cells, by a yet unknown non-classical pathway [67, 166]. Secreted galectin-3 is also often known to become a part of the cell surface and the ECM and BM [160, 163]. Galectin-3 has indeed been shown to be present in highest amounts in mice lungs and expressed constitutively on the surface of lung vascular endothelium [51]. In addition, galectin-3 secreted by cells also gets associated with the ECM by interacting with polyLacNAc ligands on ECM components such as laminin, thereby dictating adhesion and motility of cells [155]. Thus, depending upon its cellular

localization, galectin-3 is involved in a wide variety of functions. Extracellular galectin-3 mainly promotes processes like cell-cell recognition, adhesion, invasion and metastasis [157, 158, 162, 163].

Extracellular galectin-3 secreted by cells can form ordered arrays of complexes on the cell surface by binding to multivalent glycoconjugates, which triggers a cascade of transmembrane signalling events. By binding to these glycoconjugates, galectin-3 delivers signals intracellularly, as well as mediates cell–cell and cell–ECM adhesion [161]. For instance, carbohydrate dependent binding of galectin-3 to β1 integrins leads to their rapid endocytosis, thereby modulating cell adhesion [167]. Similarly, cross linking of cell surface growth factor receptors, such as EGFR by galectin-3 results in increased retention time on the cell surface, delays removal by constitutive endocytosis, thereby altering signalling [104]. Galectin-3 is often overexpressed in various human solid tumors and blood malignancies and, in many cases, this altered expression correlates with the stage of tumor progression [160]. Galectin-3 on the surface of tumour cell lines has been reported to be associated with malignant progression. Furthermore, extracellular galectin-3 also aids angiogenesis by stimulating capillary tube formation and neovascularization in vitro [168].

Tumors have been shown to secrete galectin-3 and elevated levels in the serum appear to be a potential diagnostic tool [169]. Extracellular galectin-3 has also been reported to promote arrest in the fine vessels and thus metastasis by facilitating homotypic aggregation [170]. Role of galectin-3 expressed on the target endothelial cells in mediating heterotypic interactions with carbohydrate ligands on tumour cells, leading to organ specific metastasis, has also been extensively explored. Among the reported galectin-3 ligands, recent reports implicate T/Tn antigens on O-glycans, expressed on tumor cells in mediating both homophilic, as well as heterophilic interactions with endothelial cells via galectin-3 [170,

171]. Association of T/Tn antigen with malignant progression in gastric, colorectal and breast carcinomas has been observed, where it apparently participates in many of the galectin-3 mediated processes [172]. However, galectin-3 has been reported to have highest affinity for binding to polyLacNAc residues, present on both N- and O-linked glycans. Moreover, the binding affinity of galectin-3 for polyLacNAc is 200 times higher than that of T/Tn antigens [172-174]. Levels of polyLacNAc on the cell surface have been found to increase significantly during malignant transformation [98]. Therefore, extracellular galectin-3 present on target organ endothelium can affect cancer progression and metastasis possibly through its interaction with proteins which are carriers of polyLacNAc and are expressed on the surface of tumor cells.

1.6 Rationale of the study

LAMP1 is a highly glycosylated molecule which is expressed on the cell surface in a metastatic potential dependent manner [51, 85]. Each LAMP1 carries about 17-20 N-oligosaccharides which are highly substituted with polyLacNAc [114]. Major portion of LAMP1 molecule is exposed on the lumen (of the lysosomes) or the cell surface. LAMP1 on the cell surface would thus provide high density of high affinity ligands-polyLacNAc for galectin-3 on the target organ, in the most easily accessible manner. Since, lungs express highest levels of galectin-3 on almost all the tissue compartments including the surface of its vascular endothelium [51, 175], it would be important to explore the role of LAMP1 molecule per se and its associated carbohydrates in promoting lung specific metastasis.

1.7 Model system used for the study

To understand the role of polyLacNAc substituted β1,6 branched N-oligosaccharides on LAMP1 in lung specific metastasis, low (B16F1) and high (B16F10) metastatic variants of

B16 melanoma have been chosen as the model **(Illustration 5)**. They have been chosen mainly for three reasons. Firstly, the low metastatic (B16F1) cells and the high metastatic (B16F10) cells carrying β1,6 branched N-glycans specifically colonize lungs, irrespective of the route of administration (intravenous, where lungs would be the first site; or intra-aortic, where lungs would be the last organ encountered) [176]. Second is the availability of cell lines with vastly different metastatic potential. Thirdly, quantitation of metastatic colonies in the lungs is relatively simple as the melanoma cells express the black pigment- melanin.

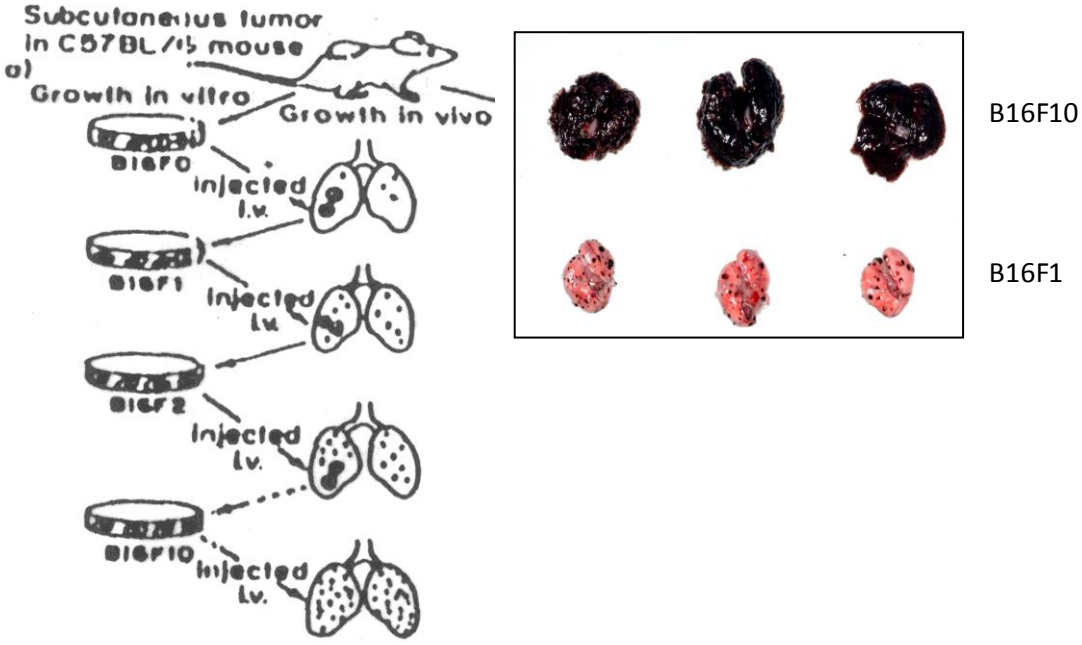

Illustration 5: The B16 murine melanoma model. Scheme of derivatization of B16F10 cells by repeated growth in vivo (in lungs) followed by in vitro culturing of melanoma cells to select metastatic phenotype. Box indicates metastatic melanoma colonies on lungs of mice injected with B16F10 (high metastatic) and B16F1 (low metastatic) cells.

1.8 Previous work done in the lab

Previous work done in the lab using the B16 murine melanoma model shows the following:

1. The expression of β1,6 branched N-oligosaccharides correlates with the metastatic potential of B16 melanoma cells.
2. Lysosome Associated Membrane Protein-1 (LAMP1) and β1 integrin were identified to be the major carriers of these oligosaccharides.
3. Expression of LAMP1 on melanoma cell surface correlates with the metastatic potential.
4. Expression of PolyLacNAc substituted β1,6 branched N-oligosaccharides on LAMP1 correlates with metastatic potential of melanoma cells.
5. PolyLacNAc is the most preferred ligand for galectin-3.
6. Lungs express highest amounts of galectin-3 compared to other organs in mice.
7. Galectin-3 is present on all the major tissue compartments of the lungs including the surface of vascular endothelium where it is constitutively expressed.
8. Galectin-3 not only aids arrest on lung vascular endothelium, but also participates in all the steps of extravasation.
9. Owing to the presence of 17-20 N-glycosylation sites highly substituted with polyLacNAc, LAMP1 was identified as a major ligand on melanoma cells for galectin-3.
10. High metastatic B16F10 cells treated with blocking antibodies to LAMP1 show significantly reduced lung metastasis.

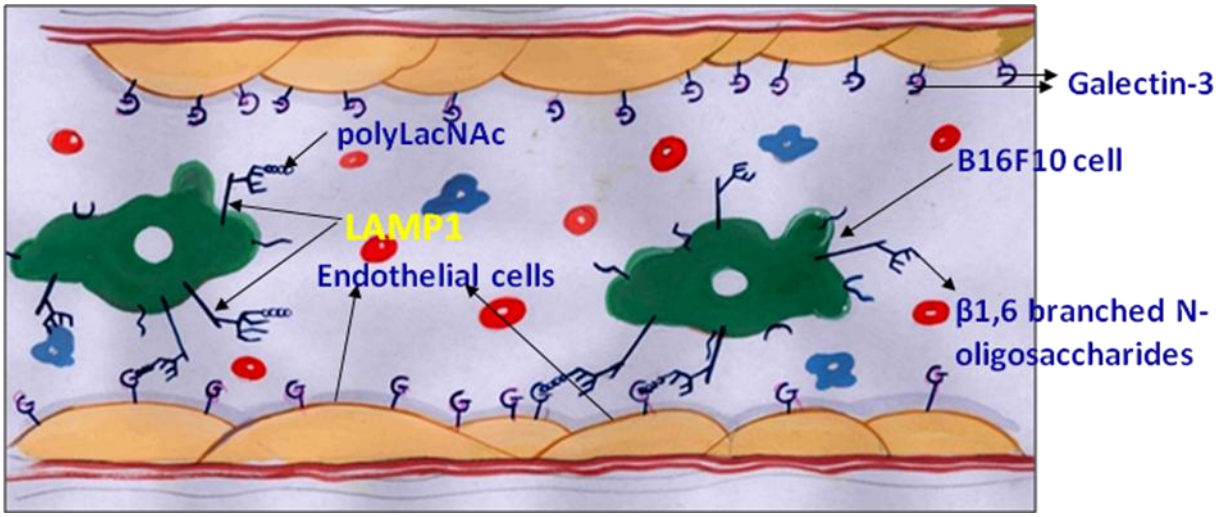

Illustration 6: Schematic diagram representing proposed hypothesis for colonization of B16 melanoma cells in lungs. B16 melanoma cells get arrested in lungs through interaction of polyLacNAc on LAMP1 of these cells with galectin-3 which is constitutively present on lung vascular endothelium.

1.9 Key Questions and Objectives of the study

From the above studies, several questions arise.

Key Questions:

- Does cell surface LAMP1 participate in any of the cellular processes important for metastasis?
- What is the contribution of sugars on LAMP1 in these processes and hence lung metastasis?
- What is the mechanism for surface translocation of LAMP1 on the surface of B16 melanoma cells?
- Would knockdown of LAMP1 in high metastatic cells affect their cellular properties important for metastasis?

Objectives:

The following objectives were proposed to answer these questions:

1. **To investigate the effect of translocating LAMP1 to cell surface in low metastatic cells, on different cellular properties important for lung metastasis.**

2. **To investigate the contribution of β1,6 branched N-oligosaccharides on LAMP1 in different cellular properties of metastatic cells.**

3. **To investigate the effect of downregulation of LAMP1 on the cellular properties of high metastatic murine melanoma cells.**

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines and reagents

Murine melanoma cell lines, B16F1 and B16F10 were obtained from National Centre for Cell Science, Pune, India. Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), L-glutamine, Antibiotic–Antimycotic solution, PCR buffer, TRIzol and Calcein AM were obtained from GIBCO, Invitrogen Corporation, USA. Aprotinin, Leupeptin, Pepstatin, PMSF, Trypsin, Dextrose, Tween-20, Bovine Serum Albumin (BSA), TEMED, β-mercaptoethanol, Glycine, Coomassie Brilliant Blue, Ponceau-S, Lactose, Divinyl Sulphone, Dialysis tubing, N-hydroxysuccinimido biotin, Lysolecithin, Phalloidin FITC, DAPI, Trypan Blue, Gelatin, PIPES, Lysozyme, RNase A, Ethidium Bromide, Agarose, Diethyl Pyrocarbonate (DEPC), Polybrene, Puromycin, Doxycycline, Mitomycin C, Swainsonine and Primers for PCR were obtained from Sigma Chemical Co, USA. Ampicillin, Yeast Extract, Tryptone and Agar powder were obtained from HIMEDIA, India. Tris, NP-40, Sodium Deoxycholate, Sodium Dodecyl Sulphate (SDS), Bisacrylamide, Isopropyl Thio D-galactopyranoside (IPTG) and Triton X-100 were obtained from USB, USA. Taq Polymerase, T4 DNA ligase buffer, ATP, dNTPs, T4 DNA ligase enzyme, Klenow Fragment enzyme and restriction enzymes were obtained from either New England Biolabs (NEB), USA or Fermentas, USA. Protoscript first strand cDNA synthesis kit was obtained from NEB, USA. Plasmid DNA extraction and DNA gel extraction kits were obtained from either Sigma or Qiagen, USA. Acrylamide, PVDF membrane and ECL plus kit were acquired from Amersham-Pharmacia Biotech Ltd., England. Fibronectin and Matrigel were purchased from BD Pharmingen, USA. Biotinylated lectins like Leuco-Phyto Haem Agglutinin (LPHA), *Lycopersicon esculentum* lectin (LEA) and vectashield mounting medium were from Vector Labs, USA. Proteinase K and Protease Inhibitor cocktail were procured from Calbiochem,

USA. Tissue culture plastic wares were obtained from BD Falcon or Nunc., USA. Sepharose 4B beads and Cyanogen bromide activated Sepharose 4B beads were procured from Pharmacia Fine Chemicals, Sweden AB. Inbred strains of C57BL/6 mice were used for the metastatic assays and other experiments. All other fine chemicals were obtained locally and were of Analytical or better grade. Water used to prepare all the reagents was of Milli-Q grade.

2.1.2 Antibodies

Rat anti mouse LAMP1 monoclonal antibody (Clone 1D4B) was obtained from BD Pharmingen, USA. Anti-β actin antibody (clone AC-74), Anti-rat HRPO, Anti-rat FITC conjugate, Extr-Avidin FITC conjugate and Avidin Peroxidase were obtained from Sigma Chemical Co, USA.

2.2 METHODS

2.2.1 Maintenance of cell lines in-vitro

B16 melanoma variants were routinely cultured in DMEM supplemented with 0.03% L-glutamine, antibiotic-antimycotic solution (100 units/ml of Penicillin, 100 µg/ml of Streptomycin and 0.25 µg/ml of amphotericin B), 0.04 mg/ml gentamycin and 10% fetal bovine serum (Complete medium). Cell suspension (1 X 10^6 cells/ml) in 8ml of complete medium was cultured in a 90 cm^2 tissue culture dish and incubated in a humidified atmosphere containing 5% CO_2 at 37ºC to achieve 90% confluency. For inhibition of glycosylation, melanoma cells were grown in presence of N-glycosylation inhibitor, swainsonine (SW) (2 µg/ml) for 48 h and harvested for in-vitro experiments on reaching 90% confluency.

2.2.2 Cell harvesting, subculturing and cryopreservation

Confluent monolayer cell culture was washed with Phosphate Buffered Saline (PBS – 10 mM Sodium Phosphate and 150 mM NaCl, pH=7.4) and cells were harvested with trypsin solution (0.25% trypsin, 0.02% EDTA and 0.05% glucose in PBS) for one minute. Action of trypsin was inhibited by addition of complete medium and it was removed by washing once with complete medium. Cell number and viability was checked by dye exclusion with 0.04% trypan blue in PBS. Cells with greater than 95% viability were used for all the assays and for subculturing. Cells of early passage were preserved in cryopreservent (FBS containing 10% DMSO) in liquid Nitrogen. Each frozen stock contained about 2-3 x 10^6 cells/ml.

2.2.3 Revival of cell lines from frozen stocks kept in liquid nitrogen

A vial from the frozen stocks of cell lines stored in liquid nitrogen was quickly thawed in water bath at 37°C followed by two washes with complete medium by centrifuging the cells at 1500 g for 5 min at 37°C. After centrifugation, cell pellet was resuspended into 5 ml of complete medium and seeded into 25 cm^2 cell culture flask and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Next day, medium was changed to remove nonviable, non-adherent cells.

2.2.4 Preparation of total cell lysate

Melanoma cells were harvested, washed with chilled PBS and solubilized in chilled lysis buffer containing 10 mM Tris chloride, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM magnesium chloride and 1 mM calcium chloride and protease inhibitor cocktail (1µg/ml of each of pepstatin, leupeptin, aprotinin and 0.3 mM PMSF). One ml of lysis buffer was used to make lysate from 15 X 10^6 cells by 3 sonication cycles of 30 seconds with 1 minute intervals at 50% output on ice. The clarified supernatant (lysate) obtained by centrifugation at 16,000 rpm for 60 minutes at 4ºC was aliquoted and stored at -80ºC.

2.2.5 Protein estimation

Protein was estimated according to Peterson's modification of Lowry method [177]. Samples were taken in 1 ml of distilled water and mixed with 1 ml of CTC reagent [equal volumes of Solution A (0.1% Copper Sulphate, 0.2% Sodium Potassium Tartarate and 10% Sodium Carbonate), 10% Sodium Dodecyl Sulphate (SDS), 0.8 N NaOH, and distilled water] and incubated for 10 minutes. 0.5 ml of six times diluted Folin and Ciocalteau's reagent was mixed and incubated for 30 minutes in dark. Absorbance of solution was measured at 750 nm using a UV Spectrophotometer. Bovine Serum Albumin was used as the standard (5 to 30 µg).

2.2.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was done as per the method of Laemelli [178]. 30% Acrylamide (29.2% acrylamide and 0.8% N,N'-methylene-bisacrylamide) was mixed with 1M Tris base (pH=8.8) and 20% SDS, in order to obtain the desired resolving gel concentration containing 0.37 M Tris-Base and 0.1% SDS. Fifty micro litres each of ammonium persulphate (20% w/v) and TEMED (Tetraethylmethyl ethylene diamine) 10% (v/v) were added and the gel was poured between sealed glass plates with spacers. A stacking gel (4.5% Acrylamide, 0.0625 M Tris pH-6.8, 0.2% SDS), was overlaid on the polymerized resolving gel. The protein samples were boiled for 5 minutes with or without β-mercaptoethanol in sample buffer (0.0625 M Tris pH-6.8, 2% SDS, 10% glycerol (w/v), 750 mM β-mercaptoethanol and 0.05% Bromo Phenol blue) and loaded into wells. Electrophoresis was done using electrode buffer (0.025M Tris-chloride, 0.2% SDS (w/v) and 0.192 M glycine) at 100 Volts (constant voltage). Finally, protein bands on the gels were detected by staining with either 0.2% Coomassie brilliant blue (in 50% methanol, 10% acetic acid and 40% distilled water), or by silver staining or proteins were transferred to polyvinylidene diflouride (PVDF) membrane for blotting.

2.2.7 Western Blotting

The transfer of proteins from the gel to a Polyvinylidene diflouride (PVDF) membrane was done as per Towbin et al [179]. The resolving gel was equilibrated in chilled transfer buffer (0.025 M Tris, 0.192 M Glycine, and 20% Methanol) for 15 minutes. The membrane was pre-treated, super-imposed on the gel and the transfer apparatus was set as per manufacturers' instructions. Transfer of proteins to the membrane was carried out with chilled transfer buffer using a constant voltage of 70 Volts for 3 hours. Extent of protein transferred to the membrane was checked by soaking in 0.2% (v/v) Ponceau–S stain in 3% trichloroacetic and 3% sulphosalicylic acid, for 2 minutes. The stain was later washed off with Tris Buffered Solution (TBS-20 mM Tris and 500 mM NaCl).

2.2.8 Probing of Western blots with specific lectins or antibodies

Cell surface oligosaccharides on glycoproteins were studied by using specific lectins. For instance, LPHA (Leukoagglutinin Phytohemagglutinin) is used for probing β1,6 branched N-linked oligosaccharides while LEA (*Lycopersicon esculentum* Agglutinin) specifically recognizes poly-N-acetyllactosamines [180]. The lectin blots were subsequently developed using Avidin Peroxidase (AVP).

For studying proteins, the PVDF membranes blotted with proteins were blocked with 3% BSA and probed with specific antibodies and secondary HRPO conjugated antibodies. The blocking conditions and working dilutions of the lectins and antibodies, along with their time of incubation are as given in **Table 1**. All incubations were carried out at room temperature under shaking conditions and the blot was developed using Enhanced Chemiluminiscence (ECL) reagent, as per manufacturers' protocol.

Table 1: Lists the biotinylated lectins and specific antibodies used for the study, their concentration and incubation time.

Lectin or antibody	Blocking	Primary antibody	Host species	Secondary antibody
Biotinylated LPHA	T-TBS (TBS with 0.1% Tween-20), 1 hour	2 µg/ml in T-TBS	----	1:30,000 Avidin Peroxidase (AVP) in T-TBS for 1 hour
Biotinylated LEA	T-TBS for 1 hour	0.5 µg/ml in T-TBS	----	1:30,000 AVP in T-TBS for 1 hour
LAMP1	3% BSA for 1 hour	0.5 µg/ml in 1% BSA	Rat	1 µg/ml Anti-Rat HRPO in 1% BSA for 1 hour
β-actin	3% BSA for 1 hour	0.1 µg/ml in 1% BSA	Mouse	0.5 µg/ml Anti-mouse HRPO in 1% BSA for 1 hour

2.2.9 Purification and characterization of recombinant human galectin-3

Expression of galectin-3 was induced in *E.coli* BL-21 [containing pET3C plasmid coding for recombinant human (rh) Galectin-3] using 50mg/litre of Isopropyl Thio D-Galactopyranoside (IPTG) as described in [181]. The culture was grown in Luria Bertani medium [prepared by dissolving tryptone (1.0%), yeast extract (0.5%) and NaCl (1.0%), pH 7.5]. The bacterial cell

lysate was obtained by sonication followed by centrifugation and purified on a Lactose-Sepharose affinity Column as per [182]. The bound protein was eluted with 150 mM lactose, dialyzed, dried and stored at -80ºC till use. The quality of protein was confirmed by Coomassie staining and immunoblotting, while its concentration was confirmed and matched with previous batches.

2.2.10 Biotin labeling of rh-galectin-3

Biotin labeling of galectin-3 was performed as per the protocol of Bayer et al [183]. One mg of rh-galectin-3 was solubilized in 1.5 ml of buffer containing 0.1 M Sodium bicarbonate ($NaHCO_3$) and 0.2 M NaCl. To this solution, 0.5 mg of N-hydroxysuccinimido biotin dissolved in dimethylformamide (DMF) (not more than 5% of final volume) was added and incubated at 25ºC for 2 hours with mild shaking. The reaction was terminated by the addition of 75 µl of 1 M NH_4Cl solution for 15 minutes at 4ºC. The sample was dialyzed overnight in 2 litres of 150 mM NaCl and subsequently against two changes of 2 litres of PBS, and stored at –20ºC.

2.2.11 FITC labeling of rh-galectin-3

FITC labeling of galectin-3 was performed as per the protocol of Goldman et al [184]. One mg of rh-galectin-3 was dissolved in 1 ml of 50 mM carbonate buffer (pH 9.5, containing 150 mM NaCl). To this solution, 40 µl of 1 mg/ml FITC prepared in carbonate buffer was added with gentle stirring and the solution was kept overnight at 4ºC. Unreacted FITC was removed by dialyzing the mixture against PBS (pH 7.4) containing 10 mM lactose and finally with PBS (pH 7.4) containing 1 mM lactose. This FITC conjugated galectin-3 was used for flow cytometric analysis.

2.2.12 Flow cytometric analysis

For flow cytometry, melanoma cells were first fixed by overnight incubation with 1% paraformaldehyde in PBS (pH 7.4) followed by lectin staining. 0.5×10^6 melanoma cells were incubated with 30 µg of biotinylated LPHA, LEA or rh-galectin-3 in 60 µl of FACS buffer (PBS, pH 7.4, containing 1% FBS) followed by Extra-Avidin-FITC diluted 1:25 in FACS buffer. Cells treated with Extra-Avidin-FITC alone served as control. For staining with FITC labeled galectin-3, 0.5 million melanoma cells were incubated with 10 µg of galectin-3-FITC in 40 µl of FACS buffer followed by three washes with PBS. Untreated cells served as control. It was ensured that the cells were maintained in suspension by intermittent tapping. Fluorescent cells were acquired at 488 nm and analyzed on FACS Calibur (BD Biosciences) using Cell Quest software. For flow cytometry of LAMP1, melanoma cells were stained with LAMP1 antibody. 1×10^6 melanoma cells were incubated with 1 µg of anti-LAMP1 (1D4B) antibody for 1 h, followed by washing and treatment with anti-rat FITC conjugate diluted in FACS buffer. The cells were again washed in PBS. All the steps were performed in cold. The cells were fixed in 1% paraformaldehyde overnight at 4ºC and acquired on FACS Calibur.

2.2.13 Designing and cloning of LAMP1 cDNA constructs

For overexpression of LAMP1 on surface of B16F1 cells, wild-type and mutant LAMP1 (mutation of Tyr^{386} of wild-type LAMP1 to Ala leading to its mislocalization to cell surface instead of lysosomes) cDNA clones were constructed. To obtain wild-type LAMP1 cDNA, total RNA was prepared from B16F10 cells using TRIzol reagent and cDNA was synthesized using cDNA synthesis kit as per manufacturer's protocol. To amplify wild-type LAMP1 from cDNA, following primers were used: Forward primer having XhoI site (represented in bold italics) followed by Flag tag (represented in bold) and mouse LAMP1 N-terminal sequence (represented in italics): 5'-

TAT***CTCGAG***ATG**GATTACAAGGATGACGATGACAAG**GAATTC*ATGGCGGCCCC*

CGGCGCC-3' and Reverse primer: 5'-

GGATCCCTAGATGGTCTGATAGCCGGCGTGACTCC-3'.

2.2.13.1 Cloning of wild-type LAMP1 cDNA in pTRIPz

The forward and reverse primer oligonucleotides were procured, reconstituted in sterile distilled water to obtain 100 μM concentration. Equal volumes (10 μl of 10 μM) of both the oligonucleotides were mixed with PCR master mix containing mouse total cDNA as a template and PCR amplified. The PCR product was digested with XhoI enzyme and digested DNA was purified using DNA gel extraction kit (Sigma) as per manufacturer's protocol.

Approximately 20 μg of lentiviral vector generated previously, pLV-K18-YFP-IRES-Puro [185] was digested with NotI enzyme, blunted using Klenow Fragment enzyme as per manufacturer's protocol and then digested with XhoI and finally digested DNA was purified using DNA gel extraction kit (Sigma) as per manufacturer's protocol. The linear vector and digested PCR product were diluted to obtain 1:12 = Vector : Insert concentration ratio and ligated using T4 DNA ligase enzyme (NEB), overnight at 22ºC as per manufacturer's protocol. The ligated plasmid (wt-LAMP1 vector) was used for transformation of ultra competent DH5α cells.

2.2.13.2 Preparation of ultra competent *Escherichia coli* DH5α cells

Reagents required:

<u>SOB (300 ml)</u>

Tryptone- 6 gm

Yeast extract- 1.5 gm

NaCl- 0.15 gm

KCl- 5.6 gm

The pH of the SOB medium was adjusted to pH 7 with 1 N NaOH, followed by the addition of 4 ml of 1 M glucose and 1 ml of 2 mM $MgCl_2$, just prior to inoculation of bacteria.

Transformation Buffer (200 ml)

PIPES- 0.6 gm

$CaCl_2$- 0.4 gm

KCl- 3.7 gm

The pH was adjusted to pH 6.7 with 10 N KOH and 2.18 gm $MnCl_2$ and sterilized by filtering it through 0.22 µm filter.

Ultra competent *E. coli* DH5α cells were prepared as described in [186]. A single colony of DH5α strain of *E. coli,* from an overnight grown LB agar plate was inoculated into 250 ml of SOB medium and incubated at 18°C with mild shaking till the optical density (OD) at 600 nm reached about 0.3 to 0.5. The culture was incubated on ice for 10 min followed by centrifugation at 2500 g for 10 min at 4°C to pellet the bacterial culture. The culture supernatant was discarded and the bacterial cells pellet was gently resuspended in 80 ml of ice cold transformation buffer. The cell suspension was incubated on ice for 10 min followed by centrifugation at 2500 g for 10 min at 4°C. The supernatant was discarded and the bacterial cell pellet was resuspended in 20 ml of ice cold transformation buffer containing 7% DMSO. This suspension of cells was incubated on ice for 10 min and subsequently aliquoted into nearly 200 µl aliquots in 1.5 ml micro centrifuge tubes, snap freezed into liquid nitrogen and immediately stored at -80°C until further use.

2.2.13.3 Transformation of ultra competent cells

Ultra competent cells were transformed with ligated wt-LAMP1 vector. Competent cells transformed with linearized vector alone, served as negative control. Briefly, competent cells

were thawed, added to the ligation mixture and kept on ice for 30 minutes. The cells were placed at 42 ºC (water bath) for exactly 90 seconds and subjected to cold shock on ice for 2-5 min. The cells were then mixed with 1ml of sterile LB broth and incubated at 37 ºC for 30 minutes in a shaker incubator. The cells were spun at 5,000 rpm for 5 minutes, pellet was resuspended in 200 µl of LB broth and spread on to low salt LB agar plate containing 100 µg/ml ampicillin and incubated for 24-36 hours at 30ºC.

2.2.13.4 Screening of recombinant colonies

Individual colonies that were grown on LB agar plates after transformation were picked up and inoculated into 1 ml LB broth and allowed to grow overnight at 37°C. Plasmids were extracted from them by alkaline lysis method and recombinant clones were screened by restriction digestion.

2.2.13.5 Isolation of plasmids by alkaline lysis method

Individual colony picked up and inoculated into 1 ml LB broth for plasmid extraction was allowed to grow overnight at 37°C. Plasmid isolation from the overnight grown culture was carried out by the alkaline lysis method [186]. The overnight grown bacterial cultures of 1.5 ml were centrifuged at 5000 rpm at 4°C for 5 min, the medium was removed and the pellets were dried. To the dried bacterial pellets, 100 µl of alkaline lysis solution I (GTE buffer: 50 mM Glucose, 25 mM Tris pH 8.0 and 10 mM EDTA) was added and vortexed till the pellets were completely dissolved. Then, 200 µl of alkaline lysis solution II (0.2 N NaOH and 1% SDS) was added, mixed gently by inverting and kept for 2 min. 150 µl of ice-cold alkaline lysis solution III (3 M potassium acetate pH 4.8 in glacial acetic acid) was then added to each tube and kept on ice for 10 min. The tubes were then centrifuged at 13000 rpm, 4°C for 15 min and the supernatants containing the renatured plasmids were transferred to fresh tubes. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to supernatant for

removal of proteins and saccharides, mixed by vortexing and centrifuged as above for 5 min. The aqueous phase was transferred to a fresh microcentrifuge tube and double the volume of absolute alcohol was added and mixed well for precipitation of plasmid DNA. Tubes were kept on ice for 15 min and centrifuged at the above conditions for 20 min. The ethanol was removed and the pellets were washed with 70% ethanol (chilled) to remove salts, centrifuged as above for 5 min and all the traces of ethanol were removed. The pellets were completely dried at 37°C for 30 min. The dried pellets were reconstituted in 20 µl of autoclaved distilled water. 0.3 µl of RNase (1µg/ml) was added to each tube and incubated at 37°C for 45 min to degrade RNA molecules.

The screening of colonies containing the wt-LAMP1 cDNA was done in purified plasmids by checking release of an insert of 1250 base pairs on double digestion with restriction enzymes XhoI and BamHI. The positive colony showing the presence of wt-LAMP1 was used for maxiprep plasmid purification to obtain high yields and quality of plasmid for transfection.

2.2.13.6 Maxi prep of plasmid DNA

Maxi prep is different from miniprep as the starting *E. coli* culture volume is 100-200 ml LB broth. Maxi prep was performed using kit based method from Sigma Aldrich. 200 ml of overnight grown culture was pelleted by centrifugation at 5000 g for 10 min. The pellet was resuspended in 12 ml resuspension solution and vortexed until the pellets were completely dissolved. Cells were lysed by adding 12 ml of lysis solution and mixed thoroughly by gently inverting the tubes for 6-8 times and the tubes were kept at RT for 3 min. 12 ml of chilled neutralization solution was added to neutralize the lysed cells resulting in the formation of a white aggregate (cell debris, proteins, lipids, SDS and chromosomal DNA). Then 9 ml of binding solution was added to the pellet and poured into the barrel of the filter syringe and were incubated for 5 min at RT so that the white aggregate floated to the top. During

incubation, the binding column was prepared by adding 12 ml of column preparation solution and was centrifuged at 5000 g for 5 min. By holding the filter syringe barrel over binding column, the plunger was gently inserted to expel the clear lysate into the column. The binding column was then spun in a swinging bucket rotor at 3000 g for 2 min and the eluent was discarded. The column was then washed with wash solution 1 and 2 and centrifuged at 5000 g for 2 min and 5 min respectively. The column was then transferred to a fresh 50 ml collection tube and 3 ml of elution solution was added and was kept at RT for 20 min and was then centrifuged at 5000 g for 5 min for the elution of DNA.

2.2.13.7 Site-directed mutagenesis of wild-type LAMP1 cDNA

To obtain mutant LAMP1 clone, site-directed mutagenesis of tyrosine^{386} of wild-type LAMP1 cDNA clone to alanine was done using Stratagene Quick Change Mutagenesis kit as per the manufacturer's protocol. Primers for site-directed mutagenesis of wt-LAMP1 were as follows:

Forward oligo: 5'-AGGAGTCACGCCGGCGCTCAGACCATCTAGGG-3'

Reverse oligo: 5'-CCCTAGATGGTCTGAGCGCCGGCGTGACTCCT-3'

The forward and reverse oligonucleotides were procured, reconstituted in sterile distilled water to obtain 100 μM concentrations. Equal volumes (10 μl of 10 μM) of both the oligonucleotides were mixed with PCR master mix containing wt-LAMP1 cDNA clone as a template and PCR amplified. The PCR product was DpnI digested and the digested plasmid was used for transformation of ultra competent DH5α cells and spread plated on low salt LB agar plates as explained in 2.2.12.3 above.

Colonies growing on ligation test plate were counted and subjected to Mini prep based plasmid extraction by alkaline lysis method as described in 2.2.12.5. The screening of

colonies containing the mutant LAMP1 cDNA was done in purified plasmids by checking release of an insert of 1250 base pairs on double digestion with restriction enzymes XhoI and BamHI. The positive colony showing the presence of mutant LAMP1 was used for maxiprep plasmid purification to obtain high yields and quality of plasmid for transfection using maxiprep plasmid purification kit (Sigma) as described in 2.2.12.6. The mutation in the plasmid was confirmed by sequencing.

2.2.13.8 Transfection, transduction & selection of clones

Culture dishes (90 mm) were seeded with exponentially growing HEK293FT cells to a confluency of approximately 50%. Mutant LAMP1 plasmid and vector control plasmid separately were co-transfected along with helper plasmids psPAX2 and pMD2.G in HEK293 FT cells in the ratio 6:5:2. Culture supernatant containing infectious viruses was collected after 48 hours of transfection and cell debris was removed by centrifugation at 5000 rpm for 20 min. Culture dishes (35 mm) were seeded with exponentially growing B16F1 cells to a confluency of approximately 50%. One ml of the supernatant along with 8 µg/ml polybrene was added to the culture dishes drop wise, the plate gently swirled and incubated for 24 hours at 37ºC. After the incubation period, the medium was removed and culture was rinsed twice using medium without serum. The cells were then grown for 24 hours in medium containing 10% FBS.

Before beginning selection, the cells were harvested and seeded into 90 mm petri dishes containing puromycin at 1 µg/ml. Medium was replaced with fresh medium every 2 days, till transduced cells formed isolated colonies. These colonies were picked up by trypsin digestion and cultured in 24 well plates and maintained as separate stocks. These clones were then checked for LAMP1 overexpression on cell surface and maintained at a puromycin concentration of 0.5 µg/ml.

2.2.14 Immunofluorescence staining

Cells to be immunostained were seeded on coverslips and grown overnight in complete medium up to 70-80% confluency. Cells were washed thrice with PBS (pH 7.4) and fixed with 2% paraformaldehyde at RT for 5 minutes. They were washed again with PBS and blocked with 3% BSA in PBS for 1 h at RT in humidified chamber and incubated with primary antibody (LAMP1) for 1 h in humidified chamber, followed by three washes with PBS to remove excess or non-specifically bound antibody. Cells were further incubated with fluorescent tagged secondary antibody (anti-rat FITC) for 1 h followed by three washes with PBS. Those incubated only with fluorescent tagged secondary antibody served as isotype control. Nuclei were stained with 5 μg/ml of DAPI in PBS for 1 minute and coverslips were mounted on slides using vectashield mounting medium. Images were acquired using a Carl Zeiss laser confocal microscope at 63x magnification.

2.2.15 Cell spreading assays

Cell spreading assays were performed on coverslips placed in 35 mm dishes, pre-coated overnight with 75 μg/ml galectin-3, fibronectin (10μg/ml) and matrigel (10μg/ml) in serum free DMEM at 4ºC. Melanoma cells were harvested, washed free of serum and seeded on the coverslips at a cell density of 0.5×10^6/ml in serum free DMEM and incubated at 37ºC for 45 minutes in a CO_2 incubator. Coverslips treated with serum free DMEM only, served as control. Non-adherent cells were removed by gentle washing with PBS (pre-warmed to 37ºC) 3-4 times.

The bound cells were fixed at 37ºC for 5 minutes in pre-warmed 4% paraformaldehyde in PBS (made by gently boiling in PBS and pH adjusted to 7, with 1 N NaOH). The fixative was washed off with PBS and cells were permeabilized with 0.5% Triton X-100 in PBS, at RT for 15 minutes. Triton X-100 was removed by washing with PBS and the F-actin organization in

cells was checked by inverting the coverslips on 10 µl of Phalloidin-FITC staining solution made in PBS (containing 1µg/ml of Lysolecithin, 10% AR grade methanol, 0.5% BSA in PBS and 2 µg/ml of Phalloidin-FITC solution in methanol) on a parafilm in a moist chamber for 15 min at 37^0C. The coverslips were inverted on 10 µl of 2% BSA solution, and incubated at RT for 10 min. Excess stain was washed with PBS at RT for four times for 5 min and the nuclei were stained with 10 µl of 5 µg/ml of 4', 6'- Diamidino-2-Phenylindole Dihydrochloride (DAPI) in PBS for 1 minute followed by 3-4 washes with PBS.

The stained cells were mounted on glass slides using Vectashield antifading agent and sealed. The images were acquired using a Carl Zeiss laser confocal microscope at 63x magnification.

2.2.16 Treatment of cells with glycosylation inhibitor

Swainsonine (SW) was added to 40-50% confluent cultures at a concentration of 2 µg/ml to inhibit complex-type N-linked oligosaccharides and harvested after 24 hours.

2.2.17 Wound healing assays

Wound healing assays were performed in 35 mm culture dishes coated overnight with 75 µg/ml of galectin-3 in serum free DMEM at 4ºC followed by blocking of non-specific sites with 2% BSA for 1 hour. Melanoma cells were harvested and seeded at a cell density of 0.75 × 10^6 cells and incubated at 37ºC for 24 hours in a CO_2 incubator. The cells were treated with 40 µg/ml mitomycin C for 3 h for inhibiting cell proliferation. A straight, uniform wound (approx. 400 µm in width) was made using a micropipette tip on the plate upon reaching 95% confluency. The dislodged cells were washed off and the cells were maintained in serum free DMEM. Wound closure of cells in response to the immobilized galectin-3 was measured for 16-20 hours by time lapse video imaging of at least three different positions across the length of the wound using a Carl Zeiss inverted microscope. Uncoated culture dishes, blocked only

with BSA served as control. Mean percent areas of wound closure for each position was calculated using Image J software of each of the image frame.

2.2.18 Experimental metastasis assay

Experimental metastasis assay was performed according to the method of I. J. Fidler [187]. Cells were harvested, washed and resuspended in serum free DMEM. 6-8 weeks old female C57BL/6 mice were injected with 100 µl of a single cell suspension of melanoma cells (1.5×10^6 cells/ml) in DMEM via the tail vein route. Mice were sacrificed after 21 days, their lungs excised and number of colonies in the lungs was counted using a dissecting microscope.

2.2.19 Immunoprecipitation of LAMP1

Total cell lysate (2 mg) was precleared, and later incubated at RT for 2 h with 20 µg of anti-LAMP1 antibody. This was followed by addition of 200 µl of protein G sepharose beads (50% suspension) and incubation overnight at 4°C. The beads were pelleted at 2000 rpm for 10 min and later washed five times with 1 ml of lysis buffer. The bound proteins were eluted by boiling the beads in 1X Laemmli sample buffer for 5 min, separated on SDS-PAGE, western blotted and probed with LAMP1 antibody and with biotinylated LPHA or LEA.

2.2.20 Designing and cloning of LAMP1 shRNA constructs

Downregulation of LAMP1 in B16F10 cells was performed using short hairpin RNA (shRNA) approach. The shRNAs against LAMP1 were designed as per guidelines outlined by [188]. Two shRNA-coding oligonucleotides against LAMP1 were designed by obtaining cDNA sequences (5'-CCCACTGTATCCAAGTACAAT-3' and 5'-GCGTTCAACATCAGCCCAAAT-3') which are unique only to mouse LAMP1, using a BLAST search. Primer sequences used for shRNA amplification are mentioned in **Table 2**. ShRNA cassette was PCR amplified using forward primer having XhoI site (represented in

bold), mir sequence followed by sense sequence (represented in bold italics) and 15 nucleotide loop sequence. The reverse primer contained EcoRI site (represented in bold), mir sequence followed by sense sequence (represented in bold italics) and 15 nucleotide loop sequence. 11 nucleotide loop sequences are complementary to each other (**Illustration 7C**).

2.2.20.1 Cloning of LAMP1 shRNA oligonucleotides in pTRIPz plasmid

The forward and reverse oligonucleotides were procured, reconstituted in sterile distilled water to obtain 100 µM concentrations. Equal volumes of both the oligonucleotides were mixed with PCR master mix and PCR amplified. PCR conditions for shRNA amplification are given in **Table 3**. The PCR product was doubly digested with XhoI and EcoRI enzymes in their respective buffers and digested DNA was purified using DNA gel extraction kit (Sigma) as per manufacturer's protocol.

Approximately 20 µg of pTRIPz plasmid (**Illustration 7A & B**) was sequentially digested with XhoI and EcoRI enzymes in their respective buffers and digested DNA was purified using DNA gel extraction kit (Sigma) as per manufacturer's protocol. The linear vector and digested PCR product were diluted to obtain 1:30 = Vector : Insert concentration ratio and ligated using T4 DNA ligase enzyme (NEB), overnight at 22ºC as per manufacturer's protocol. The ligated plasmid was used for transformation of ultra competent DH5α cells and spread plated on low salt LB agar plates as described in 2.2.12.1.

Table 2: Sequence of primers used for shRNA amplification

S. No.	shRNA amplification primer sequences (5'-3')
1	LAMP1 shRNA 1 Forward: GAA**CTCGAG**AAGGTATATTGCTGTTGACAGTGAGCG***CCCACTGTAT CCAAGTACAAT***TAGTGAAGCCACAGA Reverse: GTT**GAATTC**CGAGGCAGTAGGCA***CCCACTGTATCCAAGTACAAT***TAC ATCTGTGGCTTC
2	LAMP1 shRNA 2 Forward: GAA**CTCGAG**AAGGTATATTGCTGTTGACAGTGA***GCGGCGTTCAACA TCAGCCCAAAT***TAGTGAAGCCACAGA Reverse: GTT**GAATTC**CGAGGCAGTAGGCA***GCGTTCAACATCAGCCCAAAT***TAC ATCTGTGGCTTC

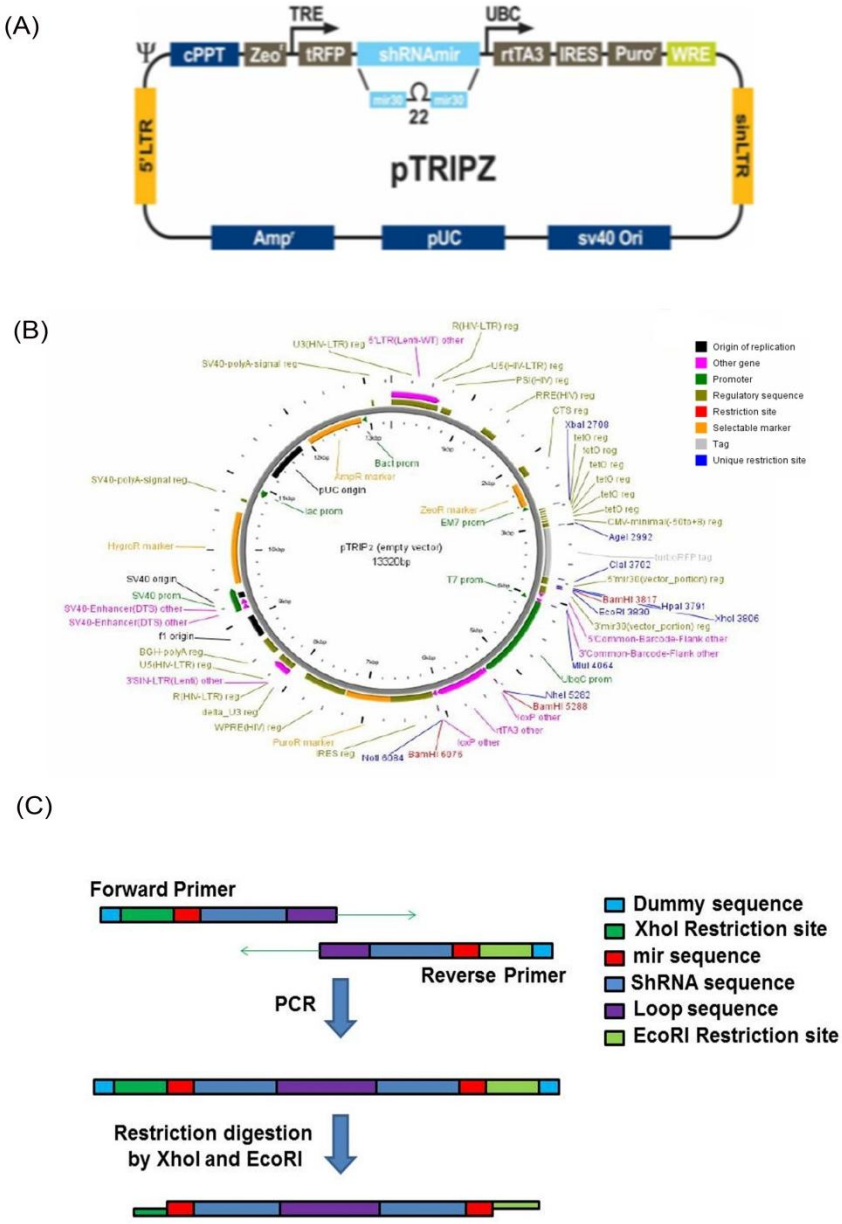

Illustration 7: (A) pTRIPz inducible lentiviral vector (B) pTRIPz inducible lentiviral vector map (C) PCR strategy.

Table 3. PCR conditions for shRNA amplification (100 µl reaction)

Temperature (°C)	Time (Min)	Components	Volume (µl)
94 (Initial denaturation)	5	Forward primer	4
94 (Denaturation)	5	Reverse primer	4
50 (Annealing)	1.30	10X buffer	10
72 (Extension)	1	DMSO (10%)	10
72 (Final Extension)	10	dNTPs (10mM)	2
No. of cycles: 30		Taq polymerase	2
		Milli-Q H_2O	68

2.2.20.2 Screening of recombinant colonies

Individual colonies that were grown on LB agar plates after transformation were picked up and inoculated into 1 ml LB broth and allowed to grow overnight at 37°C. Plasmids were extracted from them by alkaline lysis method as described in 2.2.12.5 and recombinant clones were screened by restriction digestion.

The screening of colonies containing the shRNA oligonucleotide was done in mini-prep purified plasmids by XhoI and EcoRI digestion where positive clones gave an insert release of 120 bp. Presence of positive clones was also confirmed by EcoRI and AgeI digestion where positive clones gave an insert release of 958 bp, whereas empty vector without shRNA gave an insert release of 838 bp. The positive colony showing the presence of the shRNA

oligonucleotide was used for maxiprep plasmid purification using maxiprep plasmid purification kit (Sigma) along with non-targeting shRNA as control (procured from Open Biosystems) to obtain high yields and quality of plasmid for transfection as described in 2.2.12.6.

2.2.20.3 Transfection, transduction & selection of clones

Transfection of shRNAs along with helper plasmids in HEK293FT cells and transduction in B16F10 cells was done as described in 2.2.12.8. Stable clone selection was done using puromycin (1 µg/ml) and selected clones were maintained at a puromycin concentration of 0.5 µg/ml. Later, shRNA expression was induced in selected clones by adding doxycycline at a concentration of 4 µg/ml for 96 hours and these clones were then checked for LAMP1 downregulation.

2.2.21 Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 software. For spreading and experimental metastasis assays, comparison within the group was done by performing one way ANOVA followed by Bonferroni's multiple comparison tests. For wound-healing assays, 2-way ANOVA with the Bonferroni post-test was conducted. (p value <0.05 was considered significant).

CHAPTER 3

RESULTS

3. RESULTS

Previous work in the lab has demonstrated that expression of polyLacNAc substituted β1,6 branched N-oligosaccharides on melanoma cells correlates with their metastatic potential. Moreover, inhibition of N-oligosaccharides, specifically β1,6 branching and polyLacNAc synthesis, resulted in inhibition of lung metastasis. Galectin-3 is a high affinity receptor for polyLacNAc. It is shown to be expressed at the highest levels on the lungs and found to be expressed constitutively on the lung vascular endothelium [51]. Attachment to the target organ endothelium serves as the first step in colonization of that organ by tumor cells. PolyLacNAc on melanoma cells may aid initial anchoring of cells to lung endothelium by binding to galectin-3. It has been shown that this polyLacNAc/galectin-3 pair participates in not just anchoring these cells to lung endothelium, but in all the subsequent steps of extravasation like spreading, degradation of ECM/BM and movement into organ parenchyma [175].

LAMP1 and β1 integrin were identified to be among the major carriers of polyLacNAc substituted β1,6 branched N-oligosaccharides on B16F10 cells. β1 integrin is a cell surface receptor molecule whose role in different metastatic processes is well documented. Involvement of LAMP1, a lysosomal membrane protein, in metastatic processes is plausible only if it appears on the cell surface. Expression of LAMP1 on the surface of B16 melanoma cells was indeed found to correlate with their metastatic potential [51]. Moreover, expression of polyLacNAc substituted β1,6 branched N-oligosaccharides on LAMP1 has also been shown to correlate with the metastatic potential [51]. LAMP1 contains 17-20 N-glycan sites which are often substituted with polyLacNAc. The high levels of polyLacNAc on LAMP1 on B16F10 cells may thus provide easily accessible high density of high affinity ligands for galectin-3. Previous study has also shown that pre-incubation of B16F10 cells with anti-

LAMP1 antibody significantly reduced their metastatic ability as compared to untreated cells or those treated with pre-immune IgG. Although this established that surface LAMP1 indeed plays a key role in imparting metastatic phenotype, it remains to be elucidated whether it is the LAMP1 protein or its glycosylation which is more important from the point of view of metastasis. To decipher that, LAMP1 was overexpressed on surface of low metastatic B16F1 cells and conversely, downregulated in high metastatic B16F10 cells and effect of these manipulations on cellular properties and the metastatic potential was investigated.

3.1. Surface expression of polyLacNAc substituted β1,6 branched N-oligosaccharides and LAMP1 correlates with metastatic potential of melanoma cells.

Before initiating a full-fledged investigation of how LAMP1 influences metastatic outcome, the frozen aliquots of the low and high metastatic melanoma cell lines were analyzed to confirm our previous findings. B16 melanoma cell lines were evaluated for their total as well as surface expression of these oligosaccharides and LAMP1. It was conclusively shown that the expression of β1,6 branched N-oligosaccharides indeed correlates with metastatic potential, as seen by both flow cytometry (**Fig. 1A**) as well as Western blotting **(Fig. 1C)**. Beta 1,6 branch on N-oligosaccharides is the most preferred site for polyLacNAc substitutions. Results revealed that expression of polyLacNAc correlates with the levels of β1,6 branched N-oligosaccharides on the cell surface and, in turn, with the metastatic potential of melanoma cells **(Fig. 1B & C).** LAMP1 has previously been shown to be one of the major carriers of polyLacNAc in these cells. Results showed that although the total LAMP1 levels in these cells remained same **(Fig. 1D inset)**, the surface expression of LAMP1 correlates with their metastatic potential **(Fig. 1D)**. These results confirmed that surface expression of polyLacNAc substituted β1,6 branched N-oligosaccharides and LAMP1 invariably correlates with metastatic potential of B16 melanoma variants.

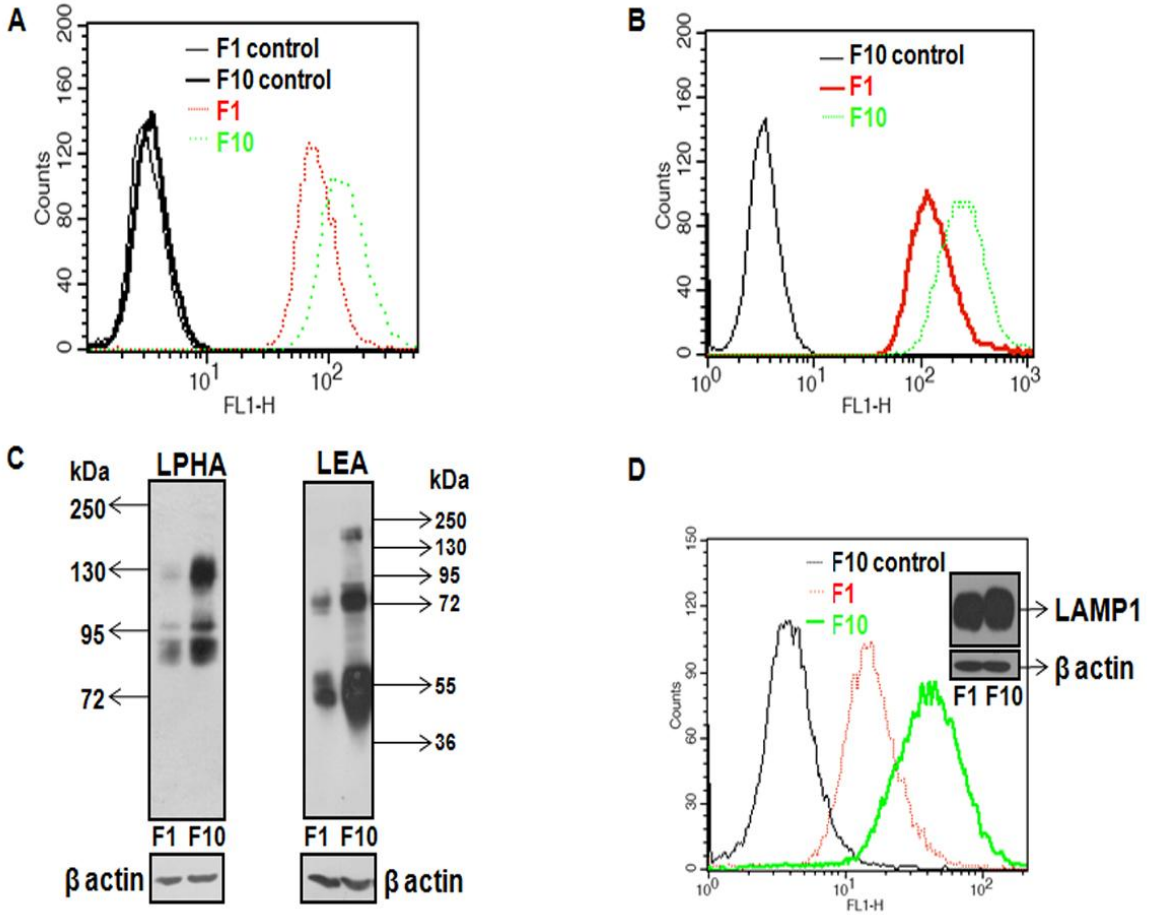

Figure 1. Analysis of expression of β1,6 branched N-oligosaccharides, polyLacNAc and LAMP1 in B16F1 and B16F10 cells. Comparison of expression of **(A)** β1,6 branched N-oligosaccharides using biotinylated LPHA, **(B)** polyLacNAc using biotinylated LEA and **(D)** LAMP1 using rat anti-LAMP1 antibody, on the surface of B16F1 (red line) and B16F10 cells (green line) using flow cytometry. B16F1 and B16F10 cells treated with either extra-avidin-FITC (in A and B) or anti-rat FITC (in D) alone served as control (black line). **(C)** Comparison of total levels of β1,6 branched N-oligosaccharides using biotinylated LPHA and polyLacNAc using biotinylated LEA in B16F1 (F1) and B16F10 (F10) cells by Western blotting. **(D inset)** Comparison of total LAMP1 in F1 & F10 cells by Western blotting. β-actin served as loading control in (C & D inset).

To investigate whether LAMP1 or its glycosylation or both are important for metastasis, LAMP1 was overexpressed on the surface of low metastatic B16F1 cells which have low levels of glycosylation. Mutation in a specific region (Tyr^{386}) of the cytoplasmic tail of LAMP1 has been shown to direct them to cell surface instead of lysosomes [132]. Such a mutant LAMP1 was generated and expressed in low metastatic B16F1 cells. Further, its effect on surface expression of LAMP1 and on the cellular properties and metastatic potential of these cells was studied.

3.2. Expression of mutant LAMP1 (Tyr^{386} to Ala^{386}) in B16F1 cells results in elevated expression of LAMP1 on the surface.

Total RNA and cDNA was synthesized from B16F10 cells as described in materials and methods (section 2.2.13). Wild-type LAMP1 (wtLAMP1) cDNA was PCR amplified from mouse cDNA using LAMP1 specific primers **(Fig. 2A).** It was then ligated into previously generated pLV-K18-YFP-IRES-Puro vector [185] after removing K18-YFP from it **(Fig. 2B)**. The presence of wtLAMP1 insert in the resulting wtLAMP1-pTRIPz vector was confirmed by using different restriction digestion strategies **(Fig. 2C)**. This vector was then used for site-directed mutagenesis to generate mutLAMP1-pTRIPz vector **(Fig. 3A)**. Sequencing of the resultant vector confirmed the mutation of tyrosine to alanine **(Fig. 3B)**. Stable expression of this mutLAMP1 by lentiviral infection of B16F1 cells **(Fig. 3C)** resulted in significantly higher surface expression of LAMP1 in both the clones (C1 & C11) as compared to either uninfected (F1) or those infected with virus with vector alone (Vector Control - VC) **(Fig. 4A & B)**. The surface expression of LAMP1 in the clones C1 and C11 was several folds higher even when compared to B16F10 (F10) cells as observed by both flow cytometry as well as immunofluorescence **(Fig. 4C & D)**.

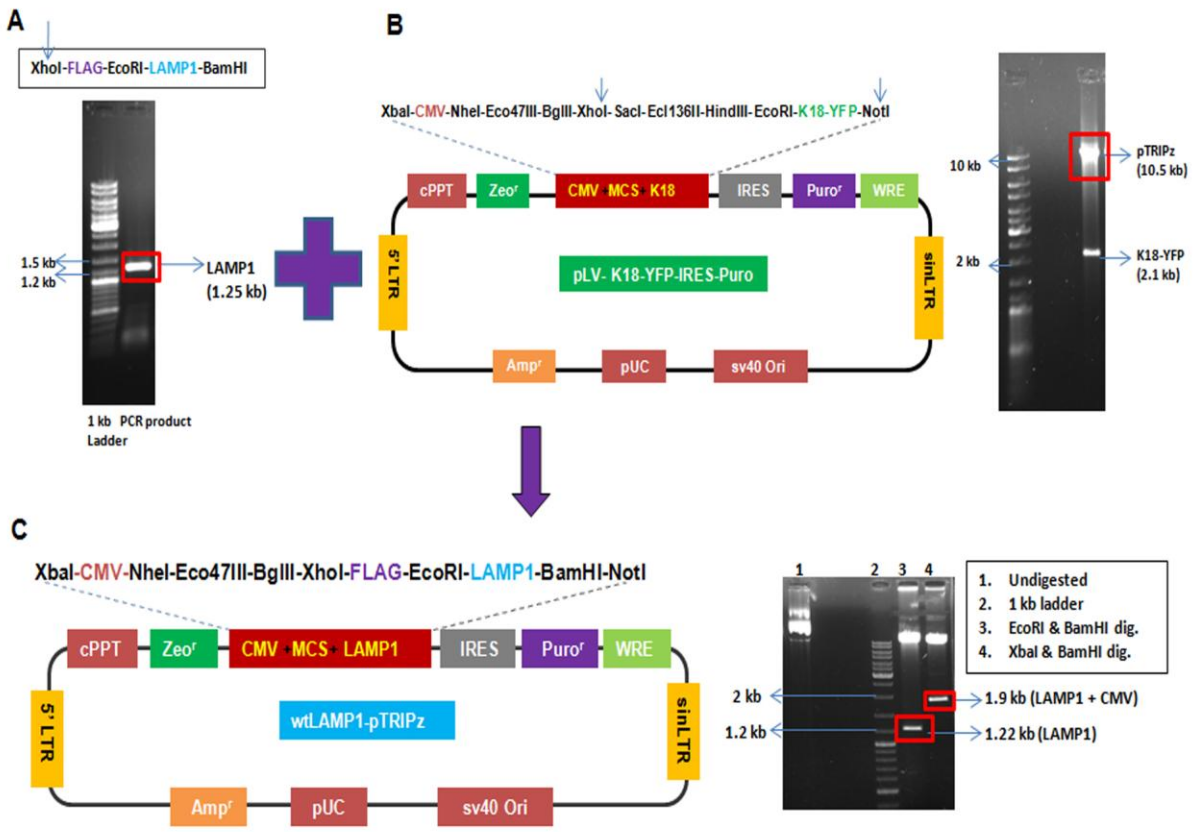

Figure 2. Generation of lentiviral vector containing wtLAMP1. (A) Agarose gel electrophoresis (AGE) of wtLAMP1 PCR amplicon amplified from cDNA synthesized from RNA of B16F10 cells. The box on top indicates the sequential sites on amplicon and the arrow on it represents the site of restriction digestion. **(B)** Features of lentiviral vector pLV-K18-IRES-Puro including its Multiple Cloning Site (MCS) and AGE of the same vector digested with XhoI and NotI showing digested vector (10.5 kb) and insert release of K18-YFP (2.1 kb). **(C)** wtLAMP1-pTRIPz generated as a ligation product of wtLAMP1 amplicon (A) and digested vector (B) and AGE of the same vector either undigested (lane 1) or doubly digested with EcoRI/BamHI (lane 3) or XbaI/BamHI (lane 4) restriction enzymes to confirm for the presence of wtLAMP1 insert. 1 kb DNA ladder served as molecular weight markers in AGE of A, B and C.

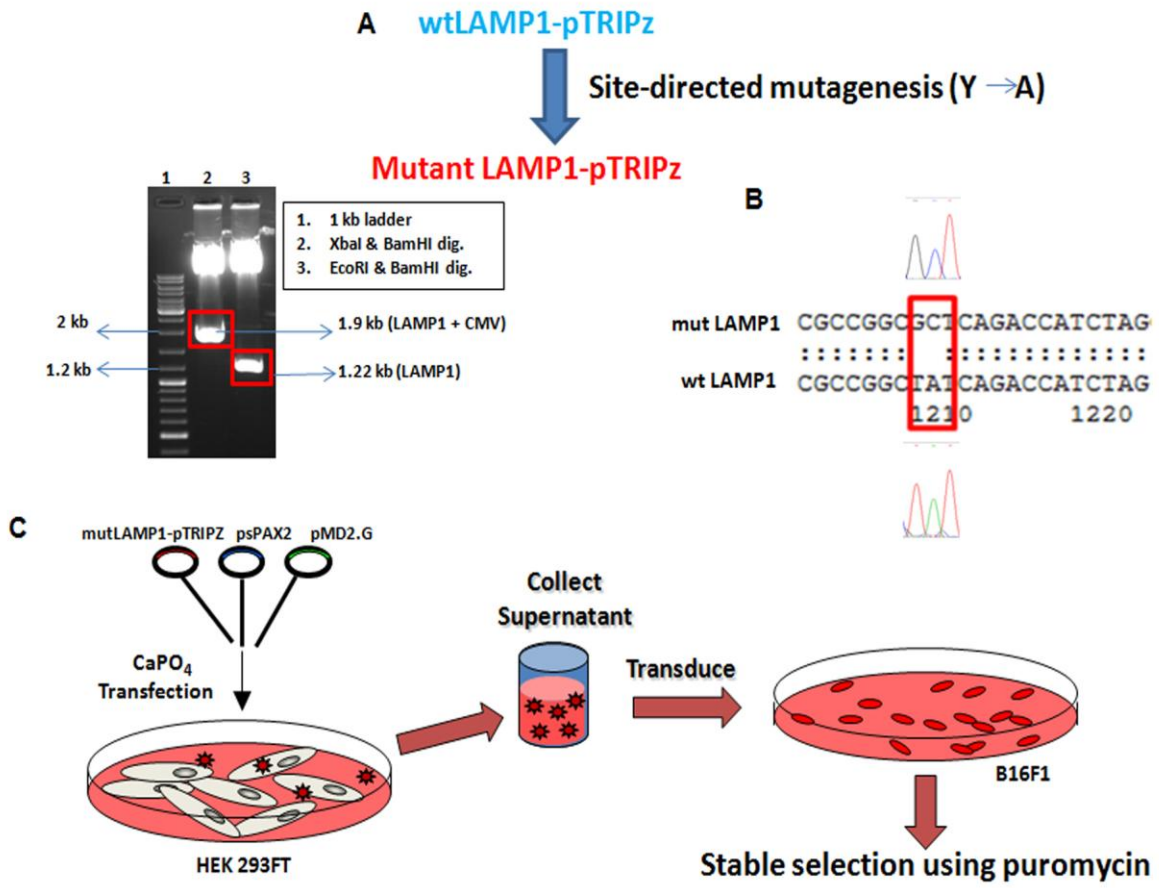

Figure 3. Site-directed mutagenesis of lentiviral vector containing wtLAMP1 and transduction of resultant mutLAMP1 vector in B16F1 cells. (A) mutLAMP1-pTRIPz vector generated by site-directed mutagenesis of wtLAMP1-pTRIPz vector and AGE of the same vector digested with XbaI/BamHI (lane 3) or EcoRI/BamHI (lane 4) restriction enzymes to confirm for the presence of mutLAMP1 insert. 1 kb DNA ladder served as molecular weight markers. **(B)** Comparison of C-terminal sequences obtained by sequencing of wtLAMP1-pTRIPz and mutLAMP1-pTRIPz using LALIGN software. Red box indicates the sequence of tyrosine (TAT) in wild-type LAMP1 mutated to alanine (GCT) in mutLAMP1 vector. **(C)** Scheme of transduction of mutLAMP1-pTRIPz in B16F1 cells and stable selection of mutLAMP1 clones.

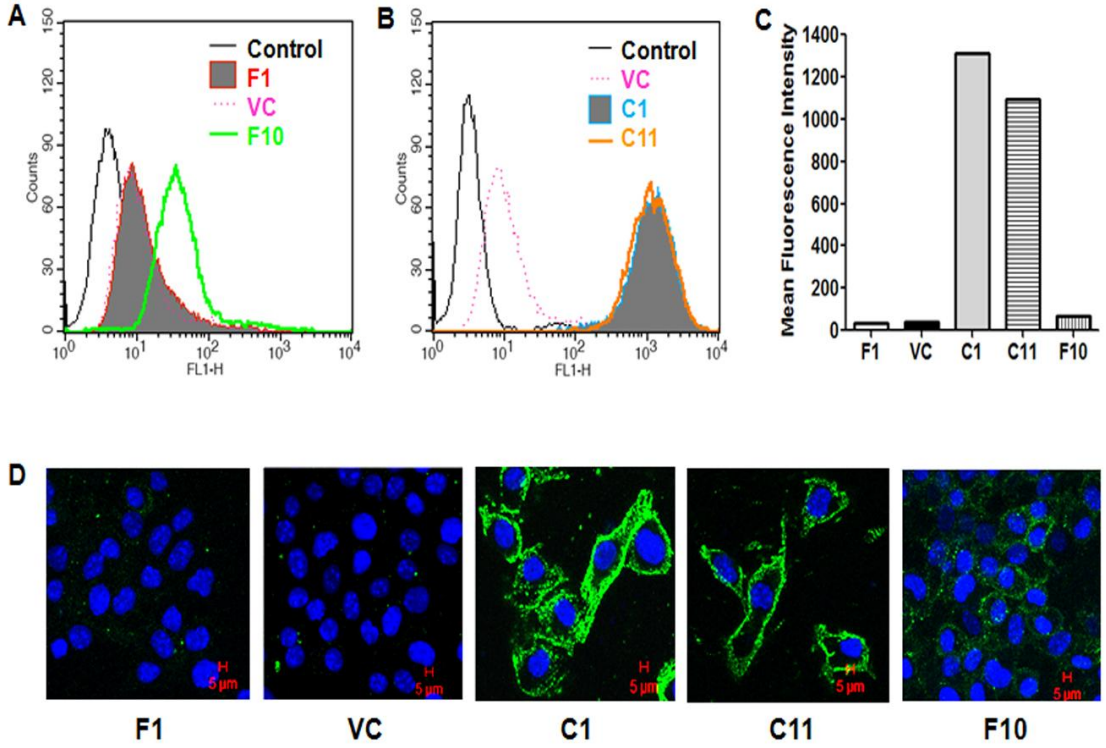

Figure 4. Analysis of LAMP1 expression on the surface of melanoma cells. Comparison of surface expression of LAMP1 by flow cytometry, in **(A)** B16F1 cells (**F1**), B16F1 cells infected with viruses having empty vector control (**…VC**) and B16F10 cells (**F10**) and in **(B)** between vector control (**…VC**) and B16F1 clones expressing mutLAMP1, (**C1**) and (**C11**). Cells treated with only anti-rat FITC served as control (—). The flow cytometry overlays have been split into two for better understanding of the data. **(C)** Graphical representation of the mean fluorescence intensities of surface LAMP1 of the cells shown in A and B. **(D)** Immunofluorescence images of the cells stained with anti-LAMP1 antibody and FITC labelled secondary antibody (green). Nuclei were stained with DAPI (blue). Scale bar = 5 µm.

3.3. Increased surface expression of LAMP1 on B16F1 cells results in significantly increased spreading and motility on fibronectin and matrigel.

Purified LAMP1 has been shown to have an affinity for ECM and BM components [189]. It is possible that the LAMP1 overexpressed on the surface is used as an alternate receptor for these components. Spreading of cells is a prerequisite for cellular motility and thus dictates the extent of movement of cells on a particular substrate. The clones overexpressing LAMP1 on the cell surface indeed showed significantly higher spreading on both fibronectin (ECM component) and matrigel (reconstituted BM) as compared to vector control (VC), as seen by laser confocal microscopic images **(Fig. 5A & B)** and by analyzing ratios of the cytoplasmic to nuclear areas **(Fig. 5C & D)**. Altered motility on ECM and BM is one of the prominent characteristics of metastatic cells. The clones also showed much higher motility on these substrates as measured by wound healing assay (**Fig. 6A & C** for fibronectin and **Fig. 6B & D** for matrigel). The results thus strongly indicated that the increased surface LAMP1 may alter the cellular properties which might eventually be important for metastasis.

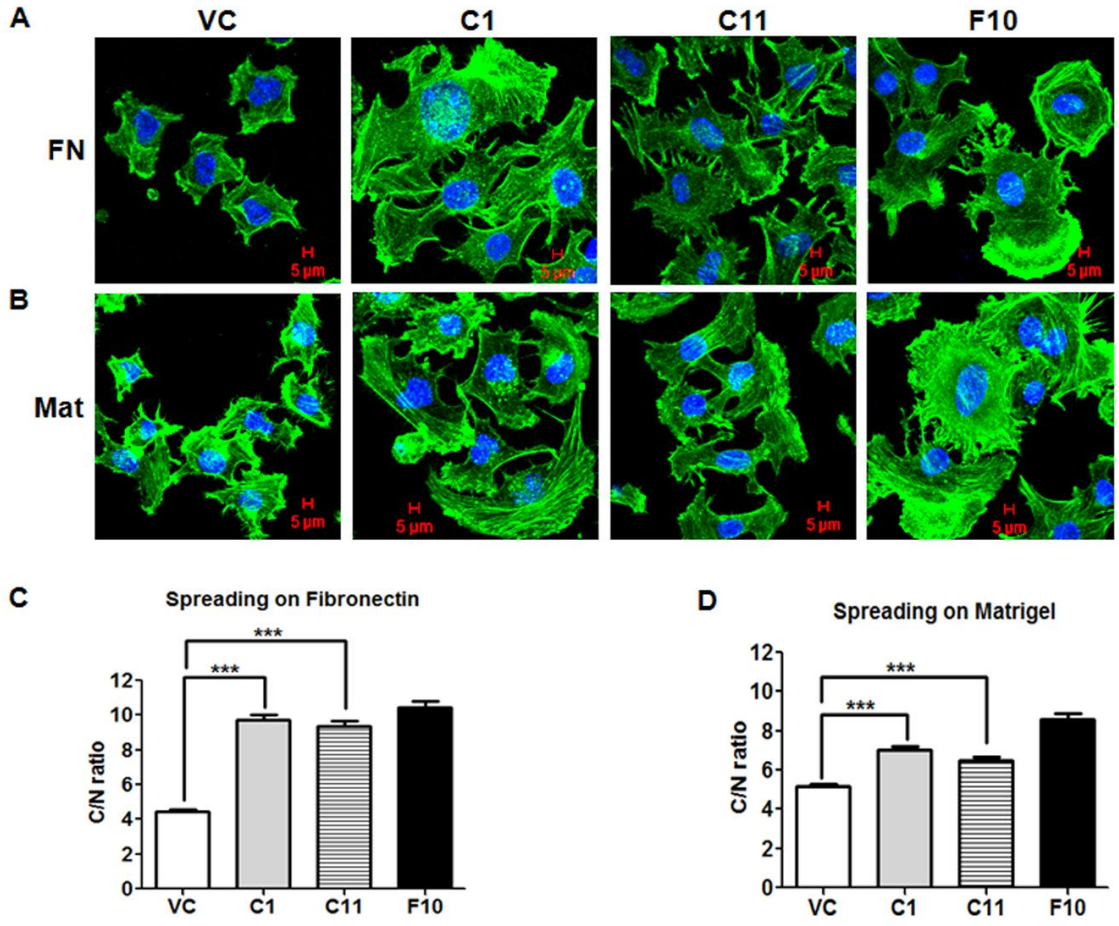

Figure 5. Effect of increased surface expression of LAMP1 on spreading of melanoma cells on fibronectin and matrigel. Spreading of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **(A)** fibronectin (FN) and **(B)** matrigel (Mat) coated coverslips as assessed by staining with Phalloidin-FITC (green). DAPI was used to stain the nuclei (blue). Scale bar = 5 µm. **(C)** and **(D)** Each bar represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments for spreading on fibronectin (C) and matrigel (D). One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ***, p value < 0.0001).

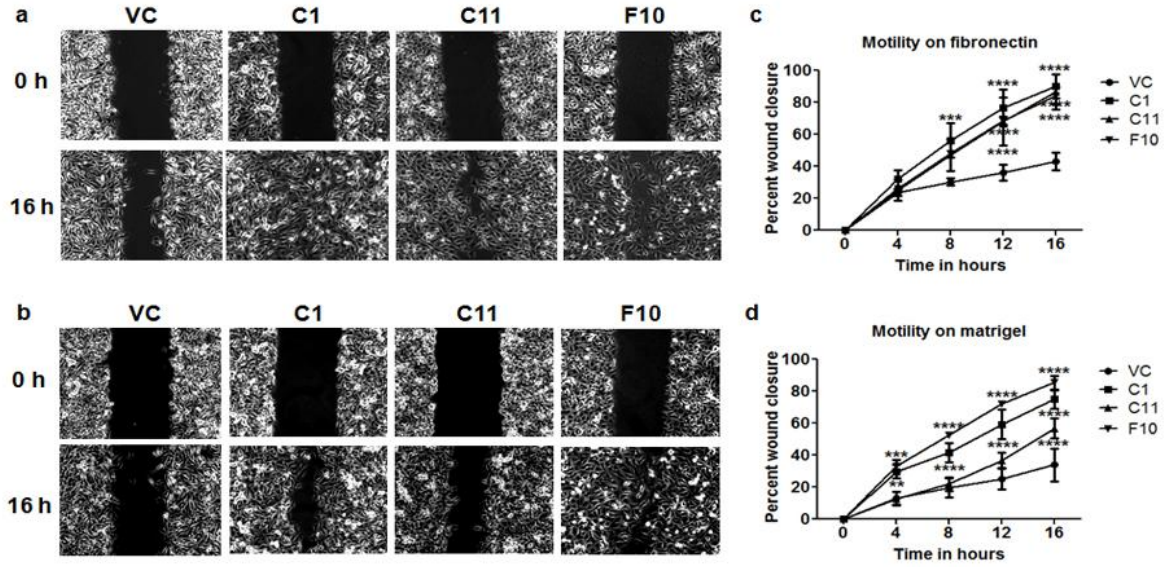

Figure 6. Motility of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **a** fibronectin and **b** matrigel coated plates as represented by time lapse video microscopy images at 0 and 16 h of wound closure. **c** and **d** represent mean percent wound closure at 4 h interval on fibronectin and matrigel respectively. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ****, p value < 0.0001, ***, p value < 0.001).

3.4. Increased expression of LAMP1 on the surface of B16F1 cells had no effect on their spreading and motility on galectin-3.

LAMP1 is a major carrier of polyLacNAc and is a known ligand for galectin-3. Secreted galectin-3 often becomes part of the ECM, BM and even the cell surface [160] and is used as a substratum for cellular adhesion, spreading and movement. Surprisingly, the increased surface expression of LAMP1 had no effect on spreading of these cells (C1 & C11) on galectin-3 as compared to the vector control cells (VC) and the spreading was very similar to that seen on uncoated coverslips, as seen by laser confocal microscopic images **(Fig. 7A & B)** and ratios of cytoplasmic to nuclear areas **(Fig. 7C & D)**. Besides, motility of these cells (C1 & C11) was also almost similar to vector control cells (VC) in presence of either BSA **(Fig. 8A & B)** or immobilized galectin-3 **(Fig. 8C & D)**. PolyLacNAc substitutions on β1,6 branched N-oligosaccharides of these melanoma cells have been shown to be the major participants in galectin-3 mediated processes [190]. B16F1 cells have lower levels of enzymes catalyzing the formation of these oligosaccharides. The lack of any effect is possibly because of low levels of polyLacNAc substitutions on each LAMP1 molecule overexpressed on the cell surface.

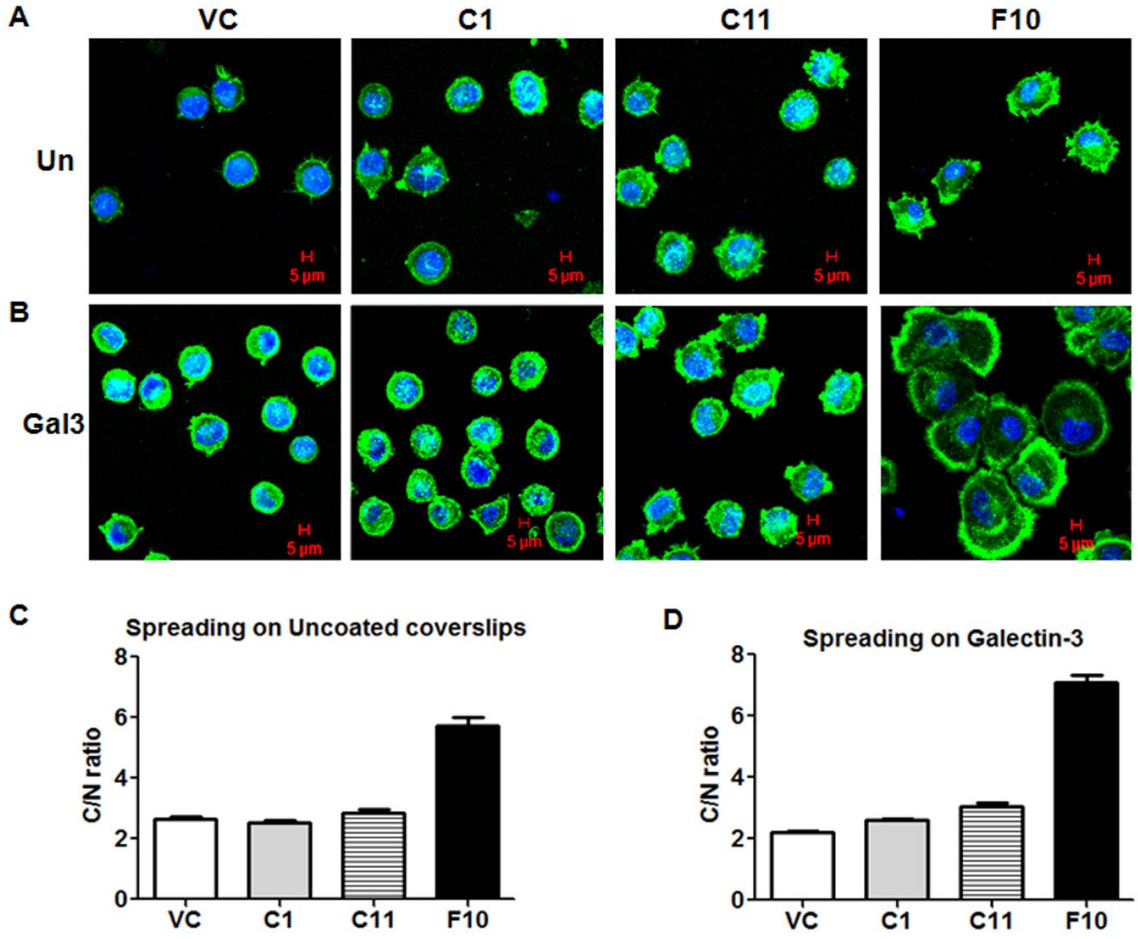

Figure 7. Effect of increased surface expression of LAMP1 on spreading of melanoma cells on galectin-3. Spreading of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **(A)** uncoated (Un) and **(B)** galectin-3 (Gal3) coated coverslips as assessed by staining with Phalloidin-FITC (green). DAPI was used to stain the nuclei (blue). Scale bar = 5 µm. **(C)** and **(D)** Each bar represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments for spreading on uncoated (C) and galectin-3 coated coverslips (D). One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance.

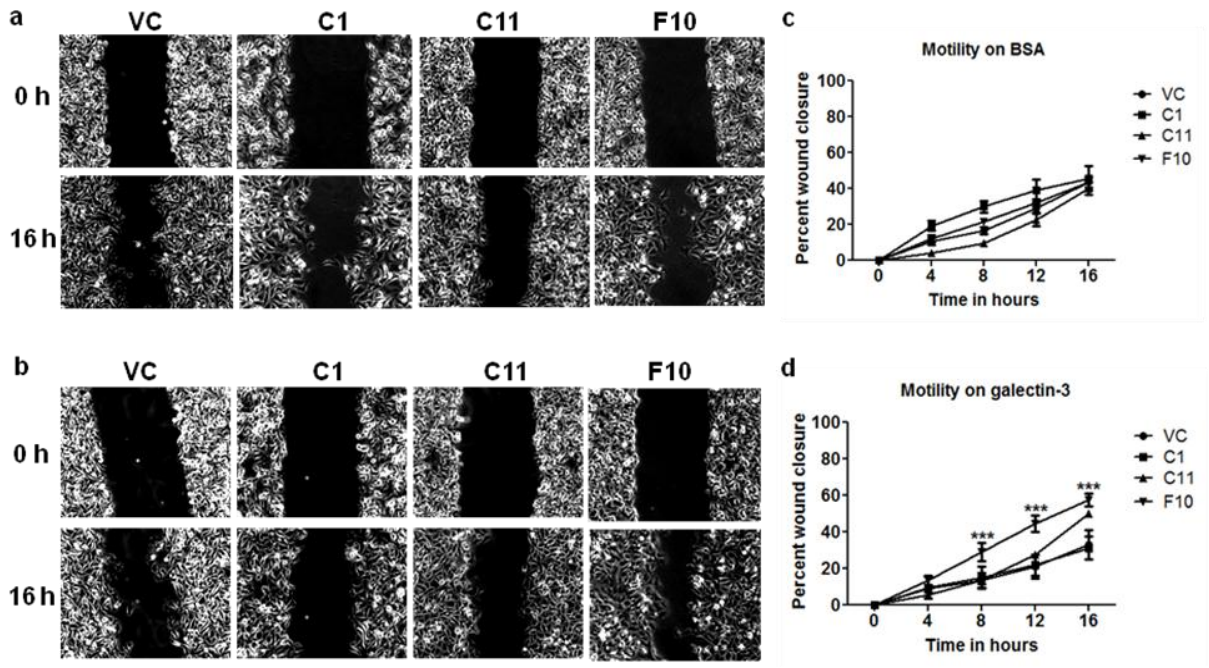

Figure 8. Effect of increased surface expression of LAMP1 on motility of melanoma cells on galectin-3. Motility of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **a** BSA and **b** galectin-3 coated plates as represented by time lapse video microscopy images at 0 and 16 h of wound closure. **c** and **d** represent mean percent wound closure at 4 h interval on BSA and galectin-3 respectively. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ***, p value < 0.001).

3.5. Increased surface expression of LAMP1 neither increases galectin-3 binding to B16F1 cells nor their metastatic potential.

LAMP1 is a highly glycosylated molecule and is a major carrier of polyLacNAc. In spite of >20 fold increase in expression of LAMP1 on the surface of clones C1 and C11 as compared to even B16F10 cells (Fig. 4), it did not result in any gain of their metastatic potential as compared to the parent B16F1 cells **(Fig. 9A)**. Further, the binding of galectin-3 to these cells remained largely unaltered **(Fig. 9B & C)**. It was possibly because of low levels of polyLacNAc substituted β1,6 branched N-oligosaccharides on each LAMP1 molecule overexpressed on cell surface. Immunoprecipitation experiment indeed showed that β1,6 branched N-oligosaccharides and polyLacNAc on LAMP1 from VC and C1 cells were not much different and were much lower as compared to that present on LAMP1 from F10 cells **(Fig. 9D)**. This suggests that carbohydrates on LAMP1 may be equally important for metastasis and hence may play a crucial role in metastasis of melanoma cells to lungs.

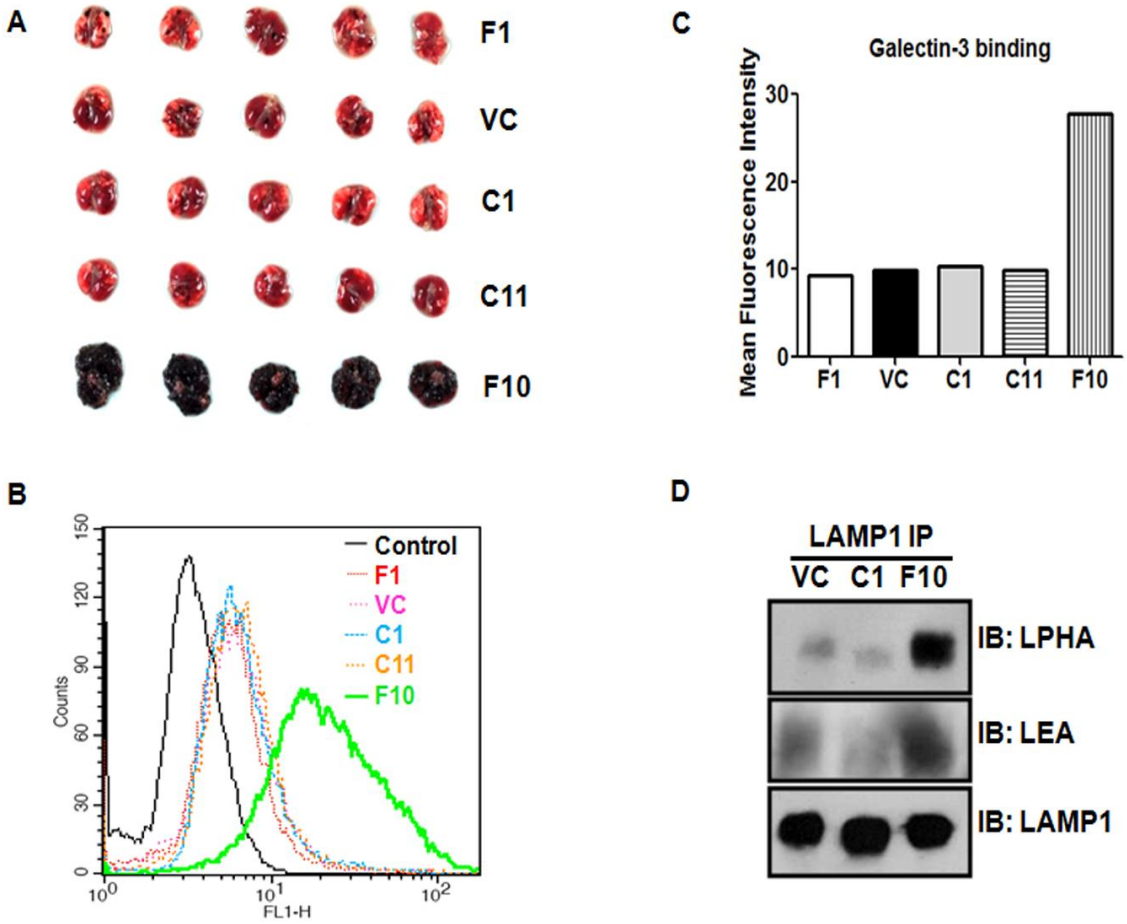

Figure 9. Increased surface expression of LAMP1 on B16F1 cells has no effect on lung metastasis. (A) Melanoma colonies on lungs of C57BL/6 mice injected with F1, VC, C1, C11 and F10 cells. **(B)** Comparison of galectin-3 binding using biotinylated galectin-3, by flow cytometry in uninfected B16F1 cells (..... **F1**) or those infected with viruses having empty vector as control (**…VC**) with B16F1 clones expressing mutLAMP1, (– – **C1**) and (–·–·– **C11**) and with B16F10 cells (— **F10**). Cells treated with only extra-avidin FITC (—) served as control. **(C)** Graphical representation of the mean fluorescence intensities of galectin-3 binding of all the cells. **(D)** Comparison of β1,6 branched N-oligosaccharides (LPHA) and polyLacNAc (LEA) on normalized amounts of immunoprecipitated LAMP1 from B16F10 (F10) cells and B16F1 cells having either empty vector (VC) or mutLAMP1 (C1), by Western blotting.

Overexpression of LAMP1 on the surface of B16F1 cells did not enhance the metastatic potential of these cells, possibly because of lower levels of glycosylation in the form of polyLacNAc on each LAMP1 molecule. LAMP1 that is substituted with high levels of polyLacNAc has been shown to have a higher residency in the Golgi [191]. In B16F10 cells, LAMP1 is highly substituted with polyLacNAc. It is possible that longer residency and accumulation of LAMP1 due to high levels of polyLacNAc, in the trans-Golgi network, results in mis-targeting of some of the LAMP1 molecules to the cell surface instead of lysosomes in these metastatic cells. In this scenario, it would be interesting to study the effect of inhibition of these oligosaccharides in B16 melanoma cells on the cell surface expression of LAMP1.

3.6. Treatment of B16F1 and B16F10 cells with swainsonine results in decreased glycosylation and consequently decreased surface expression of LAMP1.

B16F1 and B16F10 melanoma cells were treated with swainsonine, an α-mannosidase-II inhibitor, to prevent the formation of complex-type N-oligosaccharides. Effect of treatment of B16F1 and B16F10 cells with swainsonine on the levels of β1,6 branched N-oligosaccharides and polyLacNAc was determined by flow cytometry. The treatment resulted in decrease in levels of both β1,6 branched N-oligosaccharides **(Fig. 10A & 11A)** as well as polyLacNAc **(Fig. 10B & 11B)** thus confirming the inhibition of these oligosaccharides. Surprisingly, on inhibition of glycosylation, there was decrease in surface expression of LAMP1 as well **(Fig. 10C & 11C)** in these cells. Moreover, the total levels of LAMP1 remained unaltered **(Fig. 10D & 11D)** indicating that inhibition of glycosylation had no effect on stability of LAMP1 protein per se. Furthermore, the shift in molecular weight of LAMP1 of SW treated B16F1 cells in Fig. 10D & 11D indicates the loss of glycosylation on LAMP1 as a result of

swainsonine treatment. These results clearly indicate the role of glycosylation on LAMP1 in its surface translocation.

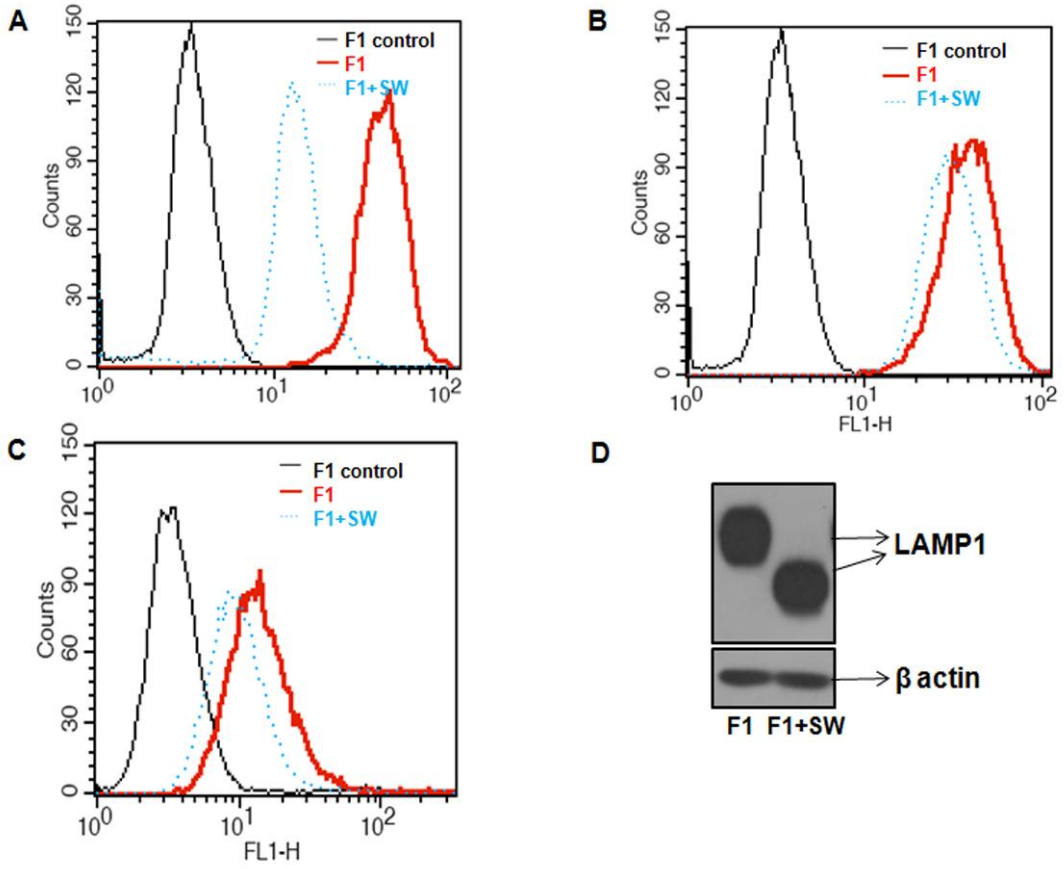

Figure 10. Analysis of surface expression of β1,6 branched N-oligosaccharides, polyLacNAc and surface as well as total LAMP1 in untreated B16F1 cells and those treated with swainsonine. Comparison of surface expression of **(A)** β1,6 branched N-oligosaccharides using biotinylated LPHA, **(B)** polyLacNAc using biotinylated LEA and **(C)** LAMP1 using rat anti-LAMP1 antibody, in untreated B16F1 cells (F1, red line) and those treated with swainsonine (F1+SW, sky blue line) using flow cytometry. B16F1 cells treated only with either extra-avidin-FITC (A and B) or anti-rat FITC (C) served as control (black line). **(D)** Comparison of total LAMP1 in F1 & F1+SW cells by Western blotting. β-actin served as a loading control.

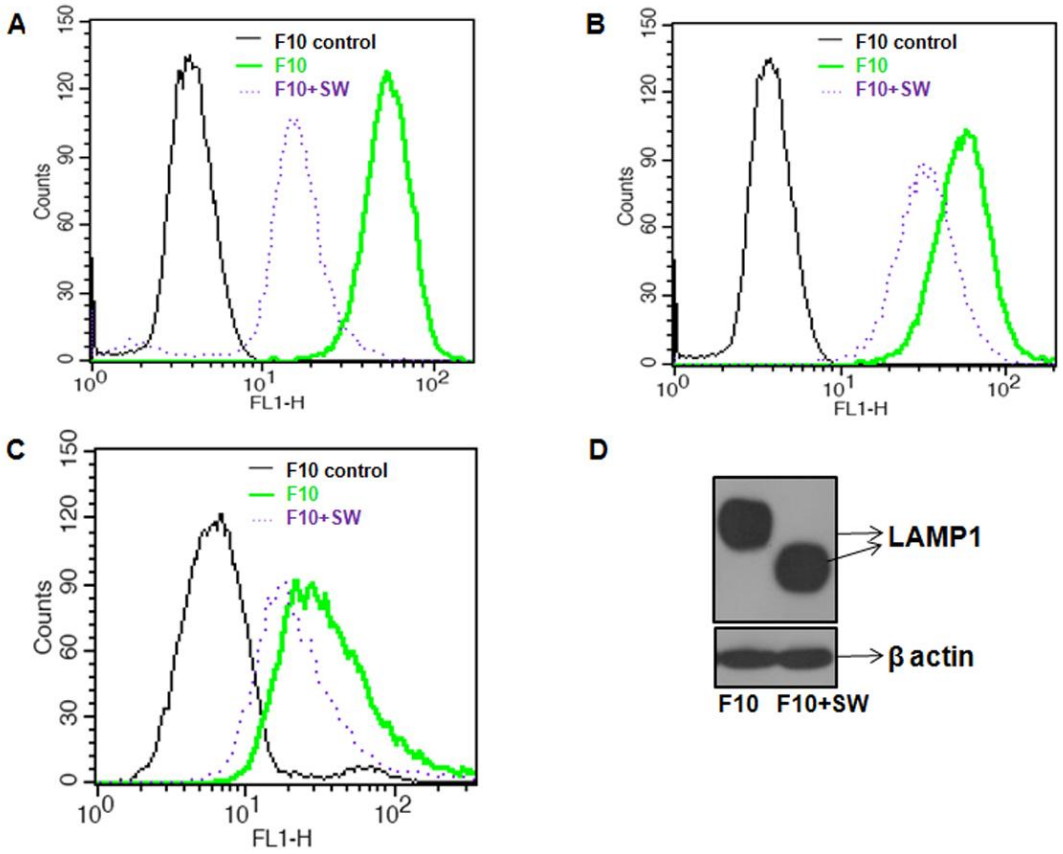

Figure 11. Analysis of surface expression of β1,6 branched N-oligosaccharides, polyLacNAc and surface as well as total LAMP1 in untreated B16F10 cells and those treated with swainsonine. Comparison of surface expression of **(A)** β1,6 branched N-oligosaccharides using biotinylated LPHA, **(B)** polyLacNAc using biotinylated LEA and **(C)** LAMP1 using rat anti-LAMP1 antibody, in untreated B16F10 cells (F10, green line) and those treated with swainsonine (F10+SW, purple line) using flow cytometry. B16F10 cells treated only with either extra-avidin-FITC (A and B) or anti-rat FITC (C) served as control (black line). **(D)** Comparison of total LAMP1 in F10 & F10+SW cells by Western blotting. β-actin served as a loading control.

Although, there was no increase in β1,6 branched N-oligosaccharides and polyLacNAc levels on LAMP1 overexpressed on surface of B16F1 cells (Fig. 9D), they could greatly alter the cellular properties on ECM and BM components. To confirm that this effect on cellular properties was glycosylation independent, the effect of inhibition of glycosylation on surface translocation of mutLAMP1 per se and in these cellular properties was investigated.

3.7. Inhibition of glycosylation has no effect on the surface expression of mutLAMP1 or on the spreading of cells expressing them on fibronectin and matrigel.

The mutLAMP1 expressed in B16F1 cells largely remains on the cell surface. These cells were treated with SW to investigate the effect of inhibition of glycosylation on mutLAMP1 surface expression and on spreading of cells on fibronectin and matrigel. Results indicated that although swainsonine treatment significantly reduced the surface levels of β1,6 branched N-oligosaccharides in these cells **(Fig. 12A & B)**, it hardly had any effect on surface expression of LAMP1 on these cells **(Fig. 12C & D)**. Reduced surface levels of β1,6 branched N-oligosaccharides also did not significantly influence their spreading on fibronectin **(Fig. 13A & C)** as well as matrigel **(Fig. 13B & D)**. These results indicate that glycosylation does not influence the surface expression of mutLAMP1. Secondly, the increased spreading on fibronectin and matrigel of cells expressing such LAMP1 also remained unaffected by glycosylation inhibition, suggesting that spreading on these substrates is largely through LAMP1 protein.

This clearly indicated that the increased surface expression of mutLAMP1 in B16F1 cells as well as their spreading on fibronectin and matrigel was independent of glycosylation and the effect was solely mediated by mutLAMP1 protein and not its glycosylation.

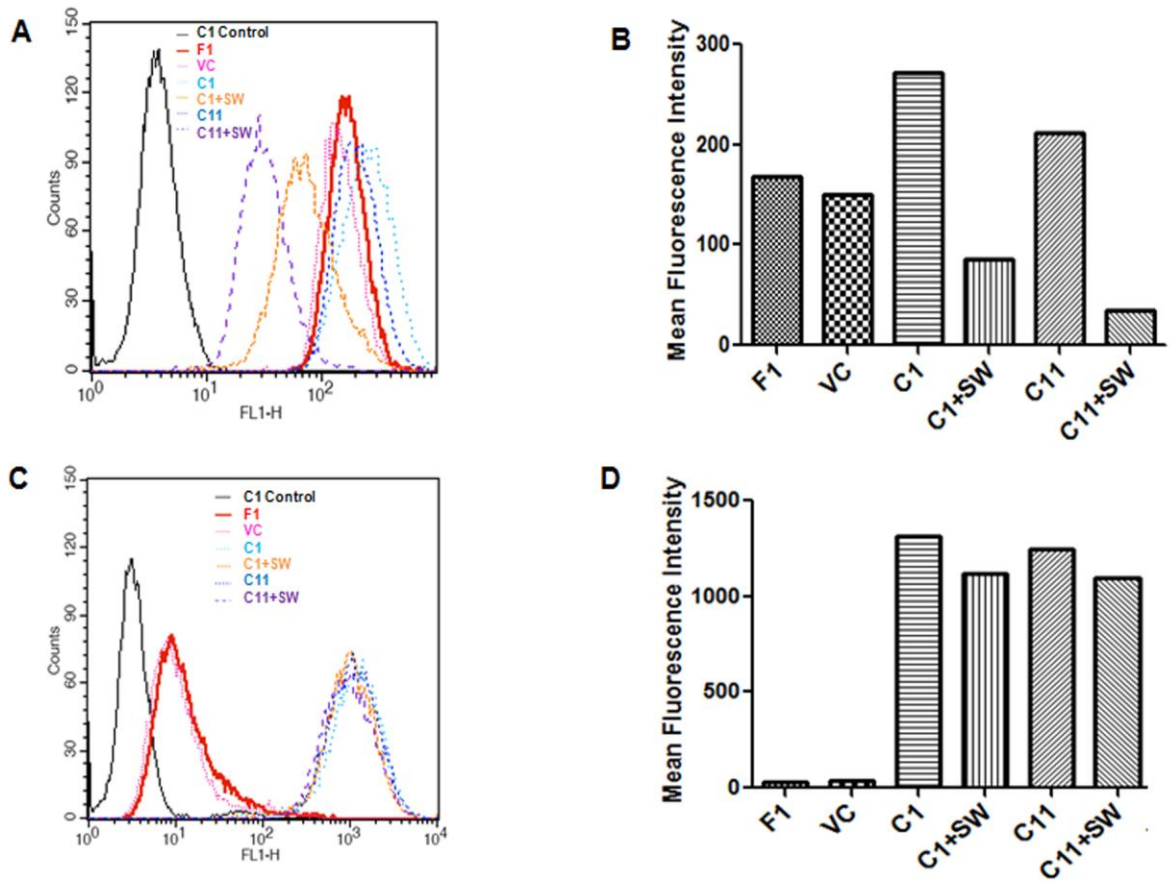

Figure 12. Analysis of surface expression of β1,6 branched N-oligosaccharides and LAMP1 in clones expressing mutLAMP1, upon swainsonine treatment. Comparison of surface expression of **(A)** β1,6 branched N-oligosaccharides using biotinylated LPHA and **(C)** LAMP1 using rat anti-LAMP1 antibody, in uninfected B16F1 cells (**F1**) or those infected with viruses having empty vector (**VC**) or expressing mutLAMP1, (**C1**) and (**C11**) and the mutLAMP1 clones treated with swainsonine (**C1+SW**, **C11+SW**) using flow cytometry. Cells treated only with either extra-avidin-FITC (A) or anti-rat FITC (C) served as control (black line). **(B)** Graphical representation of the mean fluorescence intensities of β1,6 branched N-oligosaccharides of the cells shown in A. **(D)** Graphical representation of the mean fluorescence intensities of surface LAMP1 of the cells shown in C.

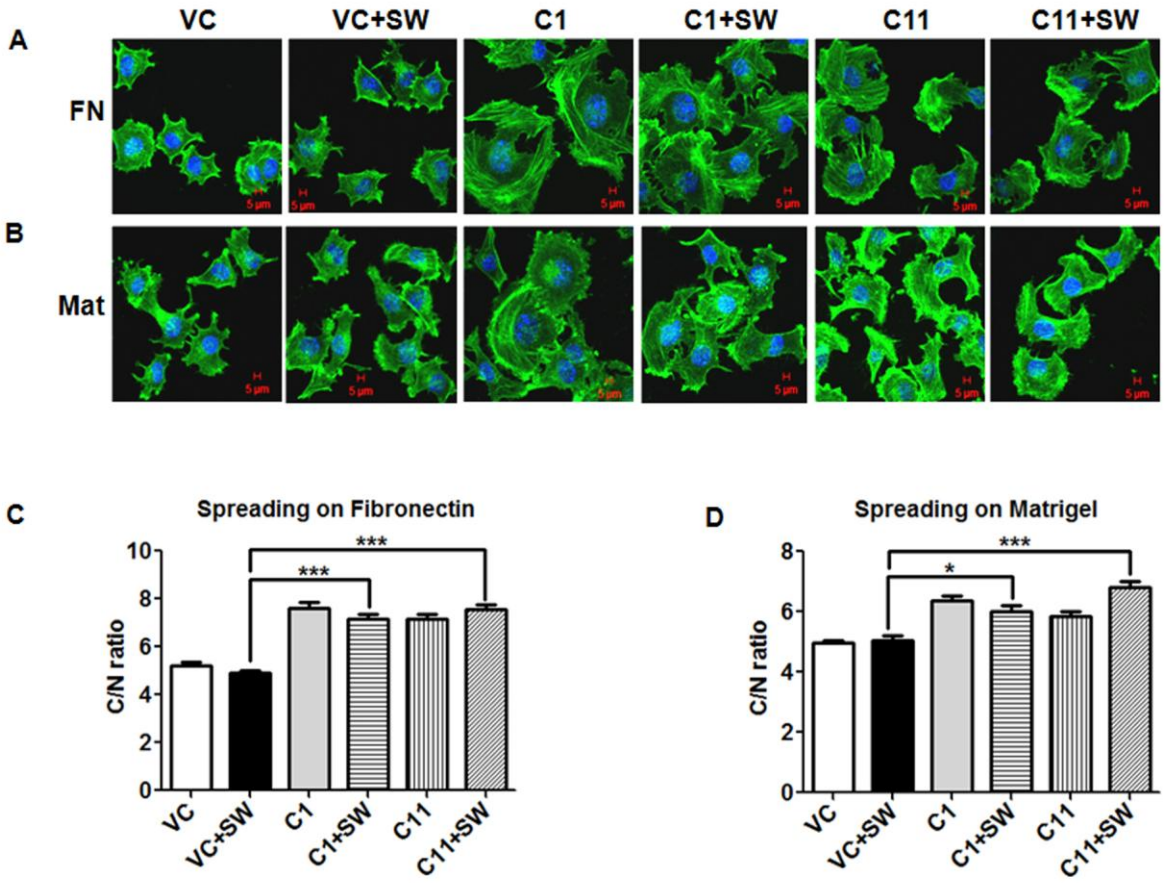

Figure 13. **Effect of swainsonine treatment on spreading of mutLAMP1 clones on fibronectin and matrigel.** Spreading of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and their swainsonine treated counterparts (VC+SW, C1+SW and C11+SW) on **(A)** fibronectin (FN) and **(B)** matrigel (Mat) coated coverslips as assessed by staining with Phalloidin-FITC (green). DAPI was used to stain the nuclei (blue). Scale bar = 5 μm. **(C)** Each bar represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments for spreading on fibronectin (FN) and **(D)** for spreading on matrigel (Mat). One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ***, p value < 0.0001, *, p value < 0.01).

Increased expression of LAMP1 on the surface of B16F1 cells using mutLAMP1 did neither enhance their galectin-3 mediated properties nor their metastatic potential. Therefore, it was futile to study the effect of inhibition of glycosylation on these properties as well as their metastatic ability.

The cellular and lysosomal morphology of cells in LAMP1-deficient mice remains unaffected and even the animals remain absolutely viable and fertile. LAMP2 appears to take over the function of LAMP1 in these cells suggesting that LAMP1 is dispensable for normal lysosomal functions [127]. Downregulating the expression of LAMP1 in high metastatic B16F10 cells thus should result in its decreased expression on the cell surface without affecting other normal cellular functions. Downregulation of LAMP1 may also result in significant reduction of polyLacNAc substituted β1,6 branched N-oligosaccharides on the cell surface. LAMP1 was therefore downregulated in B16F10 cells using shRNA approach. Further, its effect on their cellular properties including experimental metastasis was investigated.

3.8. Cloning of LAMP1 shRNAs in pTRIPz vector and its expression in high metastatic B16F10 cells results in inducible expression of LAMP1 shRNAs.

To downregulate LAMP1 in B16F10 cells, two shRNA sequences specific for LAMP1 **(Fig. 14A)** were cloned in an inducible lentiviral vector pTRIPz **(Fig. 14B)** between its XhoI and EcoRI sites. The PCR amplified and XhoI and EcoRI digested LAMP1 shRNAs **(Fig. 14C)** were ligated to the pTRIPz vector digested with the same enzymes **(Fig. 14D)**. The positive colonies screened using AgeI and EcoRI digestion **(Fig. 14E)** were then used to generate lentiviral particles in HEK293FT cells which were further transduced in B16F10 cells. Inducible expression of NT shRNA and LAMP1 shRNAs was confirmed by observing for

RFP fluorescence after induction by doxycycline **(Fig. 15)**. These cells expressing RFP were sorted on FACS Aria (BD Biosciences) to obtain a homogeneous population expressing the respective shRNAs.

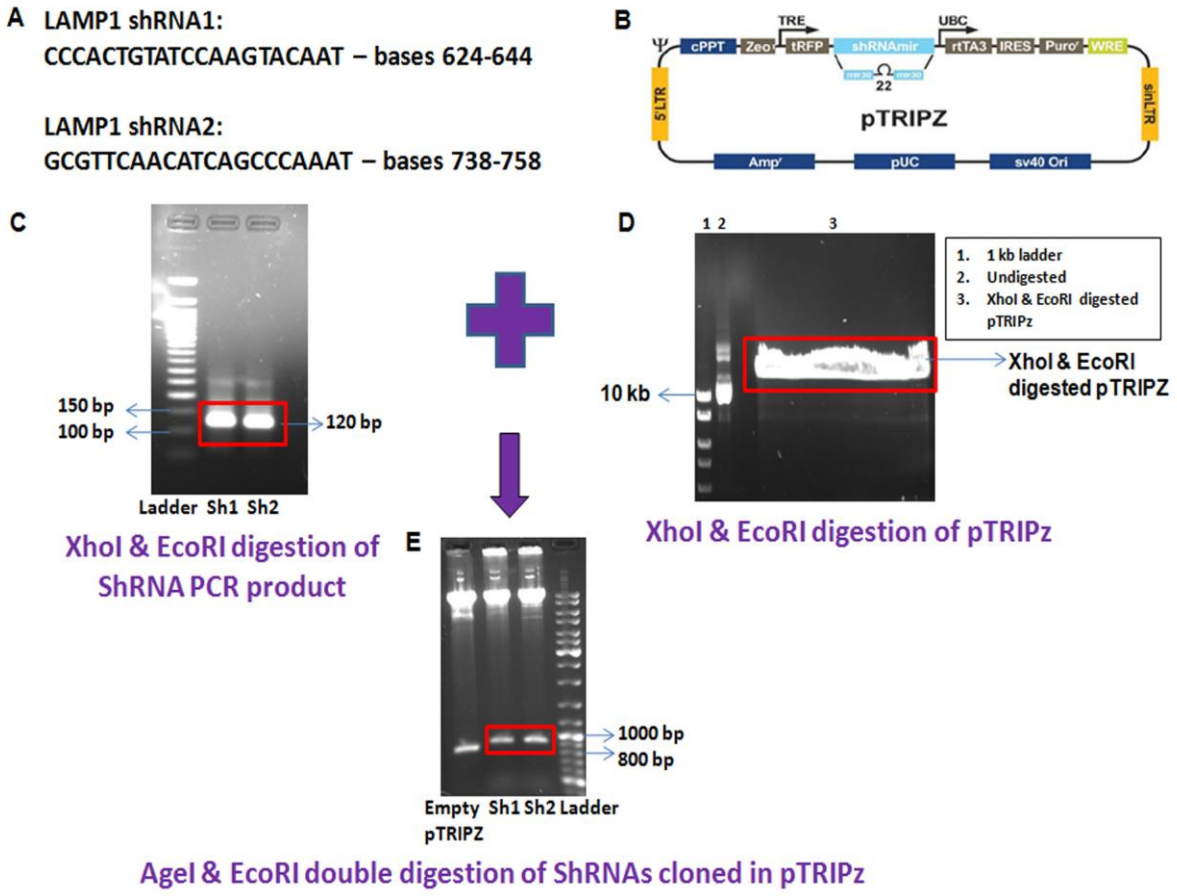

Figure 14. Cloning of LAMP1 shRNAs in pTRIPz lentiviral vector. (A) Sequences of LAMP1 shRNAs 1 and 2. **(B)** Features of pTRIPz lentiviral vector. **(C)** AGE of PCR amplified and XhoI and EcoRI digested LAMP1 shRNAs (120 bp). **(D)** AGE of XhoI and EcoRI digested pTRIPz. **(E)** AGE of EcoRI and AgeI digested LAMP1 shRNA-pTRIPz vectors.

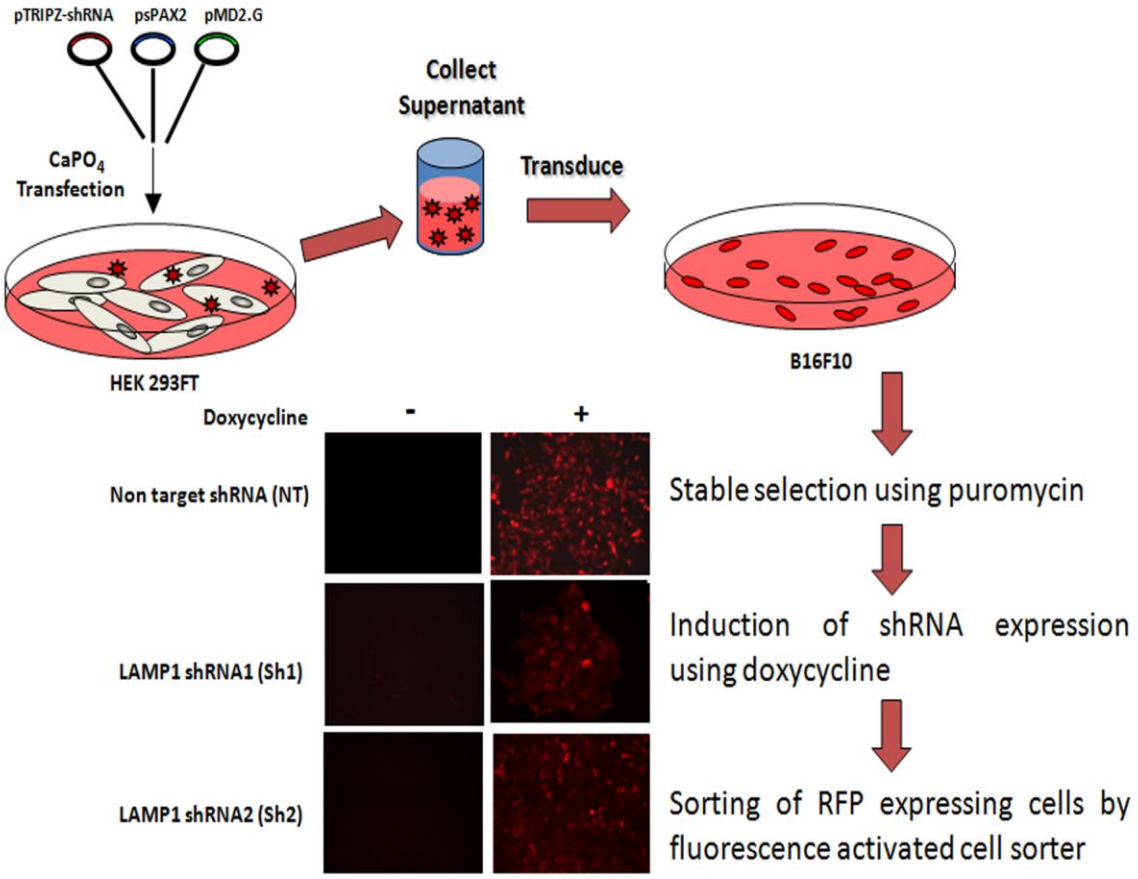

Figure 15. Transduction and expression of LAMP1 shRNAs in B16F10 cells. Scheme of transduction and inducible expression of LAMP1 shRNAs cloned in pTRIPz in B16F10 cells.

3.9. Expression of LAMP1 shRNAs in B16F10 cells results in decreased levels of total as well as surface LAMP1.

Surface expression of LAMP1, but not LAMP2 has previously been shown to correlate with metastatic potential of B16 melanoma cells [51]. However, its direct involvement in promoting metastasis still remains to be established. To conclusively ascertain its role, LAMP1 was downregulated in high metastatic B16F10 cells using two different inducible shRNAs specific for LAMP1 (Sh1 and Sh2). An inducible non-targeting shRNA (NT) was also expressed as control. The induction of non-targeting shRNA (NT) clone had no effect on total or surface levels of LAMP1 and was equal to that of untransduced B16F10 cells and thus served as an apt control **(Fig. 16A & B).** On the other hand, on induction of expression

of LAMP1 shRNAs (Sh1 & Sh2), there was significant reduction in total LAMP1 in both the shRNA clones (Sh1 & Sh2) as seen by Western blotting **(Fig. 16A)** as well as their cell surface LAMP1 as seen by flow cytometry in Sh1 **(Fig. 16C)** and Sh2 **(Fig. 16D)** clones, with Sh1 showing higher downregulation compared to Sh2 clone.

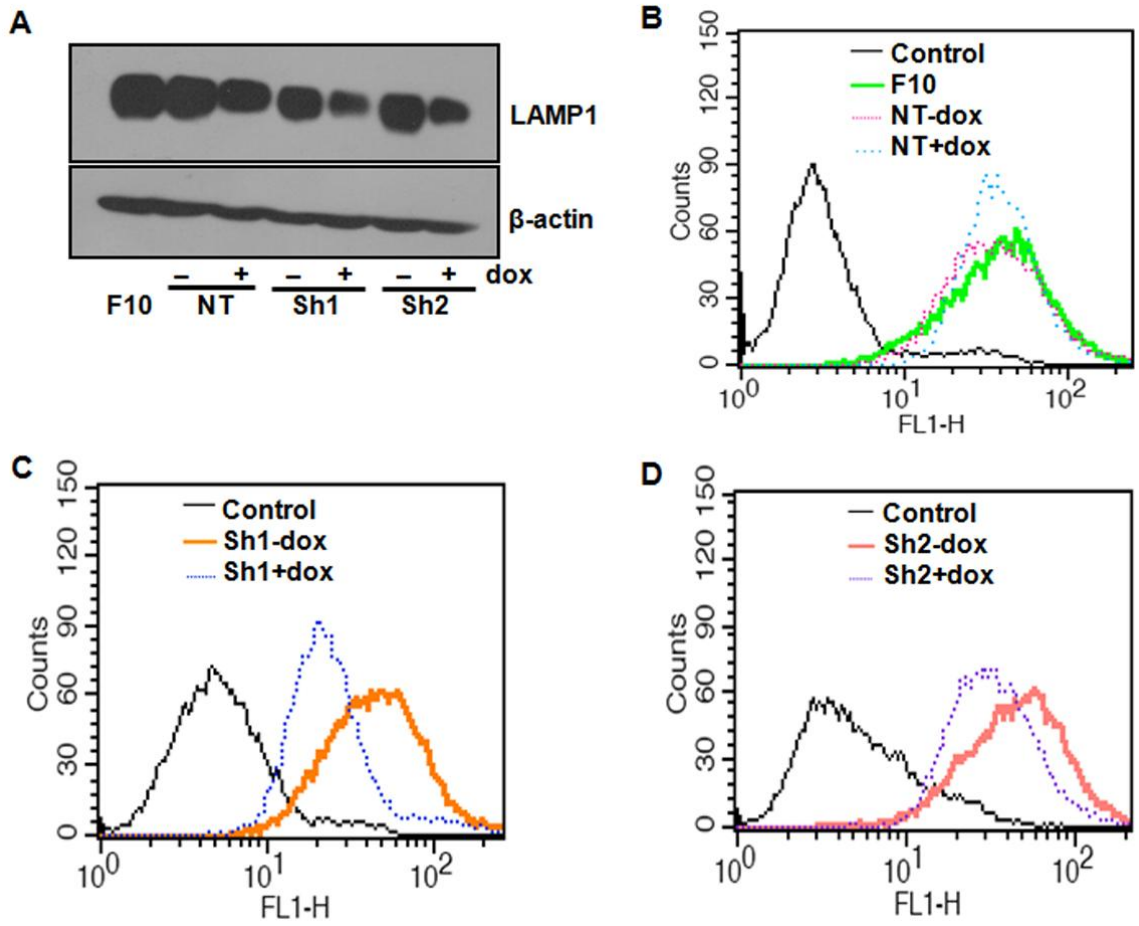

Figure 16. Analysis of total and surface expression of LAMP1 in B16F10 cells transduced with LAMP1 shRNAs. Comparison of LAMP1 expression in B16F10 cells and its clones transduced with either non-targeting shRNA (NT), shRNA1 (Sh1) and shRNA2 (Sh2) induced with doxycycline (+dox) or under uninduced condition (-dox) by **(A)** Western blotting the cell lysates (total) or by flow cytometry (surface expression), on NT **(B)**, Sh1 **(C)** or Sh2 **(D)** clones along with B16F10 (F10) cells. Cells treated with only anti-rat FITC served as Control in (B-D). Beta actin served as a loading control in (A).

3.10. Downregulation of LAMP1 in B16F10 cells does not alter their spreading and motility on ECM and BM components.

Purified LAMP1 has been shown to have an affinity for ECM and BM components [189]. Besides, when LAMP1 is overexpressed on surface of low metastatic cells, it has a profound influence on their cellular properties such as spreading and motility on ECM and BM components (Fig. 5 & 6). The effect of LAMP1 downregulation in B16F10 cells on these cellular properties was therefore investigated. Both the induced LAMP1 shRNA clones (sh1+dox & sh2+dox) did not show any significant decrease in spreading on either fibronectin **(Fig. 17A)** or matrigel **(Fig. 17B)** as compared to uninduced LAMP1 shRNAs (sh1-dox & sh2-dox) or induced non-target shRNA control (NT+dox), as seen by laser confocal microscopic images **(Fig. 17)**. The clones also did not show any significant alterations in motility on fibronectin **(Fig. 18A-F)** as well as matrigel **(Fig. 19A-F)** on doxycycline induction as measured by wound healing assays. The results strongly indicated that the decreased surface LAMP1 did not alter the cellular properties on fibronectin and matrigel.

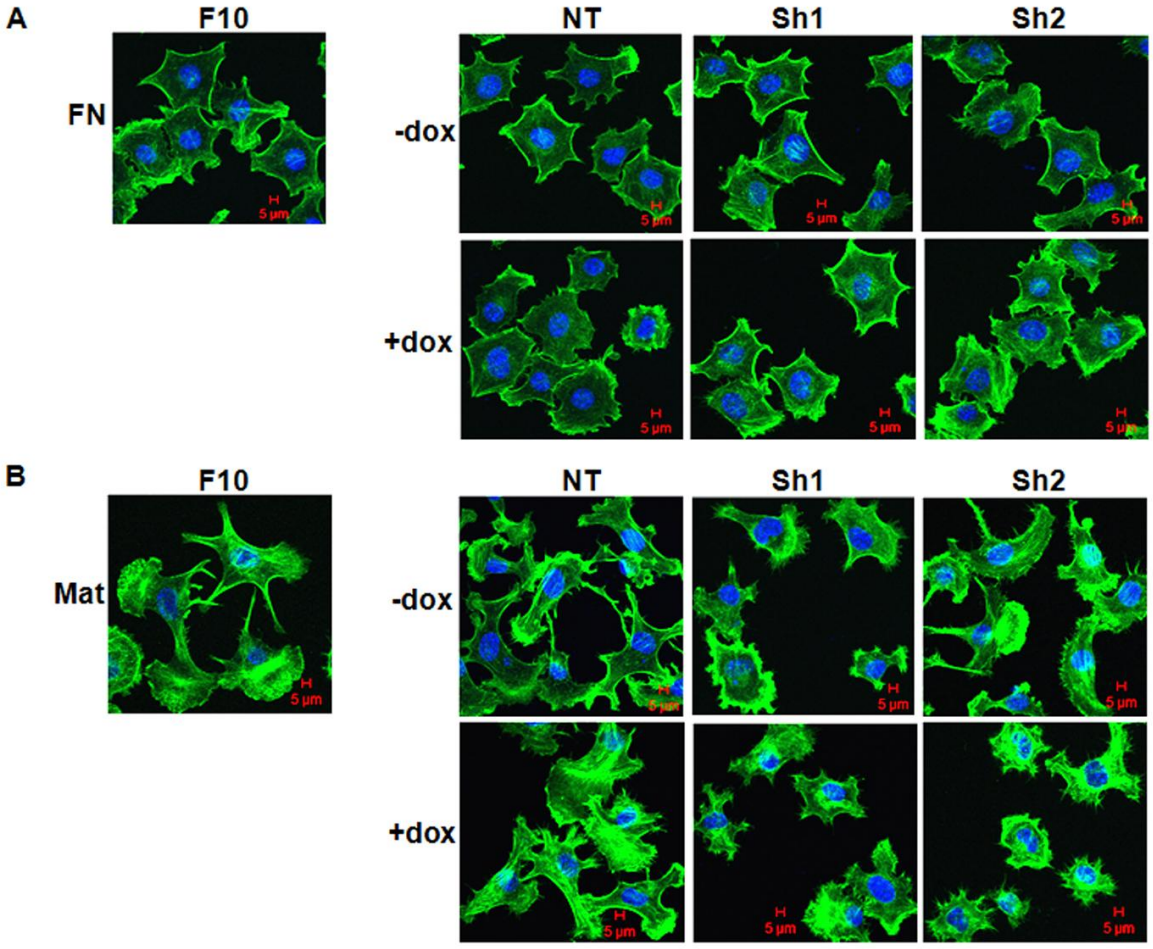

Figure 17. Effect of decreased surface expression of LAMP1 on spreading of melanoma cells on fibronectin and matrigel. Spreading of untransduced B16F10 cells (F10) and those transduced with either non-target shRNA (NT) or LAMP1 shRNAs (Sh1 & Sh2) without (-dox) or with (+dox) induction of shRNA by doxycycline on **(A)** fibronectin (FN) and **(B)** matrigel (Mat) coated coverslips as assessed by staining with Phalloidin-FITC (green). DAPI was used to stain the nuclei (blue). Scale bar = 5 μm.

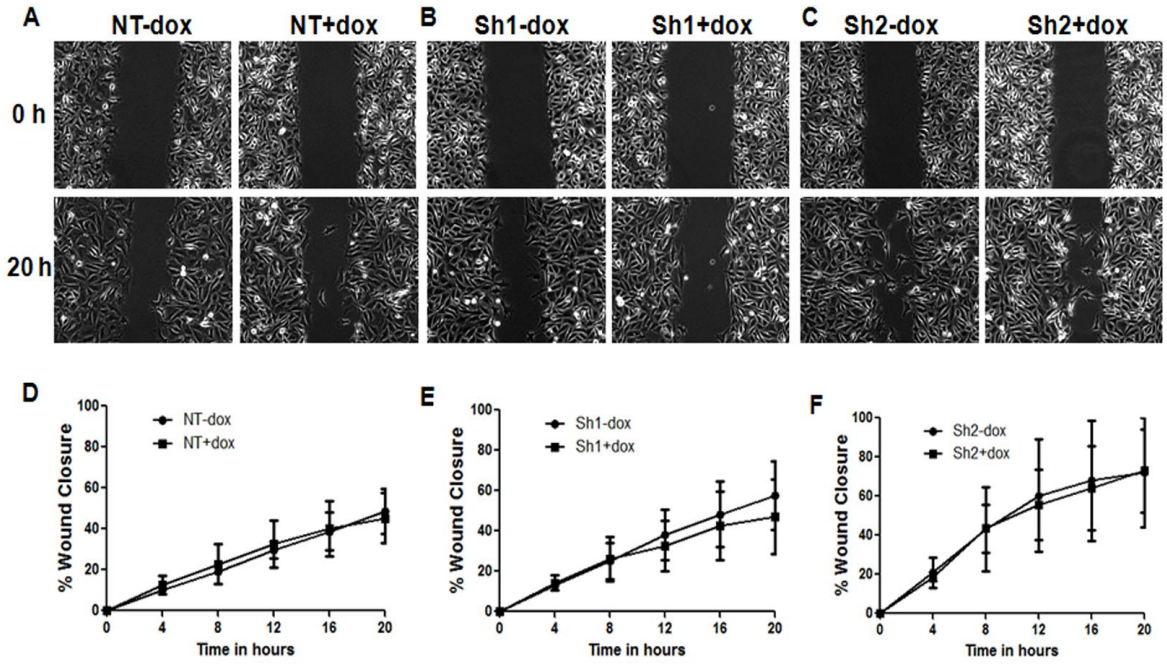

Figure 18. Effect of decreased surface expression of LAMP1 on motility of melanoma cells on fibronectin. Motility on fibronectin coated plates of B16F10 cells transduced with either **(A)** non-target shRNA (NT) or LAMP1 shRNAs, Sh1 **(B)** & Sh2 **(C)** without (-dox) or with (+dox) induction of shRNA by doxycycline as represented by time lapse video microscopy images at 0 and 20 h of wound closure. **(D)**, **(E)** and **(F)** represent mean percent wound closure at 4 h interval of NT, Sh1 and Sh2 respectively. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance.

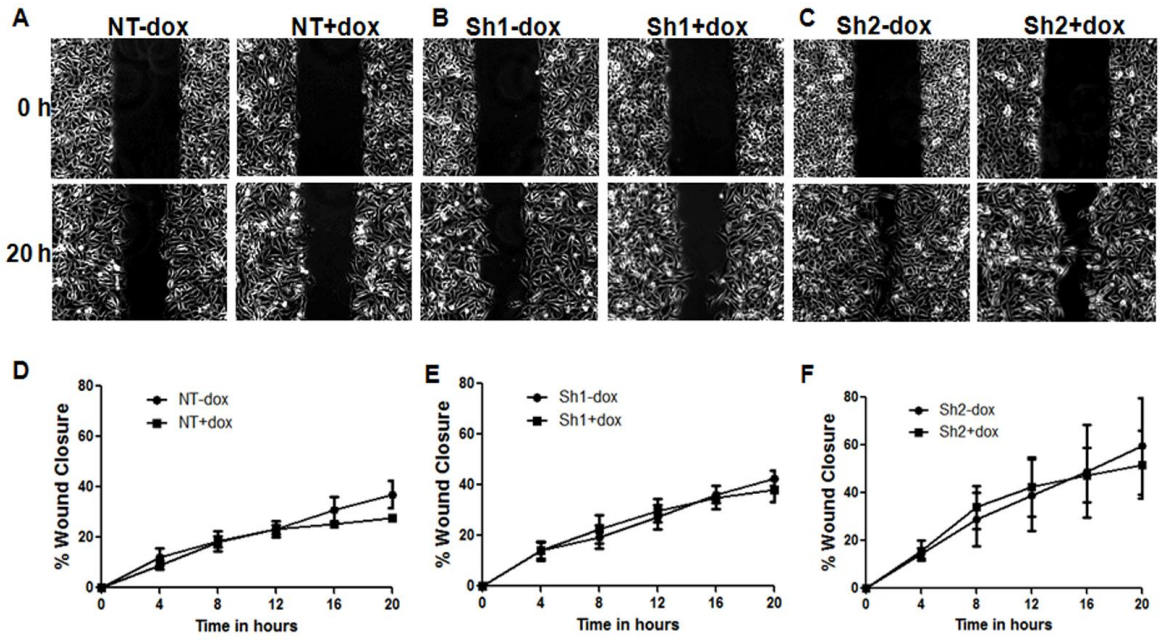

Figure 19. Effect of decreased surface expression of LAMP1 on motility of melanoma cells on matrigel. Motility on matrigel coated plates of B16F10 cells transduced with either **(A)** non-target shRNA (NT) or LAMP1 shRNAs, Sh1 **(B)** & Sh2 **(C)** without (-dox) or with (+dox) induction of shRNA by doxycycline as represented by time lapse video microscopy images at 0 and 20 h of wound closure. **(D)**, **(E)** and **(F)** represent mean percent wound closure at 4 h interval of NT, Sh1 and Sh2 respectively. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance.

3.11. Downregulation of LAMP1 in B16F10 cells results in significantly decreased spreading and motility on Galectin-3.

PolyLacNAc (specifically on N-oligosaccharides) has been shown to be the major galectin-3 ligand which mediates melanoma cell adhesion to lung vascular endothelium [190]. More recently, it was shown that blocking the availability of polyLacNAc using either truncated galectin-3 or modified citrus pectin (MCP) blocked metastasis. Moreover, inhibition of polyLacNAc using shRNAs to the enzymes involved in their synthesis was also shown to inhibit lung metastasis. In addition, besides promoting adhesion to vascular endothelium, polyLacNAc/galectin-3 interaction was shown to facilitate cellular spreading, degradation of vascular endothelium and movement into organ parenchyma [175]. Since LAMP1 is a major carrier of polyLacNAc, decreased surface expression of LAMP1 may alter the cell surface levels of polyLacNAc and hence galectin-3 binding which ultimately may also hamper all the cellular processes mediated by galectin-3. Both the LAMP1 shRNA clones showed significantly decreased spreading on galectin-3 as compared to untransduced B16F10 cells or those transduced with non-targeting shRNA (NT) in a doxycycline-inducible manner, as seen by laser confocal microscopic images **(Fig. 20A)** and by analyzing ratios of the cytoplasmic to nuclear areas **(Fig. 20B)**. The clones also showed significantly lower motility on galectin-3 on doxycycline induction as measured by wound healing assays **(Fig. 21A-F)**. The results strongly indicated that the decreased surface LAMP1 may alter the cellular properties on galectin-3 which may eventually affect lung metastasis.

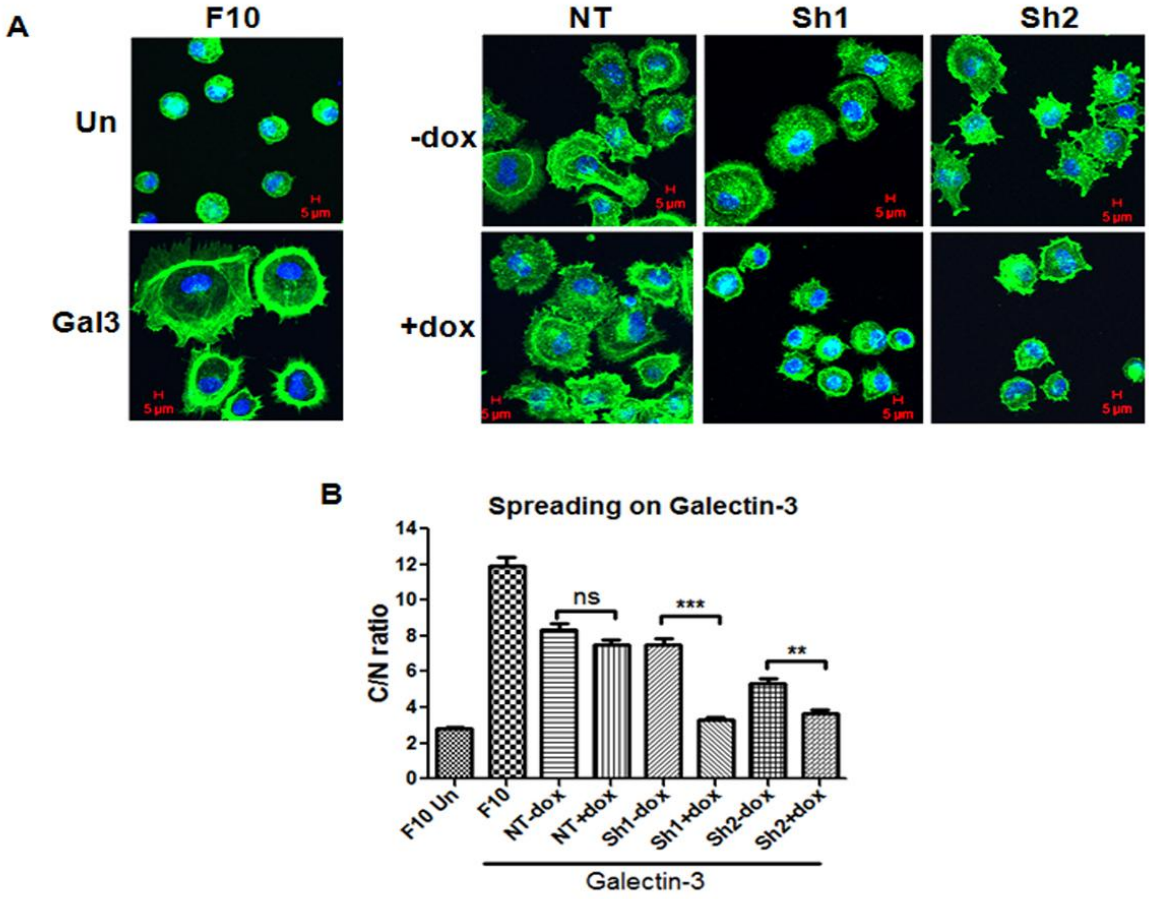

Figure 20. Effect of decreased surface expression of LAMP1 on spreading of melanoma cells on galectin-3. (A) Spreading of untransduced B16F10 cells (F10) and those transduced with either non-target shRNA (NT) or LAMP1 shRNAs (Sh1 & Sh2) without (-dox) or with (+dox) induction of shRNA by doxycycline on galectin-3 (Gal3) coated coverslips as assessed by staining with Phalloidin-FITC (green). Spreading of untransduced B16F10 cells was also seen on uncoated (Un) coverslips as control. DAPI was used to stain the nuclei (blue). Scale bar = 5 µm. **(B)** Each bar represents ratio of cytoplasmic to nuclear (C/N) area for around 50 cells from two different experiments for their spreading on uncoated or galectin-3 coated coverslips. One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ***, p value < 0.0001, **, p value < 0.001, ns, non-significant).

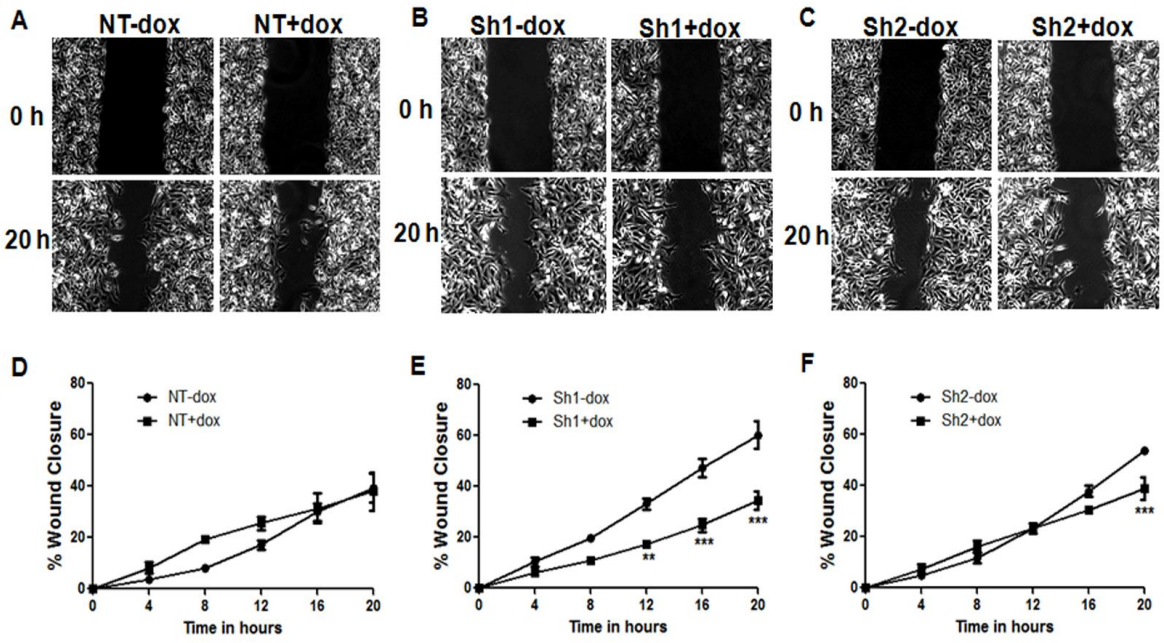

Figure 21. Effect of decreased surface expression of LAMP1 on motility of melanoma cells on galectin-3. Motility on galectin-3 coated plates of B16F10 cells transduced with either **(A)** non-target shRNA (NT) or LAMP1 shRNAs, Sh1 **(B)** & Sh2 **(C)** without (-dox) or with (+dox) induction of shRNA by doxycycline as represented by time lapse video microscopy images at 0 and 20 h of wound closure. **(D)**, **(E)** and **(F)** represent mean percent wound closure at 4 h interval of NT, Sh1 and Sh2 respectively. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ***, p value < 0.001, **, p value < 0.01).

3.12. Downregulation of LAMP1 in B16F10 cells results in decreased galectin-3 binding and significantly decreased lung metastasis of these cells.

PolyLacNAc-galectin-3 interaction has been shown to be important for lung metastasis as shRNA mediated inhibition of polyLacNAc synthesis inhibited galectin-3 mediated processes as well as lung metastasis [175]. LAMP1 being a major carrier of polyLacNAc [192], the effect of LAMP1 downregulation on binding of galectin-3 to these cells was therefore investigated. The B16F10 cells showed decreased binding of galectin-3 on induction of both the LAMP1 shRNAs, Sh1 **(Fig. 22A)** and Sh2 **(Fig. 22B)** indicating that the downregulation of LAMP1 might affect the adhesion of cells to galectin-3 present on lung endothelium. To investigate if modulation of these galectin-3 mediated properties has any effect on lung colonization, experimental metastasis assay was performed. The LAMP1 downregulated clones showed significantly decreased lung metastasis as compared to untransduced or non-targeting shRNA transduced B16F10 cells in a doxycycline inducible manner **(Fig. 22C & 22D)**. This conclusively established the role of LAMP1 and its association with galectin-3 through its polyLacNAc in mediating lung metastasis.

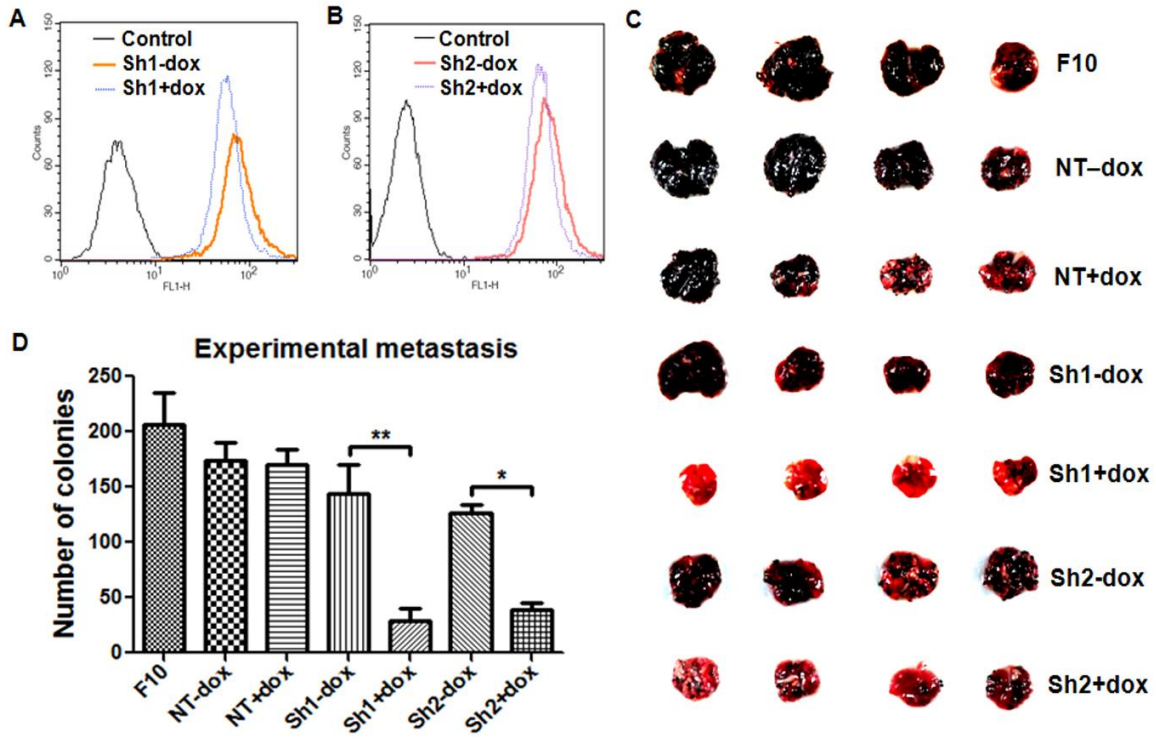

Figure 22. Effect of decreased surface expression of LAMP1 on galectin-3 binding and lung metastasis. Comparison of galectin-3 binding by flow cytometry using galectin-3-FITC on surface of B16F10 cells transduced with LAMP1 shRNA clones, Sh1 **(A)** and Sh2 **(B),** either uninduced (-dox) or induced with doxycycline (+dox**)**. Unstained cells served as Control. **(C)** Melanoma colonies on lungs of C57BL/6 mice injected with F10, NT-dox, NT+dox, Sh1-dox, Sh1+dox, Sh2-dox and Sh2+dox cells. **(D)** Graphical representation of mean number of lung colonies. One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by $**$, p value < 0.01, $*$, p value < 0.05).

CHAPTER 4

DISCUSSION

4. Discussion

Metastasis, the major cause of mortality seen in cancer patients, is a complex multi-step process [13]. For a cell to metastasize, it must modulate cell-cell and cell-ECM interactions, invade the surrounding normal tissues, intravasate into the blood vessels, survive in circulation and finally home into an organ [13, 63]. Tumor cells often get trapped in the fine vasculature of the first organ encountered. However, many tumors metastasize to very specific distant organ sites, bypassing several organs in their blood flow path [24, 26, 193]. Adhesive interactions between the molecules on the surface of tumor cells and the target organ, organ growth microenvironment and more recently, chemokines and their receptors have been shown to play a critical role in organ-specific metastasis [26, 194, 195]. Tumor cells show several membrane modifications associated with metastasis [62, 63]. Altered expression of β1,6 branched N-oligosaccharides on cell surface glycoproteins is one such consistently observed modification. Its expression on several human cancers and many human and murine tumor cell lines has been shown to correlate with their malignant potential [83, 98]. Manipulation of its expression in various cell lines has been shown to result in gain or loss of their ability to metastasize [90, 92, 196]. These evidences underline a definite association of β1,6 branched N-oligosaccharides with metastasis. Further, its association with organ-specific metastasis is outlined by the fact that a majority of cell lines carrying these oligosaccharides metastasize specifically to either the liver or to the lungs [77, 86, 87]. These oligosaccharides may mediate organ specific metastasis in two ways. Firstly, β1,6 branch serves as the preferred site for further substitutions like Lewis antigens, polyLacNAc, sialic acids and others which may serve as ligands for several endogenous lectins such as selectins, galectins, siglecs and as yet unidentified endogenous lectins [98, 99, 197]. For example, expression of sialyl Lewis antigens, the E-selectin ligands, on these branches appear to

facilitate liver metastasis possibly because of sinusoidal type of capillaries in liver which permits direct interaction of tumor cells with endothelial BM and the sub-endothelial matrix [100, 198, 199]. Similarly, expression of polyLacNAc substituted β1,6 branched N-oligosaccharides, the galectin-3 ligands, has been shown to facilitate lung metastasis [51, 190]. Secondly, the multi-antennary highly substituted bulky carbohydrate structures formed as a result of β1,6 branching may alter the structural and functional properties of proteins which carry them, thus possibly aiding organ-specific metastasis. Some of the proteins important from metastasis point of view which are known to carry these oligosaccharides include cell adhesion molecules such as cadherins, integrins, CD44, growth factor receptors such as EGFR, ECM components like laminin and others like Lysosome-Associated Membrane Proteins (LAMPs) [98, 104, 108-110, 200]. LAMPs, unlike the other cell surface proteins listed above, are lysosomal membrane proteins. They are highly glycosylated and are thought to protect themselves and the lysosomal membranes from intracellular proteolysis due to highly substituted oligosaccharides on the luminal side [114, 122]. However, for involvement of LAMPs in metastasis, their expression on the cell surface would be a prerequisite.

Using low (B16F1) and high (B16F10) metastatic variants of lung colonizing B16 murine melanoma cells [176], it was shown that surface translocation of LAMP1, but not LAMP2, correlated with the metastatic potential of these cells [51]. LAMP1 was also found to be a major carrier of polyLacNAc substituted β1,6 branched N-oligosaccharides on B16F10 cells. PolyLacNAc specifically on N- and not O-oligosaccharides has been shown to facilitate lung specific metastasis via galectin-3 present in highest amounts in mice lungs and expressed constitutively on the surface of lung vascular endothelium [51, 190]. LAMP1 being densely N-glycosylated with long chains of repeating polyLacNAc, its translocation to cell surface

could present a high density of high affinity ligands (polyLacNAc) for galectin-3 [197]. Increased surface expression of LAMP1 has also been reportedly observed on human melanoma (A2058), human fibrosarcoma (HT1080) and human colon carcinoma (CaCo-2) cells [144] and has been shown to correlate with metastatic potential of human colon carcinoma and murine melanoma cell lines [51, 85]. A transformation related increase in β1,6 branching observed in fibroblasts and metastatic cell line SP1, appeared to be associated with increased LAMP1 surface expression [201]. LAMP1 on cell surface has been shown to provide ligands in the form of sialyl-Le^x to E-selectin [50, 202] and in the form of polyLacNAc to galectin-3 [51, 144, 156].

Besides providing ligands for endogenous lectins, LAMP1 protein per se has been shown to bind to RGD peptides, ECM components like fibronectin, collagen type I, BM components like laminin and collagen type IV [189] suggesting that surface LAMP1 might as well interact with organ ECM and BM components. LAMP1 has been shown to be present on unique cell surface domains involved in cell locomotion such as membrane ruffles and microspikes (filopodia) [147]. Further, its accumulation at the edges and extensions of A2058 human metastasizing melanoma cells [144] hints towards its potential role in tumor cell spreading and motility possibly by serving as additional receptors for ECM and BM components. In addition to metastatic tumor cells, increased LAMP1 surface expression has also been observed on cells which are involved in migratory and/or invasive functions such as activated cytotoxic T-lymphocytes, platelets, natural killer cells, macrophages as well as embryonic cells [135, 137-140, 146, 203]. These evidences thus collectively point towards a possible role of LAMP1 in cellular processes like adhesion, cell spreading and motility which are important from metastasis point of view.

Our previous studies have shown that LAMP1 expression on the surface of B16 murine melanoma cells correlates with their metastatic potential **(Figure 1)** [51]. It was also shown that blocking the availability of cell surface LAMP1 on highly metastatic B16F10 cells using antibodies to LAMP1 resulted in significantly decreased lung metastasis. This established the role of cell surface LAMP1 in lung metastasis, but it did not shed any light on the possible mechanism. It remained elusive whether the effect on metastasis was because of LAMP1 protein or its glycosylation. To understand how important are LAMP1 and its glycosylation especially in the form of polyLacNAc substituted β1,6 branched N-oligosaccharides for metastasis, two different strategies were adopted. Firstly, low metastatic B16F1 cells, which have low levels of enzymes responsible for adding β1,6 branched N-glycans, were transduced with mutant LAMP1 (Tyr^{386} to Ala) to increase expression of LAMP1 on their surface **(Figures 2-4)**. Secondly, high metastatic B16F10 cells, efficient in all the glycosylation machinery, were transduced with shRNAs to LAMP1 **(Figures 14-16)** to specifically remove LAMP1 and its associated glycosylation. These served as appropriate tools to understand the processes mediated by surface LAMP1 and its glycosylation.

LAMP1 on the cell surface may promote metastasis by interacting with the molecules on the target organ or by helping the cells evade immune surveillance. For example, HeLa cells expressing LAMP1 on the surface have recently been shown to be significantly less sensitive to NK cell mediated apoptosis. Similarly, purified LAMP1 has been shown to have an affinity for several ECM and BM components like fibronectin, laminin, collagen-I and IV, and even RGD peptides [189]. On the cytoplasmic side, LAMP1 has also been shown to interact with ezrin [148] which functions as a linker between the actin cortical cytoskeleton and various membrane proteins. Owing to the affinity of LAMP1 towards several ECM and BM components on the extracellular side and to ezrin on the cytoplasmic side, it is possible

that LAMP1 on the cell surface mediates interactions with specific matrix components which play important roles in aiding metastasis. Tumor cells are known to mediate specific interactions with molecules on the endothelium as well as components of the sub-endothelial matrix and the organ extracellular matrix [53, 204]. It is possible that LAMP1 on the cell surface also mediates interactions with such molecules and promotes processes important from the point of view of metastasis.

LAMP1 has been shown to be present at the edges and extensions of metastasizing human melanoma cells [144] and on unique cell surface domains involved in motility such as membrane ruffles and filopodia [147]. Spreading of cells is a prerequisite for cellular motility and thus dictates the extent of movement of cells on a particular substrate. To our knowledge, results in this thesis, for the first time show that increasing expression of LAMP1 on the cell surface promotes significant spreading of cells on fibronectin (representative of ECM) and matrigel (reconstituted BM) **(Figure 5)**. In addition, the spreading on these substrates was independent of glycosylation emphasizing the involvement of LAMP1 protein and not its glycosylation in this process **(Figure 13)**. Increased spreading was accompanied with significant increase in motility on both these substrates as assessed by wound healing assays **(Figure 6)**. Lungs are rich in laminin, one of the major components of BM, and cells expressing receptors for laminin and other BM and ECM components home into the lungs. Peptides like YIGSR, a synthetic laminin pentapeptide, and antibodies against specific integrin receptors like α5 and β1 have been shown to inhibit lung metastasis [205, 206]. Increased surface LAMP1 thus possibly provides additional receptors that appear to utilize molecules on the target organ for their establishment.

However, in contrast to this, downregulating the expression of LAMP1 on the surface of B16F10 cells had a very marginal effect on spreading and motility on fibronectin and

matrigel **(Figures 17-19)**. Expression of mutant LAMP1 in B16F1 cells resulted in several folds (>20 fold) higher expression of LAMP1 on the surface as compared to even B16F10 cells. Such higher number of LAMP1 molecules on the surface possibly cooperated with the classical integrin receptors to impart unique cellular properties to B16F1 cells expressing mutant LAMP1.

LAMP1 may also promote interactions with molecules on the target organ via high levels of glycosylated structures on it. LAMP1 is a highly glycosylated protein. More than 60% of its weight is contributed by sugars. Each LAMP1 molecule carries 17-20 N-glycosylation sites that are often substituted further with structures like Lewis antigens and polyLacNAc. These appear to serve as ligands for endogenous lectins like selectins and galectin-3 expressed on the organ vascular endothelium. Involvement of Lewis antigens in liver metastasis has been substantiated by inhibiting or inducing their expression in tumor cells which results in their concomitant decreased or enhanced liver metastasis [100, 198]. Lungs express highest amounts of galectin-3 and express it on all the major compartments. As opposed to inducible expression of selectins on liver endothelium, the lungs have been demonstrated to express galectin-3 constitutively on the surface of its vascular endothelium [51]. Previously, polyLacNAc substituted β1,6 branched N-oligosaccharides have been shown to facilitate lung metastasis by anchoring on to galectin-3 on organ endothelium. More recently, it was also shown that this lectin carbohydrate pair may participate in not just anchoring these cells to lung endothelium, but in all the subsequent steps of extravasation like spreading to stabilize adhesion, degradation of ECM/BM and movement into organ parenchyma. It has also been shown that polyLacNAc substituted N- and not O-oligosaccharides participate in all these processes. Moreover, shRNA mediated inhibition of polyLacNAc synthesis also inhibit these processes including lung metastasis [175, 190]. PolyLacNAc is the most preferred ligand for

galectin-3 and LAMPs were shown to be the major carriers of polyLacNAc on high metastatic B16F10 cells. The expression of LAMP1 (and not LAMP2) on the cell surface was shown to correlate with the metastatic potential of B16 melanoma cells [51]. LAMP1 has also been shown to be a major ligand for galectin-3 [144, 156]. Overexpression of surface LAMP1 on B16F1 cells and downregulation of LAMP1 in B16F10 cells thus may also influence the metastatic processes mediated by galectin-3 which often gets incorporated on the endothelial cell surface, vascular BM and ECM [160].

However, B16F1 clones overexpressing LAMP1 on the cell surface showed neither enhanced spreading nor motility on galectin-3 coated surfaces **(Figures 7 and 8)**. Even the ability to metastasize to lungs remained unaltered **(Figure 9A)**. Although there was >20 fold higher surface expression of LAMP1 in the clones, binding of galectin-3 to the clones over expressing surface LAMP1 remained unaltered and was much lower as compared to B16F10 cells **(Figure 9B and C)**. Since galectin-3 mediated effects are dependent on galectin-3-polyLacNAc interactions, it was plausible to think that each LAMP1 molecule expressed on cell surface might not have adequate polyLacNAc units. Immunoprecipitation experiments confirmed that there was no increase in β1,6 branched N-glycans and polyLacNAc on LAMP1 molecules from these cells which was significantly lower as compared to that on LAMP1 from B16F10 cells **(Figure 9D)**. On the other hand, downregulating surface LAMP1 in high metastatic cells significantly altered their cellular properties such as spreading and motility on galectin-3 **(Figures 20 and 21)**. In addition, these cells also showed significantly reduced binding of galectin-3 to the cell surface **(Figure 22A and B)**. In fact, the levels of galetin-3 binding appeared to depend on the levels of LAMP1 on the cell surface in these clones. This indicated that polyLacNAc on specific glycoproteins such as LAMP1 may be an important player in mediating metastasis of tumor cells via galectin-3. This hypothesis was

further substantiated when B16F10 clones downregulated for LAMP1 showed significantly reduced lung metastasis **(Figure 22C and D)**. The reduction in metastasis also appeared to clearly depend on the levels of surface LAMP1 [207].

Metastasis is a multistep process and cells deficient in even one of the critical properties required for metastasis, are unable to metastasize which is often referred to as metastatic inefficiency [26, 208]. For circulating tumor cells to efficiently colonize a particular target organ, initial anchoring to its endothelium is the most critical rate-limiting step. The tumor cells may not be able to metastasize successfully if they are unable to anchor on to the endothelium in spite of being proficient in mediating the other downstream events. This was clearly evident from these studies which clearly outline the importance of interaction of polyLacNAc on LAMP1 with galectin-3 on the lungs in mediating organ specific metastasis.

Although, the role of LAMP1 on the surface of tumor cells was convincingly proven by these studies, the mechanism by which it gets translocated to surface of these cells remained elusive. LAMP1 has been shown to get sorted to lysosomes due to the presence of a specific tyrosine residue (Tyr^{386}) present in its cytoplasmic tail, since mutation of this single tyrosine leads to mistargeting of LAMP1 to cell surface instead of lysosomes [132]. However, the possibility of surface translocation of LAMP1 in metastatic cells using this strategy seems a distant reality, as chances of such frequent mutations are rare under physiological conditions. Another possibility of LAMP1 surface localization is by the fusion of lysosomes with cell membrane during the process of exocytosis of cytotoxic granules of cytotoxic T lymphocytes and natural killer cells during viral infection or transformation [137-139]. However LAMP2, another abundant lysosomal membrane protein, was not found to be translocated to the surface of these B16 melanoma cells [51]. Therefore, if lysosome fusion is the reason for increased LAMP1 surface expression, then even LAMP2 would have appeared on the cell

surface. Hence, surface expression of LAMP1 through lysosome fusion during exocytosis is also ruled out. In this scenario, altered levels of glycosylation which have been shown to modulate the surface expression of several cell surface receptor proteins [209-211] appear to be the more plausible mechanism of LAMP1 surface translocation.

LAMP1 is a lysosomal protein and traffics through ER and Golgi like all other membrane proteins till it reaches trans-Golgi network (TGN) where sorting occurs. LAMP1 carries high levels of polyLacNAc substituted β1,6 branched N-oligosaccharides, although their levels appear to vary depending on the cell type. B16F10 cells have higher levels of enzymes responsible for formation of β1,6 branched N-oligosaccharides and polyLacNAc [175, 190]. LAMP1 having 17-20 N-glycan sites, the residency time of LAMP1 in Golgi where these branches are added, is therefore expected to be higher in B16F10 cells. It has indeed been shown that longer residence in Golgi allows LAMP1 to acquire more polyLacNAc [191]. Thus, it is possible that during the process of acquiring more polyLacNAc, some LAMP1 molecules get mistargeted to cell surface instead of lysosomes. Treatment of cells with swainsonine (SW), an α-mannosidase-II inhibitor, results in the expression of hybrid kind of N-oligosaccharides which are not the substrates for enzymes that add β1,6 branch or polyLacNAc. As a consequence, the possible residency in Golgi and thus mistartgeting of LAMP1 molecules to the cell surface may be considerably reduced on SW treatment. Reduced expression of LAMP1 on the surface of B16F1 and B16F10 cells on SW treatment of these cells indeed confirmed the role of glycosylation in translocating LAMP1 to the cell surface **(Figures 10 and 11)** [212].

Taken together, the present study for the first time provides direct evidence for the involvement of altered N-glycosylation specially in the form of polyLacNAc substituted β1,6

branched N-oligosaccharides in regulating the surface expression of LAMP1 as well as in the modulation of metastatic processes by cell surface LAMP1 via galectin-3.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 Summary

- Surface expression of polyLacNAc substituted β1,6 branched N-oligosaccharides and LAMP1 correlates with metastatic potential of melanoma cells.
- Expression of mutant LAMP1 (Tyr^{386} to Ala) in low metastatic B16F1 cells results in significantly increased expression of LAMP1 on their surface.
- Overexpression of LAMP1 on the surface of B16F1 cells results in significantly increased cell spreading and motility of these cells on fibronectin and matrigel.
- Increased expression of LAMP1 on the surface of B16F1 cells has no effect on galectin-3 mediated spreading and motility and overall lung metastasis.
- Absence of any effect on metastasis of B16F1 cells overexpressing LAMP1 on the surface may be attributed to the absence of polyLacNAc substituted β1,6 branched N-oligosaccharides on overexpressed LAMP1.
- Glycosylation regulates the expression of LAMP1 on the melanoma cell surface. However, surface expression of mutant LAMP1 is glycosylation independent.
- The LAMP1 mediated spreading on fibronectin and matrigel is also glycosylation independent.
- Expression of LAMP1 shRNAs in high metastatic B16F10 cells show decreased total as well as surface expression of LAMP1.
- Downregulation of LAMP1 marginally affects spreading and motility on fibronectin and matrigel, whereas, it significantly affects the same on galectin-3.
- Downregulation of LAMP1 in B16F10 cells significantly reduces their lung metastatic ability.
- The decrease in lung metastasis of B16F10 cells carrying LAMP1 shRNAs and their reduced binding to galectin-3 confirmed that both LAMP1 protein and its associated carbohydrates are important for metastasis.

5.2 Conclusions

The present investigations demonstrate that LAMP1 molecules expressed on the surface of metastatic cells indeed play a key role in facilitating organ specific metastasis via galectin-3 expressed in significantly high amounts on the lungs and constitutively on its vascular endothelium. Further, it very clearly shows that, unless fully glycosylated with terminally substituted polyLacNAc, LAMP1 overexpression fails to promote metastasis. Although, overexpression of such LAMP1 molecules resulted in significant gain in the cellular interactions with ECM/BM components, it failed to augment the same on galectin-3. The results demonstrate that LAMP1 molecules on the cell surface carrying larger highly substituted (polyLacNAc) N-glycans serve as the first anchor for the circulating metastatic cells for their arrest on the lung vascular endothelium. Inhibition of expression of such anchors inhibited metastasis suggesting that the surface LAMP1 participates in a major rate-limiting step in lung metastasis by providing high affinity ligands (polyLacNAc) at high density for interaction with galectin-3 on the lungs.

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Regulation of melanoma metastasis to lungs by cell surface Lysosome Associated Membrane Protein-1 (LAMP1) via galectin-3

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ABSTRACT

Lysosome Associated Membrane Protein-1 (LAMP1), which lines the lysosomes, is often found to be expressed on surface of metastatic cells. We previously demonstrated that its surface expression on B16 melanoma variants correlates with metastatic potential. To establish the role of cell surface LAMP1 in metastasis and to understand the possible mechanism by which it facilitates lung colonization, LAMP1 was downregulated in high metastatic B16F10 cells using shRNAs cloned in a doxycycline inducible vector. This also resulted in significantly decreased LAMP1 on the cell surface. Being a major carrier of poly-*N*-acetyllactosamine (polyLacNAc) substituted β1,6 branched N-oligosaccharides, the high affinity ligands for galectin-3, LAMP1 down regulation also resulted in appreciably decreased binding of galectin-3 to the cell surface. LAMP1 has been shown to bind to Extracellular Matrix (ECM), Basement Membrane (BM) components and also to galectin-3 (via carbohydrates) which is known to get incorporated into the ECM and BM. Although, LAMP1 downregulation had a marginal effect on cellular spreading and motility on fibronectin and matrigel, it significantly altered the same on galectin-3, and ultimately leading to notably reduced lung metastasis. The results thus for the first time provide direct evidence that cell surface LAMP1 facilitates lung metastasis by providing ligands for galectin-3 which has been shown to be expressed in highest amounts on lungs and constitutively on its vascular endothelium.

1. Introduction

Lysosome Associated Membrane Protein-1 (LAMP1) (also known as CD107a) is a heavily glycosylated lysosomal membrane protein whose function is not yet clearly elucidated [1]. Due to the presence of heavily glycosylated structures on it, it is speculated to protect the lysosomal membranes from intracellular proteolysis [2]. It is often found to get translocated to surface of several migratory and/or invasive cells such as activated cytotoxic T-lymphocytes, natural killer cells, macrophages, embryonic cells and particularly metastatic tumor cells [3–7]. It has been shown to be expressed on surface of several metastatic cells such as human melanoma, colon carcinoma, fibrosarcoma and myelomonocytic leukemia cells [7,8]. Moreover, its cell surface expression has been shown to correlate with metastatic potential of human colon carcinoma and murine melanoma cell lines [9,10]. However, the functional relevance of tumor cell surface LAMP1 with respect to metastasis remains elusive.

Purified LAMP1 has been shown to bind to ECM components like fibronectin, collagen type I, BM components like laminin and collagen type IV and even to RGD peptides implicating its possible interaction with organ ECM and BM components [11]. LAMP1 has been shown to be present on membrane ruffles and filopodia, cell surface domains involved in cell locomotion, suggesting its potential role in tumor cell adhesion, spreading and motility [12]. LAMP1 is a heavily glycosylated protein carrying 17–20 N-glycan sites [1]. However, apart from the role of these carbohydrates in protecting the lysosomal membrane, nothing much is known about their role on cell surface. LAMP1 has been identified to be one of the major carriers of β1,6 branched N-oligosaccharides [10,13,14]. Expression of β1,6 branched N-oligosaccharides on several human cancers and many human and murine tumor cell lines has invariably been shown to correlate with their malignant potential [15,16]. Manipulation of its expression in various cell lines has been shown to affect their ability to invade and metastasize [17,18]. β1,6 branch serves as the preferred site for further substitutions like Lewis antigens, polyLacNAc, sialic acids and others which may serve as ligands for several endogenous lectins such as selectins, galectins, siglecs and as yet unidentified endogenous lectins [15,19–21]. Evidences indicate that LAMP1 on cell surface may predominantly

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provide ligands both in the form of sialyl-Le^x to E-selectin [22,23] and in the form of polyLacNAc to galectin-3 [7,10,24].

Galectin-3 is a multifunctional β-galactoside binding lectin. It is found to be present in the nucleus, cytoplasm as well as secreted outside the cell by a non-classical pathway and is also known to become a part of the cell surface and the ECM [25,26]. Galectin-3 has also been shown to be present in highest amounts in mice lungs and expressed constitutively on the surface of lung vascular endothelium [10]. Intracellular galectin-3 has been implicated in several processes such as cell growth and differentiation, pre-mRNA splicing, regulation of apoptosis, whereas, extracellular galectin-3 promotes processes like cell–cell recognition, adhesion, invasion and metastasis [26,27]. Galectin-3 is known to bind to several carbohydrate structures like T and Tn antigens and polyLacNAc [28,29]. However, its affinity for long chain polyLacNAc is several folds higher (>200 folds) than T/Tn antigens [29,30].

Previously, the surface expression of polyLacNAc substituted β1,6 branched N-oligosaccharides on LAMP1 has been shown to correlate with metastatic potential of B16 melanoma cells [10]. However, increasing expression of LAMP1 on the surface (LAMP1 with a mutation in the cytoplasmic tail, Tyr^{386} to Ala^{386}) of low metastatic B16F1 cells did not influence their metastasis, possibly because of the absence of galectin-3 ligands, i.e., polyLacNAc substituted β1,6 branched N-oligosaccharides on overexpressed LAMP1 [Agarwal et al., manuscript submitted]. These evidences indicate that it is possibly the carbohydrates on LAMP1, presented in an easily accessible form on the cell surface for interaction with galectin-3 that determines the extent of lung metastasis. To conclusively establish the role of LAMP1 and its associated carbohydrates in metastasis, the present paper investigates the effect of downregulation of LAMP1 in high metastatic B16F10 cells on their cellular properties and its influence on their lung metastasis.

2. Materials and methods

2.1. Cell lines and reagents

B16F10 murine melanoma cell line was obtained from National Centre for Cell Science, Pune, India. Cell culture reagents were obtained from Invitrogen, USA. Restriction enzymes and T4 DNA ligase were from Fermentas International Inc., Canada. Anti-mouse LAMP1 antibody (clone 1D4B) raised in rat was purchased from BD Biosciences, USA. PVDF membrane and ECL kit were purchased from GE Healthcare, Amersham, UK. Cultureware were obtained from Nunc and BD Falcon and Fibronectin and Matrigel from BD Biosciences, USA. *Escherichia coli* BL 21 with pET3C plasmid containing a full-length human galectin-3 was a kind gift from Dr. Hakon Leffler, Lund's University, Sweden. IPTG was obtained from USB Corporation, USA. Primers for amplification of shRNAs to LAMP1, polybrene, puromycin, paraformaldehyde, BSA, mitomycin C, FITC, Phalloidin-FITC, anti-rat HRPO and DAPI were purchased from Sigma Chemical Company, USA. Vectashield mounting medium was from Vector Labs, USA. Conjugation of purified rh-galectin-3 to FITC was conducted as described in [31]. Reagents for bacterial culture were purchased from Himedia, India, while all other chemicals were purchased locally and were of analytical grade. Inbred strains of C57BL/6 mice used for the metastatic assays and other experiments were maintained in the Institute animal house and all the animal experiments were approved by the Institutional Animal Ethics Committee.

2.2. Cell culture

Melanoma cells were routinely cultured as described previously [32].

2.3. Designing and cloning of short hairpin RNA (shRNA) constructs for downregulating LAMP1 in melanoma cells

Downregulation of LAMP1 in B16F10 cells was carried out by using the shRNAmir technology with lentiviral vectors. For the same, two shRNA sequences were designed against LAMP1 as per guidelines outlined by [33]. Two 21 nucleotide sequences (5′-CCCACTGTATCCAAGTACAAT-3′ and 5′-GCGTTCAACATCAGCCCAAAT-3′) from the open reading frame of mouse LAMP1 gene were chosen which were unique only to LAMP1. For cloning shRNAs into pTRIPz lentiviral vector, primers were designed according to pTRIPz manual (Open Biosystems). Forward primers contained XhoI site followed by mir sequence (represented in Italics), sense sequence (represented in italics bold) and loop sequence. Reverse primers contained EcoRI site followed by mir sequence (represented in Italics), sense sequence (represented in Italics bold) and loop sequence.

LAMP1 shRNA1 forward primer:

5′GAACTCGAG*AAGGTATATTGCTGTTGACAGTGAGCG****CCCACTGTATCCAAGTACAAT***TAGTGAAGCCACAGA3′.

LAMP1 shRNA1 reverse primer:

5′GTTGAATTC*CGAGGCAGTAGGCA****CCCACTGTATCCAAGTACAAT***TACATCTGTGGCTTC3′.

LAMP1 shRNA2 forward primer:

5′GAACTCGAG*AAGGTATATTGCTGTTGACAGTGAGCG****GCGTTCAACATCAGCCCAAAT***TAGTGAAGCCACAGA3′.

LAMP1 shRNA2 reverse primer:

5′GTTGAATTCCGAGGCAGTAGGCA**GCGTTCAACATCAGCCCAAAT**TACATCTGTGGCTTC3′.

Using these primers, shRNA cassettes were PCR amplified. The shRNAs were cloned in pTRIPz lentiviral vector digested with EcoRI and XhoI sites. The ligated plasmids were purified and subsequently co-transfected with helper plasmids (pMD2.G and psPAX2) in HEK293FT cells for generating virus particles which were used for transduction of B16F10 cells. The transduction of LAMP1 shRNAs along with a non-targeting shRNA (obtained from Open Biosystems) in B16F10 cells and generation and maintenance of stable clones were done exactly as described in [34]. For induction of shRNA expression, cells were cultured in complete DMEM containing doxycycline (4 μg/ml) for 96 h.

2.4. Preparation of total cell lysates, protein estimation, SDS–PAGE and Western blotting

Preparation of total cell lysates, protein estimation, SDS–PAGE and Western blotting was done exactly as described previously [10].

2.5. Flow cytometric analysis

For flow cytometry of LAMP1, melanoma cells were stained with LAMP1 antibody as described previously [10]. For determination of galectin-3 binding, melanoma cells were first fixed by overnight incubation with 1% paraformaldehyde in PBS (pH 7.4) followed by staining with FITC labeled galectin-3. 0.5 million melanoma cells were incubated with 10 μg of galectin-3-FITC in 40 μl of FACS buffer (PBS pH 7.4, containing 1% FBS) followed by three washes with PBS. Untreated cells served as control. Fluorescent cells were acquired at 488 nm and analyzed on FACS Calibur using Cell Quest software (BD Biosciences).

2.6. Purification of recombinant human galectin-3

Expression and purification of recombinant human (rh) galectin-3 was carried out exactly as described previously [10].

2.7. Cell spreading assays

Cell spreading assays were done as described in [34]. Briefly, melanoma cells were harvested, washed free of serum and seeded at a cell density of 0.5 million/ml in serum free DMEM on the coverslips coated overnight with 75 μg/ml galectin-3, 10 μg/ml of both fibronectin and matrigel in serum free DMEM at 4 °C. The cells were incubated for 45 min in a CO_2 incubator. Bound cells were fixed, permeabilized and stained with Phalloidin-FITC and DAPI as described previously [34]. The stained cells were mounted and images were acquired using a Carl Zeiss Laser Confocal Microscope at 63× magnification. The ratio of cytoplasmic/nuclear (C/N) area of cells was measured using Image J software to quantitate cell spreading.

2.8. Wound healing assays

For wound healing assays, 6 well culture dishes were coated overnight with 75 μg/ml of galectin-3, 10 μg/ml of both fibronectin and matrigel in serum free DMEM at 4 °C, followed by blocking of non-specific sites with 2% BSA for 1 h. Melanoma cells were harvested, seeded at a density of 0.5 million cells per ml of complete medium and incubated at 37 °C for 24 h in a CO_2 incubator. The cells were treated with 40 μg/ml mitomycin C for 3 h for inhibiting cell proliferation. A straight, uniform wound (approx. 400 μm in width) was made using a micropipette tip on the monolayer and the cells were maintained in serum free DMEM. Wound closure of cells in response to the immobilized galectin-3, fibronectin and matrigel was measured for 20 h by time lapse video imaging of at least three different positions across the length of the wound using a Carl Zeiss Inverted Microscope at 10× magnification.

2.9. Experimental metastasis assay

The assay was carried out as described previously [32]. For injecting doxycycline induced shRNA clones, mice were fed with doxycycline (1 mg/ml) in 5% sucrose solution 48 h prior to injection and continued until sacrificed.

2.10. Statistical analysis

All data are represented as mean ± SE unless stated. All the statistical analysis was performed using GraphPad Prism 5 software. For spreading and experimental metastasis assays, comparison within the group was done by performing one way ANOVA followed by Bonferroni's multiple comparison test. For wound-healing assays, 2-way ANOVA with the Bonferroni posttest was conducted. (p value <0.05 was considered significant).

3. Results and discussion

3.1. Downregulation of LAMP1 expression in B16F10 cells using shRNAs also results in its significantly decreased expression on the cell surface

The cellular and lysosomal morphology of cells in LAMP1-deficient mice remains unaffected and even the animals remain absolutely viable and fertile [35]. LAMP2 appears to take over the function of LAMP1 in these cells suggesting that LAMP1 is dispensable for normal lysosomal functions. Downregulating the expression of LAMP1 in B16F10 cells thus should result in its decreased expression on the cell surface without affecting other normal cellular functions. Surface expression of LAMP1, but not LAMP2 has previously been shown to correlate with metastatic potential of B16 melanoma cells [10]. However, its direct involvement in promoting metastasis still remains to be established. To conclusively ascertain its role, LAMP1 was downregulated in high metastatic B16F10 cells using two different inducible shRNAs specific for LAMP1 (Sh1 and Sh2). An inducible non-targeting shRNA (NT) was also expressed as control. The induction of non-targeting shRNA (NT) clone had no effect on total or surface levels of LAMP1 and was equal to that of untransduced B16F10 cells and thus served as an apt control (Fig. 1A and B). On the other hand, on induction of expression of LAMP1 shRNAs (Sh1 & Sh2), there was significant reduction in total LAMP1 (as seen by Western blotting) (Fig. 1A) as well as cell surface LAMP1 (as seen by flow cytometry) in Sh1 (Fig. 1C) and Sh2 (Fig. 1D) clones, with Sh1 showing higher downregulation compared to Sh2 clone. Since LAMP1 is one of the major carriers of polyLacNAc substituted β1,6 branched N-oligosaccharides in these cells [10], it would be interesting to investigate if its downregulation has any bearing on galectin-3 binding and processes mediated by galectin-3.

3.2. Downregulation of LAMP1 in B16F10 cells results in decreased galectin-3 binding and significantly decreased spreading and motility on galectin-3

PolyLacNAc (specifically on N-oligosaccharides) has been shown to be the major galectin-3 ligand which mediates melanoma cell adhesion to lung vascular endothelium [32]. More recently, it was shown that blocking the availability of polyLacNAc using either truncated galectin-3 or modified citrus pectin (MCP) blocked metastasis. Moreover, inhibition of polyLacNAc using shRNAs to the enzymes involved in their synthesis was also shown to inhibit lung metastasis. In addition, besides promoting adhesion to vascular endothelium, polyLacNAc/galectin-3 interaction was shown to facilitate cellular spreading, degradation of vascular endothelium and movement into organ parenchyma [36]. Since LAMP1 is a major carrier of polyLacNAc, decreased surface expression of LAMP1 may alter the cell surface levels of polyLacNAc and hence galectin-3 binding which ultimately may also hamper all the cellular processes mediated by galectin-3. The B16F10 cells indeed showed decreased binding of galectin-3 on induction of both the LAMP1 shRNAs, Sh1 (Fig. 2A) and Sh2 (Fig. 2B) indicating that the downregulation of LAMP1 might affect the adhesion of cells to galectin-3 present on lung endothelium. The induction of non-targeting shRNA had no effect on galectin-3 binding (data not shown). Both the LAMP1 shRNA clones also showed significantly decreased spreading on galectin-3 as compared to untransduced B16F10 cells or those transduced with non-targeting shRNA (NT) in a doxycycline-inducible manner, as seen by laser confocal microscopic images (Fig. 2C and D) and by analyzing ratios of the cytoplasmic to nuclear areas (Fig. 2E). The clones also showed significantly lower motility on galectin-3 on doxycycline induction as measured by wound healing assays (Fig. 3A–F). Purified LAMP1 has been shown to have an affinity for ECM and BM components as well [11]. Hence, the effect of LAMP1 downregulation in B16F10 cells on their cellular properties on ECM and BM components was also investigated. Both the induced LAMP1 shRNA clones (sh1+dox & sh2+dox) did not show any significant decrease in spreading as well as motility on both fibronectin (ECM component) (Supplementary Figs. S1A and S2) and matrigel (reconstituted Basement Membrane) (Supplementary Figs. S1B and S3) as compared to uninduced LAMP1 shRNAs (sh1−dox & sh2−dox) or induced non-targeting shRNA (NT+dox). Together these results clearly signify that downregulating carriers of polyLacNAc such as LAMP1 has a profound influence on galectin-3 mediated cellular processes which might eventually affect lung metastasis.

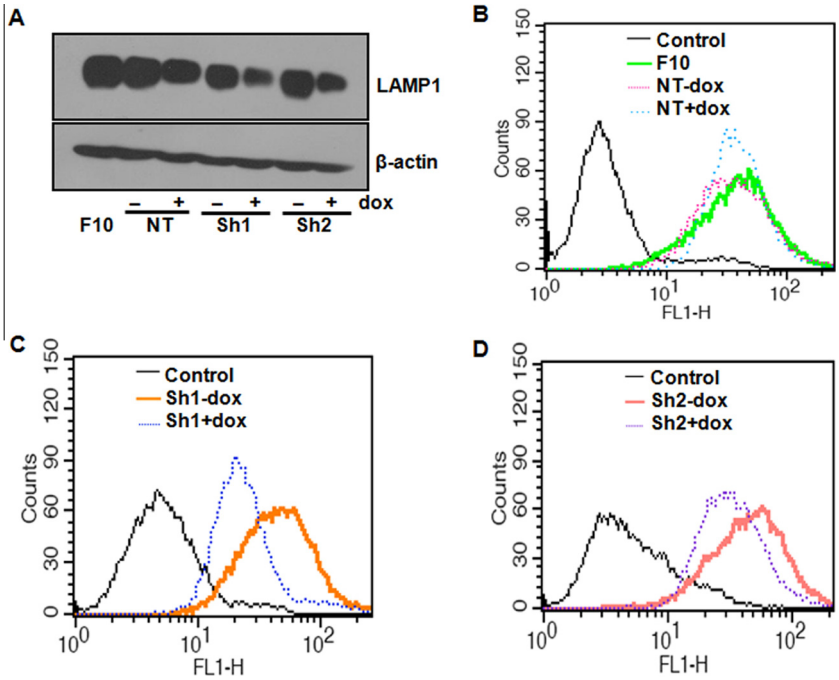

Fig. 1. Analysis of total and surface expression of LAMP1 in B16F10 cells transduced with LAMP1 shRNAs. Comparison of LAMP1 expression in B16F10 cells and its clones transduced with either non-targeting shRNA (NT), shRNA1 (Sh1) and shRNA2 (Sh2) induced with doxycycline (+dox) or under uninduced condition (−dox) by (A) Western blotting the cell lysates (total) or by flow cytometry (surface expression), on NT (B), Sh1 (C) or Sh2 (D) clones along with B16F10 (F10) cells. Cells treated with only anti-rat FITC served as Control in (B–D). Beta actin served as a loading control in (A).

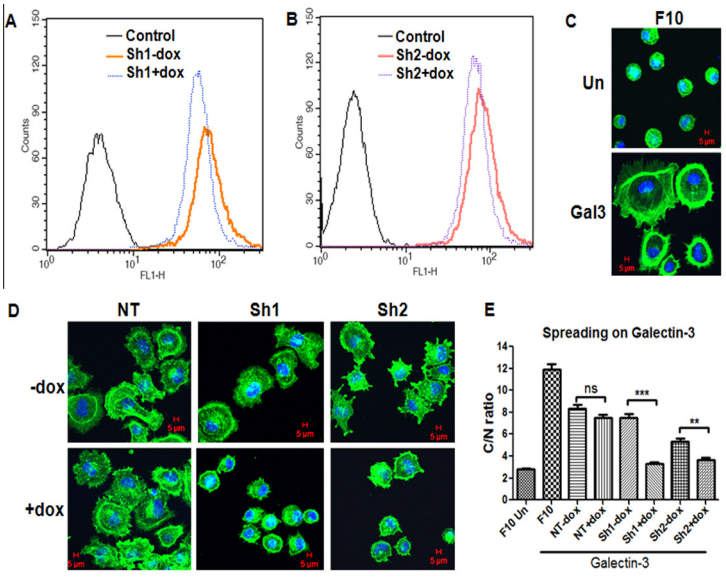

Fig. 2. Effect of decreased surface expression of LAMP1 on galectin-3 binding and spreading of melanoma cells on galectin-3. Comparison of galectin-3 binding by flow cytometry using galectin-3-FITC on surface of B16F10 cells transduced with LAMP1 shRNA clones, Sh1 (A) and Sh2 (B), either uninduced (−dox) or induced with doxycycline (+dox). Unstained cells served as Control. Spreading of (C) untransduced B16F10 cells (F10) and (D) those transduced with either non-targeting shRNA (NT) or LAMP1 shRNAs (Sh1 & Sh2) without (−dox) or with (+dox) induction of shRNA by doxycycline on galectin-3 (Gal3) coated coverslips as assessed by staining with Phalloidin-FITC (green). Spreading of untransduced B16F10 cells was also seen on uncoated (Un) coverslips as control (in C). DAPI was used to stain the nuclei (blue). Scale bar = 5 μm. (E) Each bar represents ratio of cytoplasmic to nuclear (C/N) area for around 50 cells from two different experiments for their spreading on uncoated or galectin-3 coated coverslips. One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by $^{***}p$ value < 0.0001, $^{**}p$ value < 0.001, ns, non-significant). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Downregulation of LAMP1 in B16F10 cells results in significantly decreased lung metastasis of these cells

To investigate if modulation of galectin-3 mediated properties has any effect on lung colonization, experimental metastasis assay was performed. The LAMP1 downregulated clones showed significantly decreased lung metastasis as compared to untransduced or non-targeting shRNA transduced B16F10 cells in a doxycycline inducible manner (Fig. 4A and B). Moreover, the extent of metastasis of the clones appeared to depend on the levels of surface LAMP1

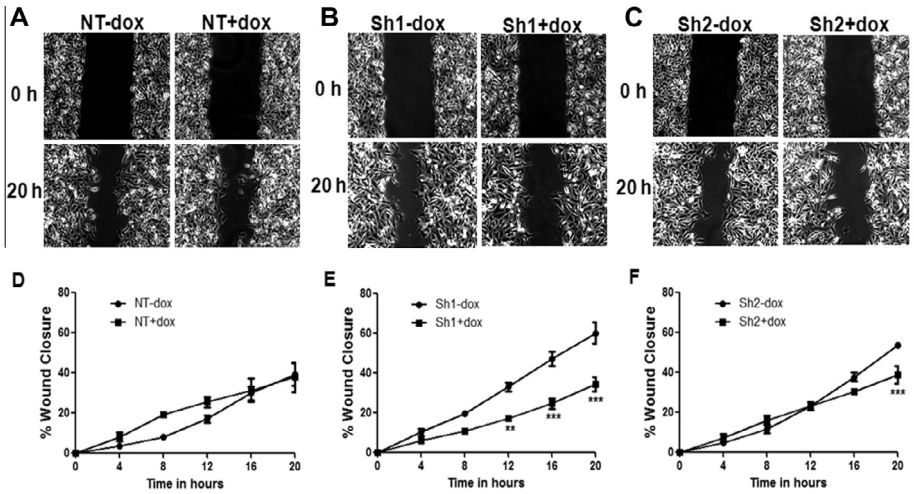

Fig. 3. Effect of decreased surface expression of LAMP1 on motility of melanoma cells on galectin-3. Motility on galectin-3 coated plates of B16F10 cells transduced with either (A) non-target shRNA (NT) or LAMP1 shRNAs, Sh1 (B) & Sh2 (C) without (−dox) or with (+dox) induction of shRNA by doxycycline as represented by time lapse video microscopy images at 0 and 20 h of wound closure. (D), (E) and (F) represent mean percent wound closure at 4 h interval of NT, Sh1 and Sh2, respectively. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by $^{***}p$ value < 0.001, $^{**}p$ value < 0.01).

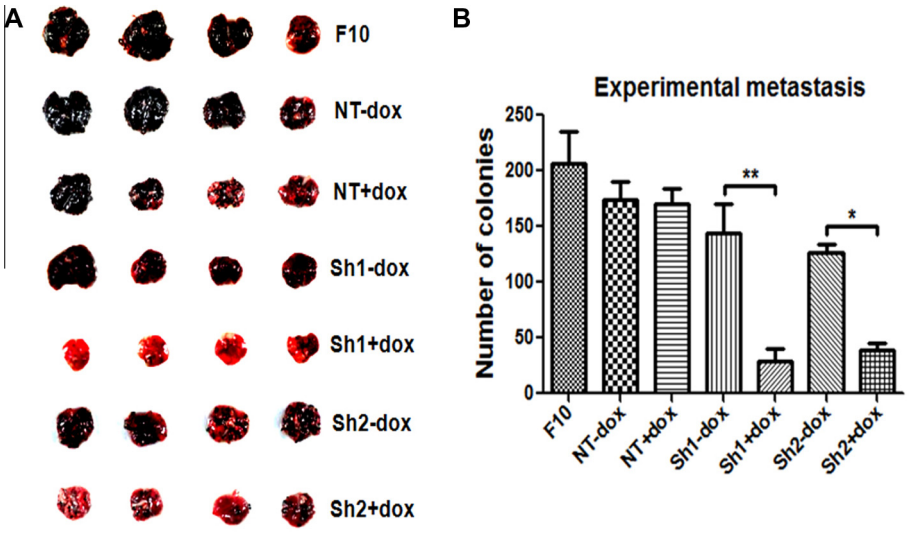

Fig. 4. Effect of decreased surface expression of LAMP1 on lung metastasis. (A) Melanoma colonies on lungs of C57BL/6 mice injected with F10, NT−dox, NT+dox, Sh1−dox, Sh1+dox, Sh2−dox and Sh2+dox cells. (B) Graphical representation of mean number of lung colonies. One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by $^{**}p$ value < 0.01, $^{*}p$ value < 0.05).

and binding of galectin-3, thus conclusively establishing the role of LAMP1 in mediating lung metastasis through its association with galectin-3.

In conclusion, metastasis is a multistep process and cells deficient in even one of the critical properties required for metastasis, are unable to metastasize [37]. For circulating tumor cells to efficiently colonize a particular target organ, initial anchoring to its endothelium is the most critical rate-limiting step. The tumor cells may not be able to metastasize successfully if they are unable to anchor on to the endothelium in spite of being proficient in mediating the other downstream events. This was clearly evident from these studies. To our knowledge, this is the first report which shows that downregulating LAMP1 may severely affect galectin-3 mediated processes and in turn lung metastasis.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.028.

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Glycosylation regulates the expression of Lysosome Associated Membrane Protein-1 (LAMP1) on the cell surface

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ABSTRACT:
Lysosome Associated Membrane protein-1 (LAMP1) which lines the lysosomes, is often found to appear on the surface of several cells involved in migratory and/or invasive functions including metastatic tumor cells. However, the mechanism of its surface translocation in these cells is still poorly understood. Glycosylation, one of the major post-translational modifications of membrane proteins, regulates a variety of functions of such glycoproteins. The levels of poly-*N*-acetyllactosamine (polyLacNAc) substituted β1,6 branched N-oligosaccharides on B16 melanoma variants has previously been shown to correlate with the metastatic potential of these cells. Lysosomal protein LAMP1 is one of the major carriers of these oligosaccharides and its expression on the cell surface also correlates with metastatic potential of B16 murine melanoma cells. To investigate whether these oligosaccharides have any role in increasing surface expression of LAMP1, low (B16F1) and high metastatic (B16F10) variants of B16 melanoma cells were treated with N-glycosylation inhibitor, swainsonine (SW). SW treatment resulted in significantly decreased expression of polyLacNAc substituted β1,6 branched N-oligosaccharides on these cells. This was also accompanied with significantly reduced expression of LAMP1 on the cell surface, although total levels of LAMP1 in these cells remained unaffected. This points towards a possibility that glycosylation modulates the surface expression of LAMP1 on tumor cells. The present study thus clearly underscores a novel role of N-glycosylation in regulating the surface translocation of a lysosomal membrane protein, LAMP1.

KEYWORDS:
β1,6 branched N-oligosaccharides, cell surface LAMP1, poly-*N*-acetyllactosamine, swainsonine

1. INTRODUCTION:

Lysosome Associated Membrane Protein-1 (LAMP1) is a heavily glycosylated lysosomal membrane protein with 17-20 N-glycosylation sites [1]. The function of LAMP1 (also known as CD107a) is not yet clearly understood. However, due to the presence of heavy glycosylation on LAMP1, it is thought to protect itself and the lysosomal membranes from intracellular proteolysis [2]. Although majorly present in lysosomal membranes, LAMP1 is also found to be expressed on surface of several cells involved in migratory and/or invasive functions such as cytotoxic T-lymphocytes, natural killer cells, macrophages, embryonic cells as well as metastatic tumor cells [3-7]. It is found to be expressed on surface of several metastatic cells such as human melanoma (A2058), human colon carcinoma (CaCo-2), human fibrosarcoma (HT1080) and human myelomonocytic leukemia cells (HL-60 and U937) [7, 8]. In addition, its cell surface expression has been found to correlate with metastatic potential of human colon carcinoma and murine melanoma cell lines [9, 10].

LAMP1 has been found to be one of the major carriers of β1,6 branched N-oligosaccharides [10-12]. β1,6 branch serves as the preferred site for further substitutions like Lewis antigens, polyLacNAc, sialic acids and others which may serve as ligands for several endogenous lectins such as selectins, galectins, siglecs and as yet unidentified endogenous lectins [13-16]. Evidences indicate that LAMP1 on cell surface may provide ligands both in the form of sialyl-Le^x to E-selectin [17, 18] and in the form of polyLacNAc to galectin-3 [7, 10, 19]. The Lewis antigens have been shown to aid the recruitment of granulocytes and macrophages

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to the inflamed endothelium and the tumor cells to the endothelium of the organs like liver and bones [17]. Moreover, inhibition of surface LAMP1 was shown to significantly reduce galectin-3 binding and in turn, attenuate metastasis of B16F10 murine melanoma cells [Agarwal *et al*, manuscript submitted]. These evidences point towards a definite role of glycosylated LAMP1 present on surface of tumor cells in promoting processes such as metastasis. However, the mechanism by which it gets translocated to surface of these cells remains elusive.

Glycosylation which is one of the most common post-translational modifications of proteins is known to modulate a variety of biological activities, including protein folding, trafficking, stability and the expression of proteins on the cell surface [20-24]. The glycosylation of N-linked proteins starts in the rough endoplasmic reticulum (RER) and progresses in the Golgi. The chaperones in the RER prevent further maturation and trafficking of proteins which are improperly glycosylated, to the next compartment. Maturation of glycosylated structures on membrane proteins occurs in the Golgi, which often depends on the availability of substrates (sugars), respective enzymes in the Golgi of the cells, the accessibility of the glycosylation sites on the proteins and the period of residency of proteins in the Golgi [20]. Proteins that are substituted with high levels of polyLacNAc have a higher residency in the Golgi [25]. LAMP1 has 17-20 N-glycosylation sites that are highly substituted with polyLacNAc. The low and high metastatic variants have been shown to differ in the levels of expression of the enzymes responsible for adding these substitutions in the Golgi resulting in proteins expressing different levels of polyLacNAc substituted structures [26, 27]. This would also result in different residency periods in the Golgi. It is possible that longer residency and accumulation of LAMP1 in the trans-Golgi network results in mis-targeting of some of the LAMP1 molecules to the cell surface instead of lysosomes.

Expression of LAMP1 on the surface of B16F1 and B16F10 has been shown to correlate with their metastatic potential [10]. In addition, the surface expression of LAMP1 was also found to correlate with the levels of poly-*N*-acetyllactosamine (polyLacNAc) substituted β1,6 branched N-oligosaccharides in these B16 melanoma variants [10]. These metastatic variants thus offer an excellent model to explore if the levels of expression of polyLacNAc substituted N-oligosaccharides dictate the levels of expression of the lysosomal protein LAMP1 on the cell surface.

2. MATERIALS AND METHODS:

2.1. Cell lines and reagents

B16F1 and B16F10 murine melanoma cell lines were obtained from National Centre for Cell Science, Pune, India. Cell culture reagents were obtained from Invitrogen, USA. Anti-mouse LAMP1 antibody (clone 1D4B) raised in rat was purchased from BD Biosciences, USA. PVDF membrane and ECL kit were purchased from GE Healthcare, Amersham, UK. Cultureware were obtained from Nunc and BD Falcon, USA. Swainsonine, paraformaldehyde, BSA, β-actin antibody, Phalloidin-FITC, anti-rat HRPO and anti-rat FITC were purchased from Sigma Chemical Company, USA. All other chemicals were purchased locally and were of analytical grade.

2.2. Cell culture

Melanoma cells were routinely cultured as described previously [26]. Briefly, melanoma cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10%

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fetal bovine serum (FBS), 0.03% L-glutamine, 10 units/ml of Penicillin G-sodium, 10 µg/ml of Streptomycin sulphate and 25 µg/ml of Amphotericin B.

2.3. Treatment of cells with swainsonine

For inhibition of glycosylation, cells were treated with swainsonine as described in [28]. Briefly, melanoma cells were grown in presence of N-glycosylation inhibitor, swainsonine (SW) (2 µg/ml) for 48 h and harvested for in-vitro experiments on reaching 90% confluency.

2.4. Preparation of total cell lysates, protein estimation, SDS-PAGE and western blotting

Preparation of total cell lysates, protein estimation, SDS-PAGE and western blotting was done exactly as described previously [10].

2.5. Flow cytometric analysis

For flow cytometry of LAMP1, melanoma cells were stained with LAMP1 antibody as described previously [10]. For staining with LPHA or LEA, melanoma cells were first fixed by overnight incubation with 1% paraformaldehyde in PBS (pH 7.4) followed by staining with biotinylated lectins as described previously [10]. Briefly, 0.5 million melanoma cells were incubated with 30 µg of biotinylated lectins in 40 µl of FACS buffer (PBS pH 7.4, containing 1% FBS) followed by incubation with extra-avidin-FITC diluted 1:25 in FACS buffer. Cells treated with extra-avidin-FITC alone served as control. Fluorescent cells were acquired at 488 nm and analyzed on FACS Calibur using Cell Quest software (BD Biosciences).

3. RESULTS AND DISCUSSION:

3.1. Expression of polyLacNAc substituted β1,6 branched N-oligosaccharides correlates with surface expression of LAMP1 and metastatic potential of melanoma cells.

To confirm the correlation of expression of polyLacNAc substituted β1,6 branched N-oligosaccharides with metastatic potential and cell surface LAMP1, the melanoma cell lines were evaluated for the expression of these oligosaccharides and LAMP1 on their cell surface. It was conclusively shown that the expression of β1,6 branched N-oligosaccharides (recognized by lectin LPHA) indeed correlates with metastatic potential, as seen by both flow cytometry (Fig. 1A) as well as Western blotting (Fig. 1C). Beta 1,6 branch on N-oligosaccharides is the most preferred site for polyLacNAc substitutions. Results showed that total as well as the cell surface expression of polyLacNAc (recognized by lectin LEA) also correlates with the metastatic potential of melanoma cells (Fig. 1B & 1C). LAMP1 was previously shown to be one of the major carriers of polyLacNAc in these cells. Results clearly showed that the surface expression of LAMP1 also correlates with metastatic potential of these cells (Fig. 1D), although the total LAMP1 levels in these cells remained same (Fig. 1D inset). Since both cell surface expression of LAMP1 as well as the expression of polyLacNAc substituted β1,6 branched N-oligosaccharides correlates with metastatic potential, it would be interesting to investigate whether increased glycosylation in these cells has any influence on the translocation of LAMP1 to cell surface.

3.2. Treatment of B16F1 cells with swainsonine results in decreased glycosylation and consequently decreased surface expression of LAMP1.

To determine the role of glycosylation in surface translocation of LAMP1, B16F1 murine melanoma cells were treated with swainsonine, an α-mannosidase-II inhibitor which prevents the formation of complex-type N-oligosaccharides. Effect of treatment of B16F1 cells with swainsonine on the levels of β1,6 branched N-oligosaccharides and polyLacNAc was determined by flow

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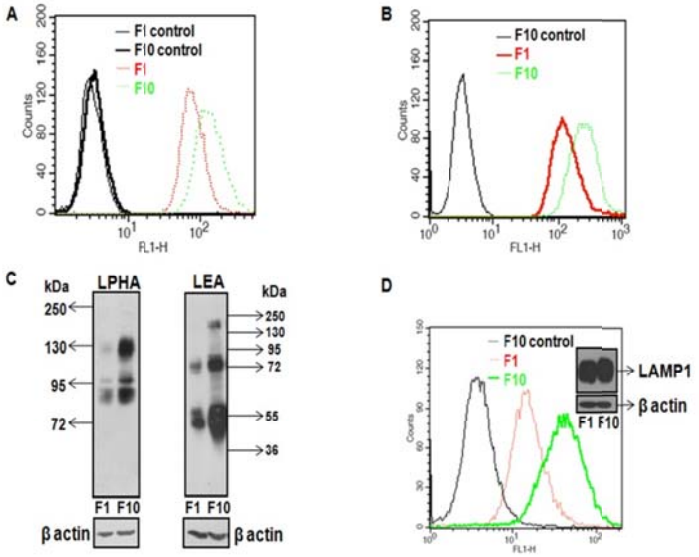
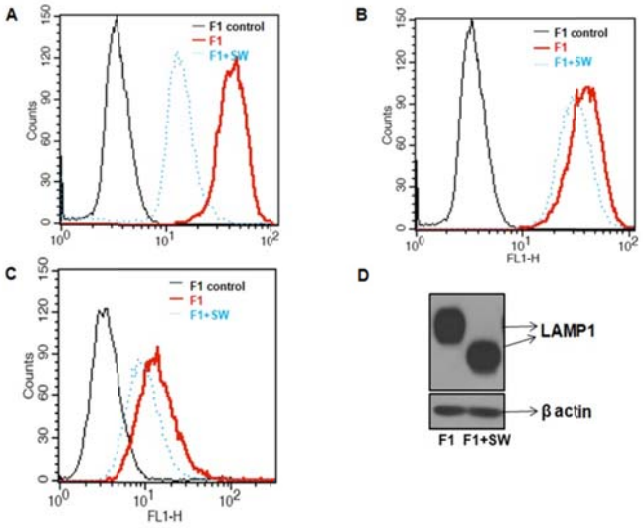
Figure 1.



Analysis of expression of β1,6 branched N-oligosaccharides, polyLacNAc and LAMP1 in B16F1 and B16F10 cells. Comparison of expression of **(A)** β1,6 branched N-oligosaccharides using biotinylated LPHA, **(B)** polyLacNAc using biotinylated LEA and **(D)** LAMP1 using rat anti-LAMP1 antibody, on the surface of B16F1 (red line) and B16F10 cells (green line) using flow cytometry. B16F1 and B16F10 cells treated only with either extra-avidin-FITC (A and B) or anti-rat FITC (D) served as control (black line). **(C)** Comparison of total expression of β1,6 branched N-oligosaccharides using biotinylated LPHA and polyLacNAc using biotinylated LEA in B16F1 (F1) and B16F10 (F10) cells by Western blotting. **(D, inset)** Comparison of total LAMP1 in F1 & F10 cells by Western blotting. β-actin served as loading control in (C & D, inset).

Figure 2.



Analysis of surface expression of β1,6 branched N-oligosaccharides, polyLacNAc and surface as well as total LAMP1 in B16F1 cells and B16F1 cells treated with swainsonine. Comparison of surface expression of **(A)** β1,6 branched N-oligosaccharides using biotinylated LPHA, **(B)** polyLacNAc using biotinylated LEA and **(C)** LAMP1 using rat anti-LAMP1 antibody, in B16F1 cells (F1, red line) and B16F1 cells treated with swainsonine (F1+SW, sky blue line) using flow cytometry. B16F1 cells treated only with either extra-avidin-FITC (A and B) or anti-rat FITC (C) served as control (black line). **(D)** Comparison of total LAMP1 in F1 & F1+SW cells by Western blotting. β-actin served as loading control.

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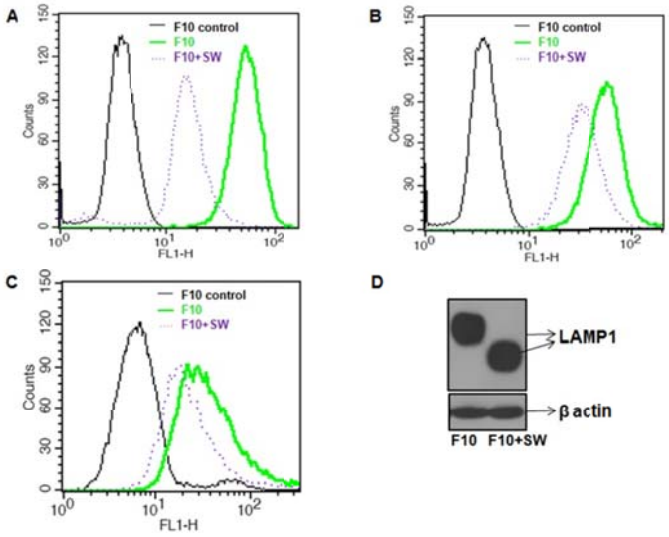
Figure 3.



Analysis of surface expression of β1,6 branched N-oligosaccharides, polyLacNAc and surface as well as total LAMP1 in B16F10 cells and B16F10 cells treated with swainsonine. Comparison of surface expression of **(A)** β1,6 branched N-oligosaccharides using biotinylated LPHA, **(B)** polyLacNAc using biotinylated LEA and **(C)** LAMP1 using rat anti-LAMP1 antibody, in B16F10 cells (F10, green line) and B16F10 cells treated with swainsonine (F10+SW, purple line) using flow cytometry. B16F10 cells treated only with either extra-avidin-FITC (A and B) or anti-rat FITC (C) served as control (black line). **(D)** Comparison of total LAMP1 in F10 & F10+SW cells by Western blotting. β-actin served as loading control.

cytometry. The treatment resulted in decrease in levels of both β1,6 branched N-oligosaccharides (Fig. 2A) as well as polyLacNAc (Fig. 2B) thus confirming the inhibition of these oligosaccharides. The effect of their inhibition on cell surface expression of LAMP1 was then explored. Surprisingly, there was decrease in surface expression of LAMP1 as well (Fig. 2C) in these cells.

Moreover, the total levels of LAMP1 remained unaltered (Fig. 2D) indicating that inhibition of glycosylation had no effect on stability of LAMP1 protein per se. Furthermore, the shift in molecular weight of LAMP1 of SW treated B16F1 cells in Fig. 2D indicates the loss of glycosylation on LAMP1 as a result of swainsonine treatment.

However, as the levels of surface LAMP1 and the associated carbohydrates are comparatively low in B16F1 cells, the effect of swainsonine treatment was also investigated in high metastatic B16F10 cells which have considerably higher expression of LAMP1 as well as polyLacNAc substituted β1,6 branched N-oligosaccharides on their surface.

3.3. Treatment of B16F10 cells with swainsonine results in decreased glycosylation and consequently decreased surface expression of LAMP1.

Similar to that observed in B16F1 cells, treatment of B16F10 cells with swainsonine also resulted in decrease in levels of both β1,6 branched N-oligosaccharides (Fig. 3A) as well as polyLacNAc (Fig. 3B) confirming the inhibition of these oligosaccharides. Their inhibition resulted in significantly decreased levels of LAMP1 on surface of B16F10 cells as well (Fig. 3C) without affecting the total levels (Fig. 3D). This further confirmed the participation of glycosylation in regulating the surface translocation of LAMP1.

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LAMP1, a lysosomal membrane protein has been shown to get sorted to lysosomes due to the presence of a specific tyrosine residue (tyr^{386}) present in its cytoplasmic tail since mutation of this single tyrosine leads to mistargeting of LAMP1 to cell surface instead of lysosomes [29]. However, the possibility of surface translocation of LAMP1 in metastatic cells using this strategy seems a distant reality, as chances of such frequent mutations are rare under physiological conditions. Another possibility of LAMP1 surface localization is by the fusion of lysosomes with cell membrane during the process of exocytosis of cytotoxic granules of cytotoxic lymphocytes and natural killer cells during viral infection or transformation [3, 4, 30]. However LAMP2, another abundant lysosomal membrane protein, was not found to be translocated to the surface of these B16 melanoma cells [10]. However, if lysosome fusion is the reason for increased LAMP1 surface expression, then even LAMP2 would have appeared on the cell surface. Hence, surface expression of LAMP1 through lysosome fusion during exocytosis is also ruled out. In this scenario, altered levels of glycosylation which have been shown to modulate the surface expression of several cell surface receptor proteins [31-33] appear to be the more plausible mechanism.

LAMP1 is a lysosomal protein and traffics through ER and Golgi like all other membrane proteins till it reaches trans-Golgi network (TGN) where sorting occurs. LAMP1 carries high levels of polyLacNAc substituted β1,6 branched N-oligosaccharides, although their levels appear to vary depending on the cell type. B16F10 cells have higher levels of enzymes responsible for formation of β1,6 branched N-oligosaccharides and polyLacNAc [26, 27]. LAMP1 having 17-20 N-glycan sites, the residency time of LAMP1 in Golgi where these branches are added, is therefore expected to be higher in B16F10 cells. It has indeed been shown that longer residence in Golgi allows LAMP1 to acquire more polyLacNAc [25]. Thus, it is possible that during the process of acquiring more polyLacNAc, some LAMP1 molecules get mistargeted to cell surface instead of lysosomes. On the contrary, SW treatment results in the expression of hybrid kind of N-oligosaccharides which are not the substrates for enzymes that add β1, 6 branch or polyLacNAc. As a consequence, the possible residency in Golgi and thus mistartgeting of LAMP1 molecules to the cell surface is considerably reduced.

4. CONCLUSIONS:

Taken together, these results for the first time provide direct evidence for the involvement of altered N-glycosylation specially in the form of polyLacNAc substituted β1,6 branched N-oligosaccharides in regulating the surface expression of LAMP1.

Conflict of Interest

The authors have no conflict of interest.

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ORIGINAL ARTICLE – CANCER RESEARCH

Role of tumor cell surface lysosome-associated membrane protein-1 (LAMP1) and its associated carbohydrates in lung metastasis

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Abstract
Purpose Expression of lysosome-associated membrane protein-1 (LAMP1) on the surface correlates with metastatic potential of B16 melanoma cells. Downregulation of their expression in high metastatic (B16F10) cells reduced their surface expression and metastatic potential. Present investigations explore if overexpression of LAMP1 on the surface of low metastatic (B16F1) cells augment their metastatic ability, and if so, how?
Methods B16F1 cells were transduced with lentiviral vector carrying mutant-LAMP1 (Y386A) (mutLAMP1). Surface expression of LAMP1 and carbohydrates was analyzed by flow cytometry, immunofluorescence and/or immunoprecipitation and Western blotting. Cell spreading and motility were assessed on components of extracellular matrix (ECM) (fibronectin) and basement membrane (BM) (matrigel), and galectin-3-coated coverslips/plates. Metastatic potential was assessed using experimental metastasis assay.
Results Pre-incubation with anti-LAMP1 antibodies significantly reduced lung metastasis of B16F10 cells. Overexpression of mutLAMP1 significantly increased its surface expression on B16F1 cells, resulting in increased cellular spreading and motility on fibronectin and matrigel. LAMP1 is the major carrier of poly-*N*-acetyllactosamine (polyLacNAc) on B16F10 cells. However, significantly higher expression of mutLAMP1 had no effect on galectin-3 binding on cell surface or on spreading or motility of cells on galectin-3-coated coverslips/plates. These cells also failed to show any gain in metastatic ability. This could be because LAMP1 from these cells carried significantly lower levels of polyLacNAc in comparison with B16F10 cells.
Conclusions PolyLacNAc on B16F10 cells and galectin-3 on lungs are the major participants in melanoma metastasis. Although surface LAMP1 promotes interactions with organ ECM and BM, carbohydrates on LAMP1 play a decisive role in dictating lung metastasis.

Keywords Cell surface LAMP1 · Organ-specific metastasis · β1,6 branched N-oligosaccharides · Poly-*N*-acetyllactosamine · Galectin-3 · Motility

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Introduction

Metastasis, the major cause of mortality seen in cancer patients, is a complex multistep process involving detachment from the primary, invasion and intravasation, survival in circulation, extravasation and organ homing (Valastyan and Weinberg 2011). Most of the regional spread of tumors can be explained by anatomical/mechanical

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mode of metastasis (Weiss 1992). However, the distant metastasis is generally organ-specific (Fidler 2003; Gupta and Massagué 2006; Nguyen et al. 2009). This involves one or all of the following factors, viz, adhesive interactions between the molecules on the surface of tumor cells and the target organ, organ growth microenvironment and, more recently, chemokines and their receptors that have been shown to play a critical role in organ-specific metastasis (Fidler 2003; Irmisch and Huelsken 2013; Poste and Nicolson 1980). Tumors often show several metastasis-associated cell surface modifications (Brooks et al. 2010; McGary et al. 2002). Expression of lysosome-associated membrane protein-1 (LAMP1) on the cell surface is one such modification where a lysosomal protein LAMP1 gets increasingly translocated to the surface of several metastatic tumor cells. LAMP-1 is a highly glycosylated protein which decorates the luminal side of lysosomes. Owing to the presence of highly substituted oligosaccharides, it is thought to protect itself and the lysosomal membranes from intracellular proteolysis (Fukuda 1991; Kundra and Kornfeld 1999). It has been shown to be expressed on surface of human melanoma, human colon carcinoma, human fibrosarcoma, human myelomonocytic leukemia and macrophage–melanoma fusion hybrid cells (Chakraborty et al. 2001; Mane et al. 1989; Sarafian et al. 1998). Its cell surface expression has been shown to correlate with metastatic potential of human colon carcinoma and murine melanoma cell lines (Krishnan et al. 2005; Saitoh et al. 1992). In addition to metastatic tumor cells, increased expression of LAMP1 (also known as CD107a) on the surface has also been observed on cells that are involved in migratory and/or invasive functions such as activated cytotoxic T lymphocytes, natural killer cells, platelets and macrophages as well as embryonic cells (Alter et al. 2004; Betts et al. 2003; Chakraborty et al. 2001; Cohnen et al. 2013; Febbraio and Silverstein 1990; Kannan et al. 1996; McCormick et al. 1998). However, the mechanism by which cell surface LAMP1 may mediate these functions is largely unknown.

Purified LAMP1 has been shown to bind to RGD peptides, ECM components such as fibronectin and collagen type I and BM components such as laminin and collagen type IV (Laferté and Dennis 1988), suggesting that surface LAMP1 might as well interact with organ ECM and BM components. Besides, LAMP1 has also been found to be a major carrier of poly-*N*-acetyllactosamine (polyLacNAc)-substituted β1,6 branched N-glycans (Dennis et al. 1987; Fukuda 1991; Krishnan et al. 2005). A transformation-related increase in β1,6 branching observed in fibroblasts, metastatic cell line SP1 and macrophage–melanoma fusion hybrids appeared to be associated with increased LAMP1 surface expression (Chakraborty et al. 2001; Heffernan et al. 1989). LAMP1 on cell surface has been shown to provide ligands in the form of sialyl-Le^x to E-selectin (Sawada et al. 1993; Tomlinson et al. 2000) and in the form of polyLacNAc to galectin-3 (Inohara and Raz 1994; Krishnan et al. 2005; Sarafian et al. 1998). LAMP1 has also been shown to be present on unique cell surface domains involved in cell locomotion such as membrane ruffles and microspikes (filopodia) (Garrigues et al. 1994). Further, its accumulation at the edges and extensions of A2058 human metastasizing melanoma cells (Sarafian et al. 1998) hints toward its potential role in tumor cell spreading and motility possibly by serving as additional receptors for molecules on ECM, BM and endothelium.

Using low (B16F1) and high (B16F10) metastatic variants of lung colonizing B16 murine melanoma cells (Hart and Fidler 1980), polyLacNAc-substituted β1,6 branched N-oligosaccharides were shown to promote metastasis of B16F10 cells to the lungs via galectin-3. In addition, LAMP1 was found to be a major carrier of these oligosaccharides in B16 melanoma cells. It was also shown that surface translocation of LAMP1, but not LAMP2, correlated with their metastatic potential (Krishnan et al. 2005). Recently, it was further shown that downregulation of LAMP1 significantly reduced expression of LAMP1 on the surface of B16F10 cells resulting in significant loss of their metastatic potential (Agarwal et al. 2014). LAMP1 has been shown to be a ligand for galectin-3 which is present in highest amounts in mice lungs and expressed constitutively on the surface of lung vascular endothelium (Krishnan et al. 2005).

However, it remains to be elucidated whether expression of LAMP1 protein alone on the cell surface is necessary and sufficient or it also requires glycosylation on LAMP1 especially in the form of polyLacNAc-substituted β1,6 branched N-oligosaccharides for efficient metastasis. The present paper, therefore, aims to investigate the effect of overexpression of LAMP1 on the surface of low metastatic B16F1 cells (deficient in glycosylation machinery) on their spreading and movement on components of ECM and BM together with that on galectin-3, and its bearing on their lung metastasis.

Materials and methods

Cell lines and reagents

B16F1 and B16F10 murine melanoma cell lines were obtained from National Centre for Cell Science, Pune, India. Cell culture reagents were obtained from Invitrogen,

USA. *Escherichia coli* BL 21 with pET3C plasmid containing a full-length human galectin-3 was a kind gift from Dr. Hakon Leffler, Lund University, Sweden. Purified rhgalectin-3 was biotinylated as described in (Bayer and Wilchek 1990). Inbred strains of C57BL/6 mice used for the metastatic assays and other experiments were maintained in the Institute Animal House, and all the animal experiments were approved by the Institutional Animal Ethics Committee.

Cell culture and experimental metastasis assay

Melanoma cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % fetal bovine serum (FBS), 0.03 % glutamine, 10 units/ml of Penicillin G-sodium, 10 μg/ml of streptomycin sulfate and 25 μg/ml of amphotericin B. For treatment of cells with LAMP1 antibody, melanoma cells were harvested with 0.25 % trypsin, washed in serum-free DMEM and incubated with 10 μg/ml of LAMP1 antibody for 1 h at 4 °C. For metastasis assays, it was ensured that the cells existed as single cell suspension and had greater than 95 % viability and the assay was performed exactly as described in (Reddy and Kalraiya 2006). Briefly, cells (0.1 million for F10 + anti-LAMP1 experiment and 0.15 million for F1 + mutLAMP1 experiment, contained in 100 μl) were injected intravenously (i.v.) in inbred strains of female C57BL/6 mice via the lateral tail vein. The animals were sacrificed after 21 days, and melanoma colonies on the surface of the lungs were counted using a dissecting microscope.

Generation of mutLAMP1 (Y386A) clones and their transduction in B16F1 cells

Total RNA was prepared from B16F10 cells using TRIzol reagent, and cDNA was synthesized using cDNA synthesis kit (New England Biolabs, USA) as per manufacturer's protocol. LAMP1 was amplified from cDNA using forward primer having XhoI site (represented in bold italics) followed by Flag tag (represented in bold) and mouse LAMP1 N-terminal sequence (represented in italics): 5′-TAT***CTCGAG*** ATG**GATTACAAGGATGACGATGACAAG**GAATTC*AT GGCGGCCCCCGGCGCC*-3′ and reverse primer: 5′-GGAT CCCTAGATGGTCTGATAGCCGGCGTGACTCC-3′. The lentiviral vector generated previously, pLV-K18-YFP-IRES-Puro (Sehgal et al. 2012), was digested with XhoI and NotI restriction enzymes to remove K18-YFP and was either self-ligated to obtain empty vector control or ligated to the amplified mouse LAMP1 to generate wild-type LAMP1 (wtLAMP1) vector. The wtLAMP1 vector was further used for site-directed mutagenesis of tyrosine^{386} of wtLAMP1 to alanine to get mutant-LAMP1 (mutLAMP1) vector using site-directed mutagenesis kit (Stratagene, USA) as per manufacturer's protocol. The primers used for site-directed mutagenesis included: 5′-AGGAGTCACGCCGGC GCTCAGACCATCTAGGG-3′ and 5′-CCCTAGATGGTCT GAGCGCCGGCGTGACTCCT-3′. The vector control plasmid, the wtLAMP1 plasmid and the mutLAMP1 plasmid were purified and subsequently co-transfected with helper plasmids (pMD2.G and psPAX2) in HEK293FT cells, and infectious viruses containing empty vector control, wtLAMP1 and mutLAMP1 were generated as described previously (Ranjan and Kalraiya 2013) which were used for transduction of B16F1 cells using 8 μg/ml polybrene. The clones were stably selected using puromycin (1 μg/ml). One vector control clone (VC), one wtLAMP1 clone and two mutLAMP1 clones (C1 and C11) growing in the form of isolated colonies were selected. The selected clones were maintained at a concentration of 0.5 μg/ml puromycin.

Flow cytometric analysis of galectin-3 binding and surface expression of LAMP1

For flow cytometry of LAMP1, melanoma cells were stained with LAMP1 antibody (clone 1D4B, BD Biosciences, USA) as described previously (Krishnan et al. 2005). For determination of galectin-3 binding, melanoma cells were first fixed by overnight incubation with 1 % paraformaldehyde in PBS (pH 7.4) followed by galectin-3 staining as described previously for biotinylated LPHA (Krishnan et al. 2005). Briefly, 0.5 million melanoma cells were incubated with 30 μg of biotinylated rhgalectin-3 in 40 μl of FACS buffer (PBS pH 7.4, containing 1 % FBS) followed by extra-avidin-FITC (Sigma) diluted 1:25 in FACS buffer. Cells treated with extra-avidin-FITC alone served as control. Fluorescent cells were acquired at 488 nm and analyzed on FACSCalibur using CellQuest software (BD Biosciences).

Detection of expression of LAMP1 on the cell surface by immunofluorescence staining

Immunofluorescence staining was done as described in (Ranjan et al. 2014). Briefly, melanoma cells were seeded on coverslips and grown overnight in complete medium up to 70–80 % confluency. Cells were washed thrice with PBS (pH 7.4) and fixed with 2 % paraformaldehyde at RT for 5 min. They were washed again with PBS, blocked with 3 % BSA in PBS for 1 h at RT in humidified chamber and incubated with primary antibody (LAMP1) for 1 h in humidified chamber, followed by three washes with PBS to remove excess or non-specifically bound antibody. Cells were further incubated with

fluorescence-tagged secondary antibody (anti-rat FITC) for 1 h followed by three washes with PBS. Those incubated only with fluorescence-tagged secondary antibody served as isotype control. Nuclei were stained with 5 μg/ml of 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 1 min, and coverslips were mounted on slides using vectashield mounting medium. Images were acquired using a Carl Zeiss laser confocal microscope at 63× magnification.

Purification of recombinant human galectin-3

Expression and purification of recombinant human (rh) galectin-3 was carried out exactly as described previously (Krishnan et al. 2005).

Cell spreading assays

Cell spreading assays were done as per (Lagana et al. 2006). Briefly, melanoma cells were harvested, washed free of serum and seeded at a cell density of 0.5 million/ml in serum-free DMEM on the coverslips coated overnight with 75 μg/ml galectin-3, 10 μg/ml fibronectin and matrigel (BD Biosciences, USA) in serum-free DMEM at 4 °C. The cells were incubated for 45 min in a CO_2 incubator. Coverslips treated with serum-free DMEM only served as control. Bound cells were fixed in 4 % paraformaldehyde, permeabilized with 0.5 % Triton X-100 for 15 min and stained with 2 μg/ml Phalloidin-FITC staining solution made in PBS (containing 1 μg/ml of lysolecithin, 10 % methanol, 0.5 % BSA) (Lagana et al. 2006) for 15 min at 37 °C. Nuclei were stained with 5 μg/ml of DAPI in PBS for 1 min. The stained cells were mounted, and images were acquired using a Carl Zeiss laser confocal microscope at 63× magnification.

Wound-healing assays

For wound-healing assays, six well culture dishes were coated overnight with 75 μg/ml of galectin-3, 10 μg/ml of fibronectin and matrigel in serum-free DMEM at 4 °C, followed by blocking of non-specific sites with 2 % BSA for 1 h. Melanoma cells were harvested, seeded at a density of 0.5 million cells per ml of complete medium and incubated at 37 °C for 24 h in a CO_2 incubator. The cells were treated with 40 μg/ml mitomycin C (Sigma) for 3 h for inhibiting cell proliferation. A straight, uniform wound (approx. 400 μm in width) was made using a micropipette tip on the monolayer, and the cells were maintained in serum-free DMEM. Wound closure of cells in response to the immobilized galectin-3, fibronectin and matrigel was measured for 16 h by time lapse video imaging of at least three different positions across the length of the wound using a Carl Zeiss inverted microscope at 10x magnification. Uncoated wells, blocked only with BSA, served as control.

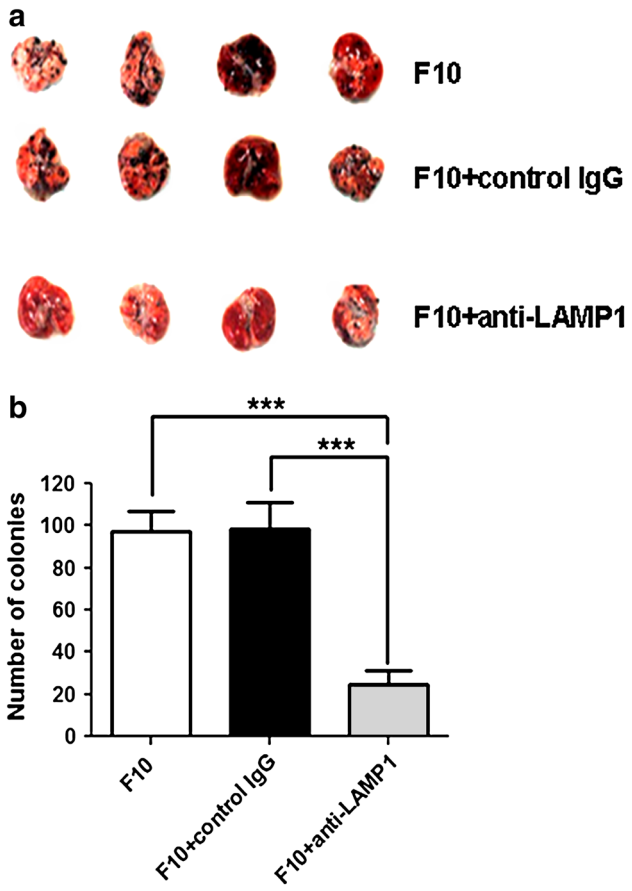

Fig. 1 Cell surface LAMP1 plays an important role in lung-specific metastasis. **a** Melanoma colonies on lungs of C57BL/6 mice injected with untreated B16F10 cells (F10), and those treated with 10 μg/ml of either control rat IgG (F10 + control IgG) or blocking antibodies to LAMP1 (F10 + anti-LAMP1). Four mice were taken in each group. **b** Graphical representation of mean number of lung colonies. One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ***p value <0.001)

Preparation of total cell lysates, protein estimation, SDS-PAGE and Western blotting

Preparation of total cell lysates, protein estimation, SDS-PAGE and Western blotting was done exactly as described previously (Krishnan et al. 2005).

Immunoprecipitation of LAMP1

Total cell lysate (2 mg) was precleared and later incubated at RT for 2 h with 20 μg of anti-LAMP1 antibody. This was followed by addition of 200 μl of protein G

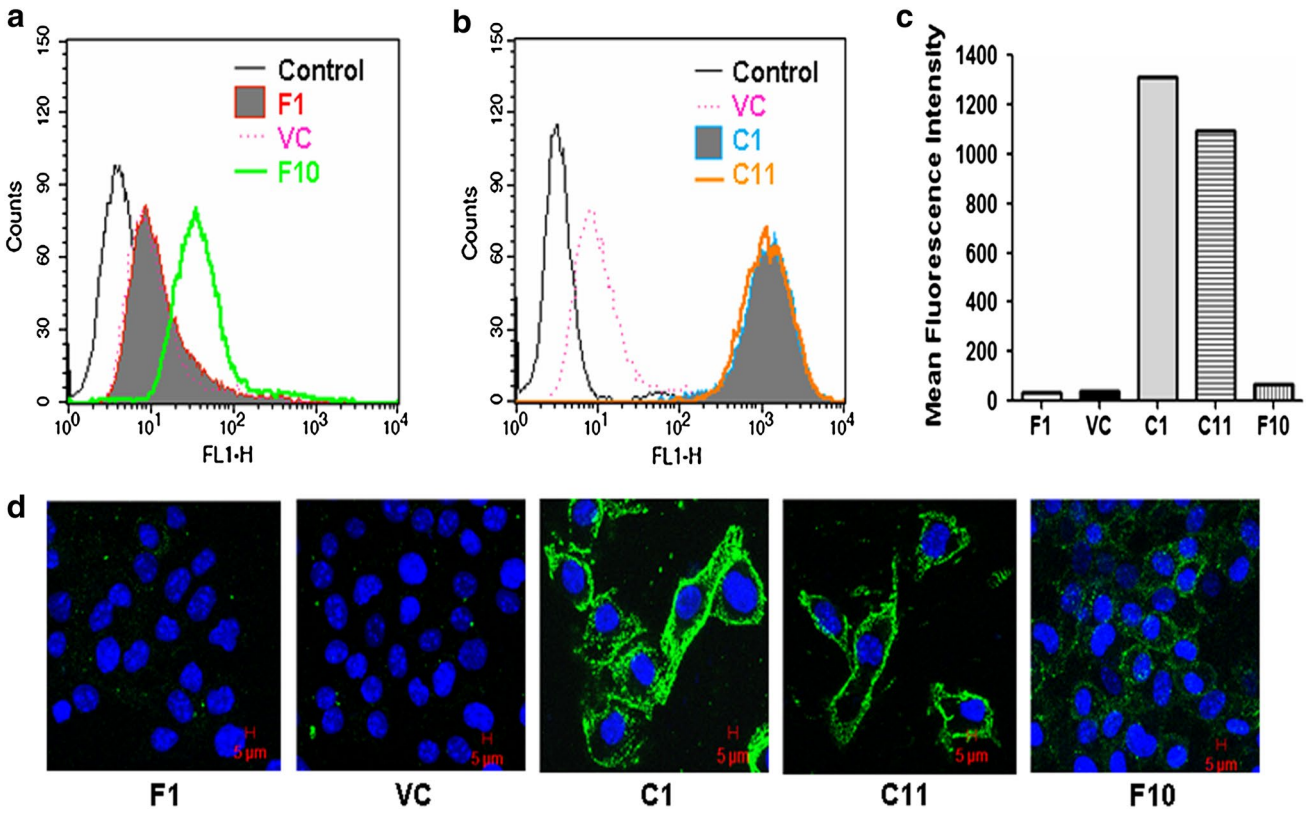

Fig. 2 Analysis of LAMP1 expression on the surface of melanoma cells. Comparison of surface expression of LAMP1 by flow cytometry, in **a** B16F1 cells (*gray shade* F1), B16F1 cells infected with viruses having empty vector control (*pink dotted lines* VC) and B16F10 cells (*green solid lines* F10) and **b** comparison of the same between vector control (*pink dotted lines* **VC**) and B16F1 clones expressing mutLAMP1 (*gray shade* C1) and (*orange solid lines* C11). Cells treated with only anti-rat FITC served as control (—). The flow cytometry overlays have been split into two for better understanding of the data. **c** Graphical representation of the mean fluorescence intensities of surface LAMP1 of the cells shown in **a** and **b**. **d** Immunofluorescence images of the cells stained with anti-LAMP1 antibody and FITC-labelled secondary antibody (*green*). Nuclei were stained with DAPI (*blue*). *Scale bar* 5 μm

Sepharose beads (50 % suspension) (GE Healthcare, Amersham, UK) and incubation overnight at 4 °C. The beads were pelleted at 2,000 rpm for 10 min and later washed five times with 1 ml of lysis buffer. The bound proteins were eluted by boiling the beads in 1× Laemmli sample buffer for 5 min, separated on SDS-PAGE, Western blotted and probed with LAMP1 antibody and with biotinylated LPHA or LEA (Vector Labs, USA) as described previously (Krishnan et al. 2005).

Statistical analysis

All the data are represented as mean ± SD unless stated. All the statistical analysis was performed using GraphPad Prism 5. For spreading and experimental metastasis assays, comparison within the group was done by performing one-way ANOVA followed by Bonferroni's multiple comparison test. For wound-healing assays, two-way ANOVA with the Bonferroni posttest was conducted. (p value <0.05 was considered significant).

Results

Blocking cell surface LAMP1 with specific antibodies inhibits lung metastasis of B16F10 cells

Expression of LAMP1 on the cell surface correlates with metastatic potential of B16 melanoma cells, and down-regulation of their expression in high metastatic B16F10 cells inhibited their metastasis. To understand how surface LAMP1 possibly participates in metastasis, B16F10 cells were pre-treated with LAMP1-specific antibodies to make it unavailable for interaction, and its effect on metastasis was assessed. Untreated cells or those treated with pre-immune IgG served as controls. Pre-treatment with anti-LAMP1 antibody significantly reduced the metastatic ability of cells as compared to controls (Fig. 1a, b) confirming that surface LAMP1 indeed plays a key role in imparting metastatic phenotype. To explore the possible mechanism by which LAMP1 facilitates interactions with molecules on lungs, B16F1 cells were transduced with mutLAMP1 (Y386A).

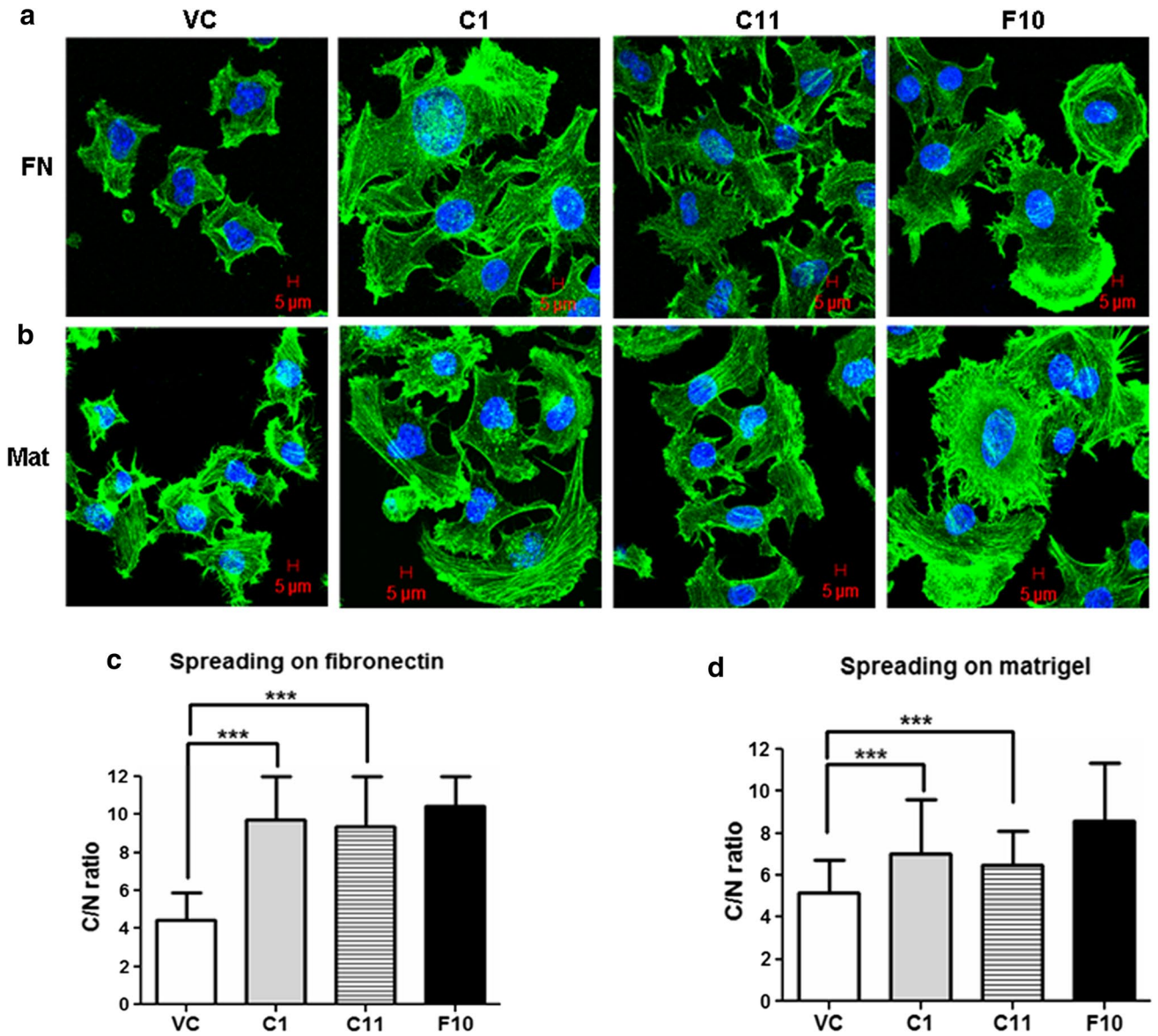

Fig. 3 Effect of increased surface expression of LAMP1 on spreading of melanoma cells on fibronectin and matrigel. Spreading of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **a** fibronectin (FN) and **b** matrigel (Mat)-coated coverslips as assessed by staining with Phalloidin-FITC (*green*). DAPI was used to stain the nuclei (*blue*). *Scale bar* 5 µm. **c** Each *bar* represents ratio of cytoplasmic to nuclear (*C*/*N*) area for around 100 cells from two different experiments for spreading on fibronectin (FN) and **d** for spreading on matrigel (Mat). One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance (denoted by ***p value <0.001)

Expression of mutLAMP1 (Y386A) in B16F1 cells results in elevated expression of LAMP1 on the cell surface

Mutation in a specific region (Tyr^{386}) of the cytoplasmic tail of LAMP1 has been shown to direct them to cell surface instead of lysosomes (Williams and Fukuda 1990). Stable expression of this mutLAMP1 (Tyr^{386} to Ala^{386}) by lentiviral infection of B16F1 cells resulted in significantly higher surface expression of LAMP1 in both the clones (C1 and C11) as compared to either uninfected (F1) or those infected with virus with vector alone (vector control—VC). The surface expression of LAMP1 in the clones C1 and C11 was several folds higher even when compared to B16F10 (F10) cells as observed by both flow cytometry (Fig. 2a–c) and immunofluorescence (Fig. 2d). The impact of increased surface expression of LAMP1 on the cellular properties, important from the point of view of metastasis, was explored.

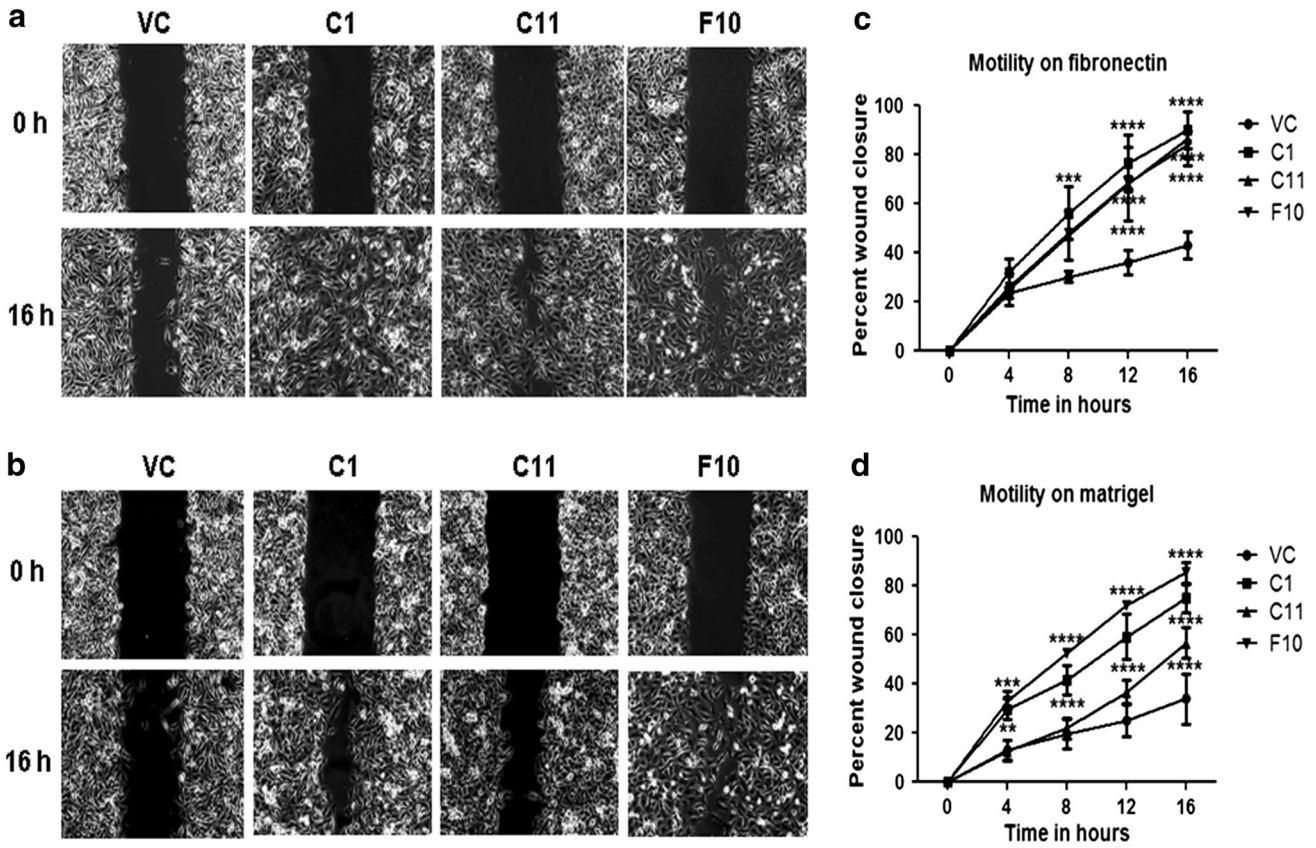

Fig. 4 Effect of increased surface expression of LAMP1 on motility of melanoma cells on fibronectin and matrigel. Motility of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **a** fibronectin and **b** matrigel-coated plates as represented by time lapse video microscopy images at 0 and 16 h of wound closure. **c**, **d** Represent mean percent wound closure at 4-h interval on fibronectin and matrigel, respectively. Area of wound closure was measured by Image J software and each image from two different experiments was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance from VC (denoted by ****p value <0.0001, ***p value <0.001)

Increased surface expression of LAMP1 on B16F1 cells results in significantly increased spreading and motility on fibronectin and matrigel

Purified LAMP1 has been shown to have an affinity for ECM and BM components (Laferté and Dennis 1988). It is possible that the LAMP1 overexpressed on the surface is used as an alternate receptor for these components. The clones overexpressing LAMP1 on the cell surface indeed showed significantly higher spreading on both fibronectin (ECM component) and matrigel (reconstituted BM) as compared to vector control (VC), as seen by laser confocal microscopic images (Fig. 3a, b) and by analyzing ratios of the cytoplasmic to nuclear areas (Fig. 3c, d). The clones also showed much higher motility on these substrates as measured by wound-healing assay (Fig. 4a, c for fibronectin, Fig. 4b, d for matrigel). The results strongly indicate that the increased surface LAMP1 may alter the cellular properties of cells which might eventually be important for metastasis. Wild-type LAMP1 (WT) was also overexpressed in B16F1 cells (Supplementary Fig. S1). But since its expression did neither affect the surface expression of LAMP1 (Supp Fig. S1a and b) nor affect the spreading of melanoma cells on fibronectin (Supp Fig. S1c) to a significant extent as compared to VC, it was not used for further studies.

Increased expression of LAMP1 on the surface of B16F1 cells had no effect on their spreading and motility on galectin-3

LAMP1 is a major carrier of polyLacNAc and is a known ligand for galectin-3. Secreted galectin-3 often becomes part of the ECM, BM and even the cell surface (Liu and Rabinovich 2005) and is used as a substratum for cellular adhesion, spreading and movement. Surprisingly, the increased surface expression of LAMP1 had no effect on spreading of these cells (C1 and C11) on galectin-3 as

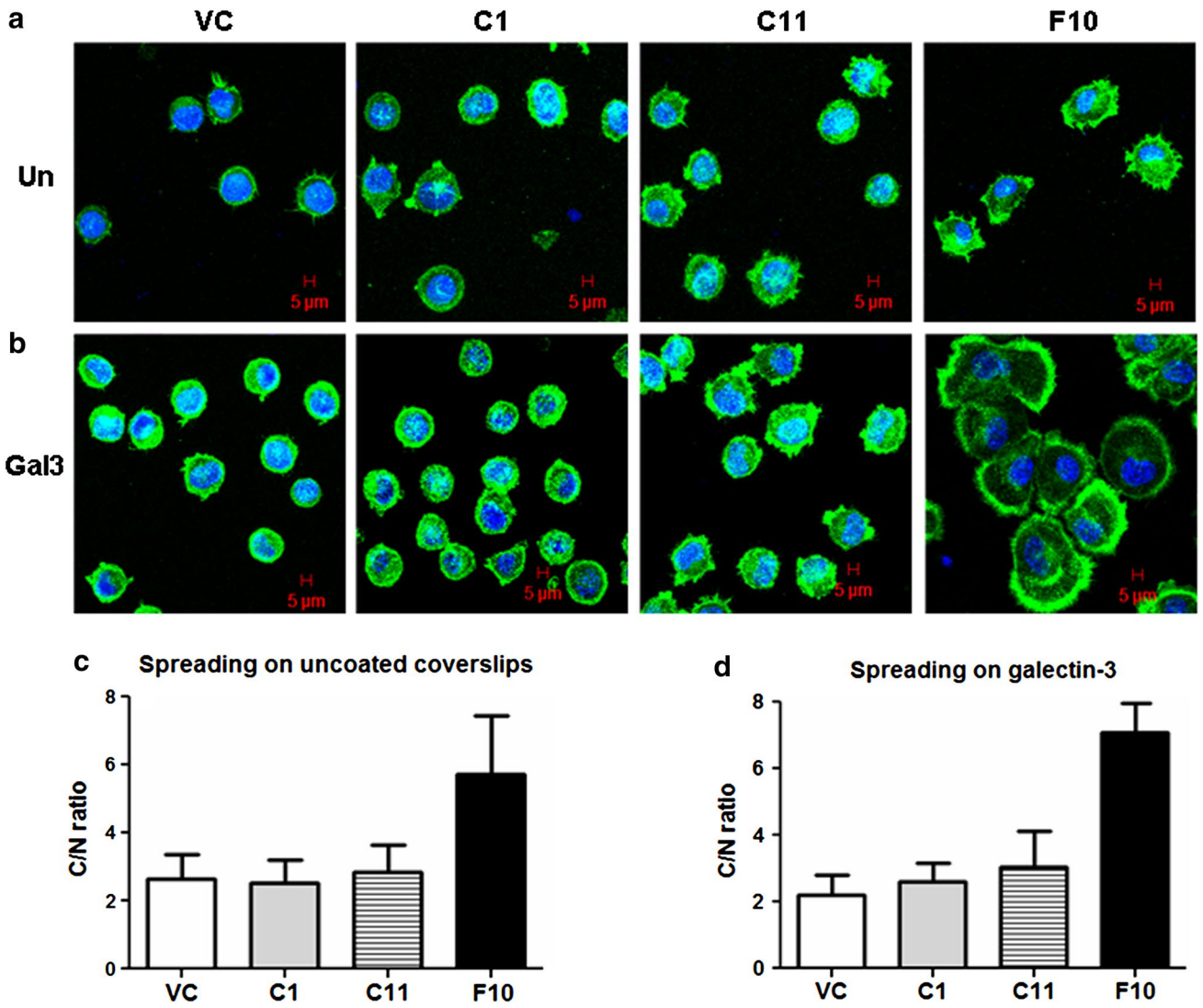

Fig. 5 Effect of increased surface expression of LAMP1 on spreading of melanoma cells on galectin-3. Spreading of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **a** uncoated (Un) and **b** galectin-3 (Gal3)-coated coverslips as assessed by staining with Phalloidin-FITC (*green*). DAPI was used to stain the nuclei (*blue*). *Scale bar* 5 µm. **c**, **d** Each *bar* represents ratio of cytoplasmic to nuclear (*C/N*) area for around 100 cells from two different experiments for spreading on uncoated **c** and galectin-3-coated coverslips **d**. One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance

compared to the vector control cells (VC), and the spreading was very similar to that seen on uncoated coverslips, as seen by laser confocal microscopic images (Fig. 5a, b) and quantitated by ratios of cytoplasmic to nuclear areas (Fig. 5c, d). Besides, motility of these cells (C1 and C11) was also almost similar to vector control cells (VC) in the presence of either BSA (Fig. 6a, c) or immobilized galectin-3 (Fig. 6b, d). The lack of any effect is possibly because of low levels of polyLacNAc substitutions as they have been shown to be the major participants in galectin-3-mediated processes.

Increased surface expression of LAMP1 neither increases galectin-3 binding to B16F1 cells nor increases their metastatic potential

LAMP1 is a highly glycosylated molecule and is a major carrier of polyLacNAc. In spite of >20-fold increase in expression of LAMP1 on the surface of clones C1 and C11 as compared to even B16F10 cells (Fig. 2c), it did not result in any gain of their metastatic potential as compared to the parent B16F1 cells (Fig. 7a). Moreover, the binding of galectin-3 to these cells also remained largely unaltered

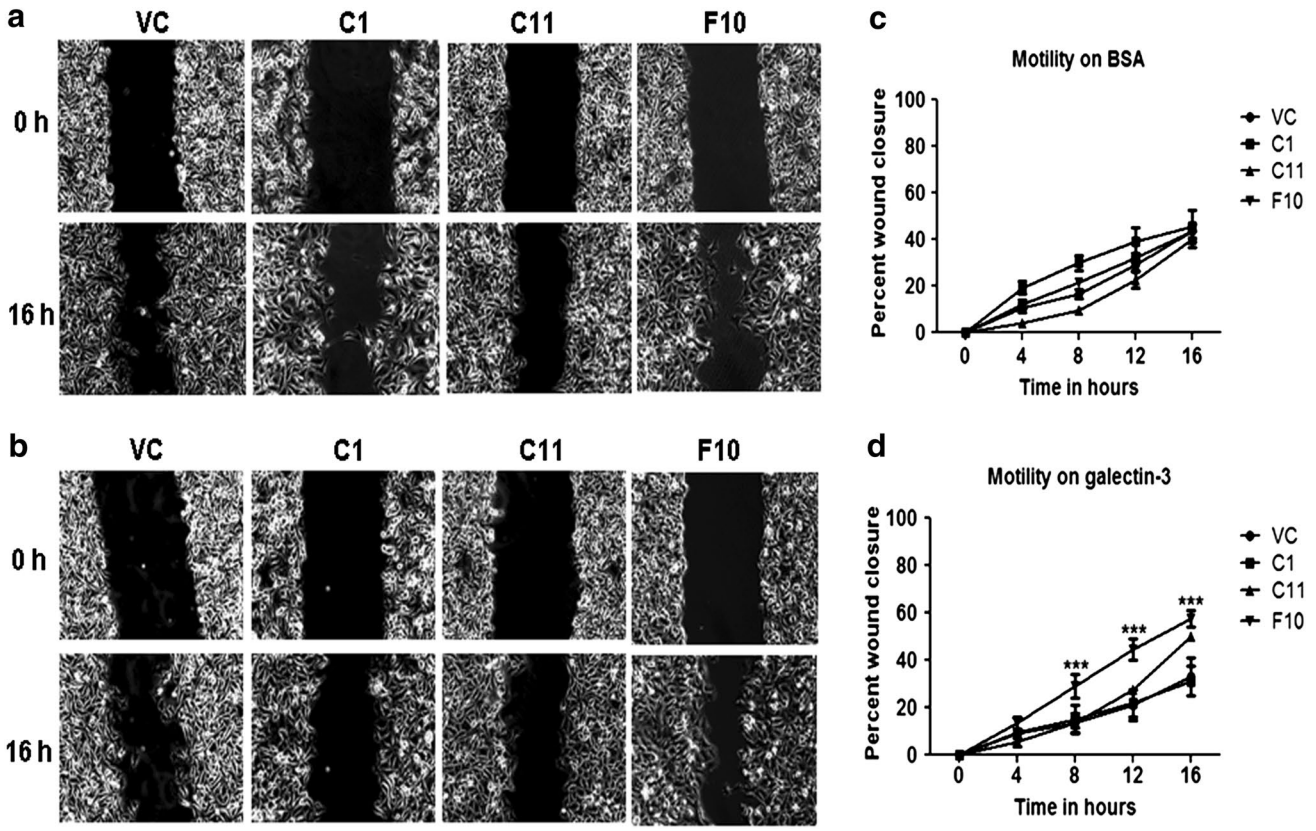

Fig. 6 Effect of increased surface expression of LAMP1 on motility of melanoma cells on galectin-3. Motility of B16F1 cells infected with viruses having empty vector (VC) or those having mutLAMP1 (C1 and C11) and F10 cells on **a** BSA and **b** galectin-3-coated plates as represented by time lapse video microscopy images at 0 and 16 h of wound closure. **c**, **d** Represent mean percent wound closure at 4-h interval on BSA and galectin-3, respectively. Area of wound closure was measured by Image J software and each image from two different experiments was analyzed at three different positions. Two-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare significance from VC (denoted by ***p value <0.001)

(Fig. 7b, c). Immunoprecipitation experiment revealed that β1,6 branched N-oligosaccharides and polyLacNAc (probed with LPHA and LEA, respectively) on LAMP1 from VC and C1 cells were comparable and were much lower as compared to that present on LAMP1 from F10 cells (Fig. 7d). This clearly suggests that carbohydrates on LAMP1 may play a crucial role in lung metastasis.

Discussion

Expression of LAMP1 on the cell surface correlates with metastatic potential of B16 melanoma cells, and down-regulation of its expression results in its decreased surface expression and concomitantly decreased metastasis (Agarwal et al. 2014; Krishnan et al. 2005). LAMP1 on the cell surface possibly facilitates metastasis by providing high density of high affinity ligands for galectin-3 expressed in highest amounts on the lungs and constitutively on its vascular endothelium, or by interacting with specific ECM/BM molecules on the lungs. Its importance in the metastatic process was further confirmed when B16F10 cells pre-incubated with LAMP1-specific antibodies showed significantly reduced metastatic potential (Fig. 1). Binding of these antibodies possibly prevented LAMP1 from interacting with such molecules on the lungs.

Although our previous results point toward the role of polyLacNAc-substituted N-oligosaccharides on LAMP1 in the metastatic process (Agarwal et al. 2014), purified LAMP1 has also been shown to have an affinity for several ECM and BM components such as fibronectin, laminin, collagen-I and IV and even RGD peptides (Laferté and Dennis 1988). On the cytoplasmic end, LAMP1 has been shown to interact with ezrin (Federici et al. 2009) which functions as a linker between the actin cortical cytoskeleton and various membrane-bound molecules (Bretscher et al. 2002; Neisch and Fehon 2011). This may influence cellular properties important for motility. It is thus possible that both LAMP1 protein and the carbohydrates on it contribute in influencing the metastatic process.

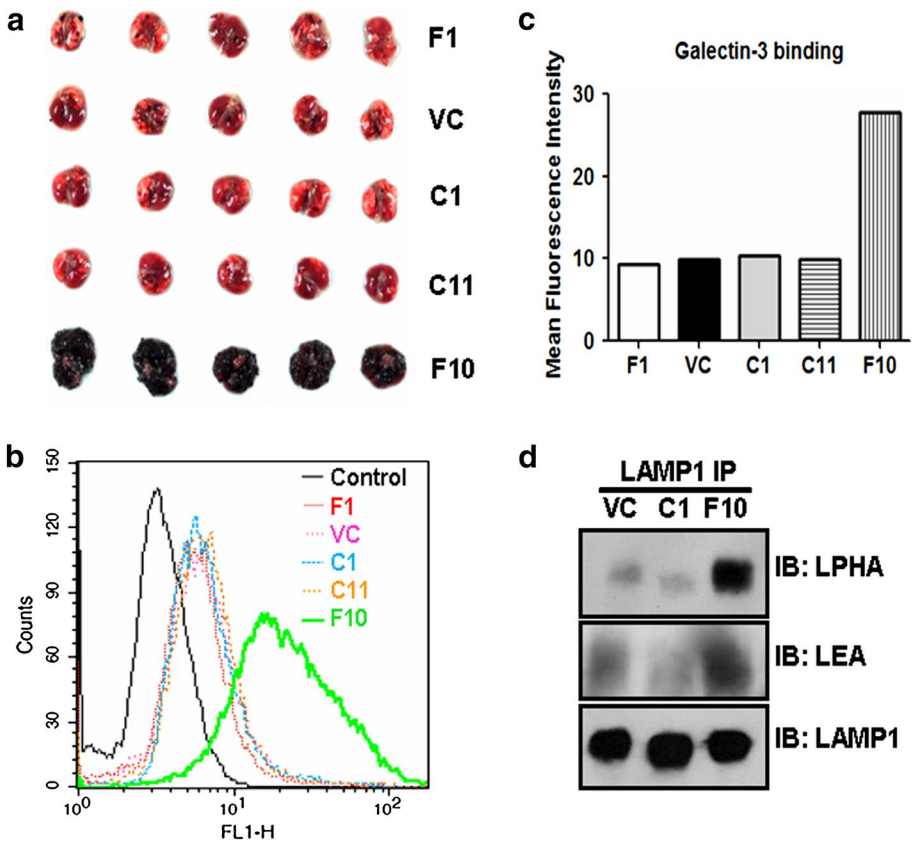

Fig. 7 Increased surface expression of LAMP1 on B16F1 cells has no effect on lung metastasis. **a** Melanoma colonies on lungs of C57BL/6 mice injected with F1, VC, C1, C11 and F10 cells. Five mice were taken in each group. **b** Comparison of galectin-3 binding by flow cytometry using biotinylated galectin-3 in uninfected B16F1 cells (*red dotted lines* F1) or those infected with viruses having empty vector as control (*pink dotted lines* VC) with B16F1 clones expressing mutLAMP1 (*blue dotted lines* C1) and (*orange dotted lines* C11) and with B16F10 cells (*green solid line* F10). Cells treated with only extra-avidin FITC (—) served as control. **c** Graphical representation of the mean fluorescence intensities of galectin-3 binding of all the cells. **d** Comparison of β1,6 branched N-oligosaccharides (LPHA) and polyLacNAc (LEA) on normalized amounts of immunoprecipitated LAMP1 from B16F10 (F10) cells and B16F1 cells having either empty vector (VC) or mutLAMP1 (C1), by Western blotting

The low metastatic B16F1 cells that express much lower levels of both surface LAMP1 and polyLacNAc-substituted β1,6 branched N-oligosaccharides provide a perfect system to investigate the role of LAMP1 and its associated oligosaccharides, in the metastatic process. Transduction of mutLAMP1 in B16F1 cells provided an ideal model to explore the mechanism by which LAMP1 may influence metastasis, as it resulted in significantly higher expression on the cell surface. These cells expressed >20-fold higher expression of LAMP1 on the cell surface as compared to even B16F10 cells (Fig. 2). Considerably increased expression of LAMP1 on cell surface resulted in significant increase in their spreading and motility on both fibronectin (ECM) and matrigel (BM) (Figs. 3, 4). Although integrins are the known receptors for such components, this is the first report which shows that LAMP1 expressed in such higher amounts on the surface could influence these cellular properties and the underlying mechanism would be worth investigating.

Alternatively, LAMP1 may also promote interactions with molecules on the target organ via high levels of glycosylated structures on it. LAMP1 is a highly glycosylated protein. More than 60 % of its weight is contributed by carbohydrates. Each LAMP1 molecule carries 17–20 N-glycosylation sites that are often substituted further with structures such as Lewis antigens and polyLacNAc (Fukuda 1991). As major portion of LAMP1 is extracellular, it may provide ligands for endogenous lectins such as selectins and galectin-3 expressed on the organ vascular endothelium, in an easily accessible manner (Häuselmann and Borsig 2014).

Lungs express highest amounts of galectin-3 and express it on all the major compartments of the lungs including constitutive expression on the surface of its vascular endothelium (Dange et al. 2014; Krishnan et al. 2005). Previously, polyLacNAc-substituted β1,6 branched N-oligosaccharides have been shown to facilitate lung metastasis by anchoring

on to galectin-3 on organ endothelium (Krishnan et al. 2005). More recently, we showed that this lectin carbohydrate pair may participate in not just anchoring, but in all the subsequent steps of extravasation such as spreading to stabilize adhesion, degradation of ECM/BM and movement into organ parenchyma (Dange et al. 2014). It has also been shown that polyLacNAc-substituted N- and not O-oligosaccharides participate in all these processes and shRNA-mediated inhibition of polyLacNAc synthesis inhibits these processes including lung metastasis (Dange et al. 2014; Srinivasan et al. 2009).

PolyLacNAc is the most preferred ligand for galectin-3, and LAMP1 was shown to be one of the major carriers of polyLacNAc on high metastatic B16F10 cells. Moreover, the levels of polyLacNAc-substituted β1,6 branched N-oligosaccharides on LAMP1 per se have been shown to correlate with the metastatic potential of melanoma cells (Krishnan et al. 2005). Further, glycosylation in these cells has also been shown to modulate the surface expression of LAMP1 (Agarwal and Kalraiya 2014). Downregulation of LAMP1 in B16F10 cells has been shown to affect the galectin-3-mediated cellular processes and their metastatic potential (Agarwal et al. 2014). Overexpression of surface LAMP1 on B16F1 cells thus may also influence galectin-3-mediated metastatic processes.

However, B16F1 clones overexpressing LAMP1 on the cell surface showed neither enhanced spreading nor motility on galectin-3-coated surfaces (Figs. 5, 6). Even the ability to metastasize to lungs remained unaltered (Fig. 7a). In spite of >20-fold higher surface expression of LAMP1, binding of galectin-3 to the clones overexpressing surface LAMP1 remained unaltered and was much lower as compared to B16F10 cells (Fig. 7b, c). Since galectin-3-mediated effects are dependent on galectin-3-polyLacNAc interactions, it was plausible to think that each LAMP1 molecule expressed on cell surface might not have adequate polyLacNAc units. Immunoprecipitation experiments indeed confirmed that there was no increase in β1,6 branched N-glycans and polyLacNAc (Fig. 7d) on LAMP1 molecules from these cells which was significantly lower as compared to that on LAMP1 from B16F10 cells. The low levels of polyLacNAc-substituted β1,6 branched N-oligosaccharides on LAMP1 in these cells could be due to limitation in availability of enzymes that add β1,6 branch and polyLacNAc in B16F1 cells (Dange et al. 2014; Srinivasan et al. 2009). Although increasing expression of such enzymes in B16F1 cells may increase their metastatic potential, it would be difficult to attribute it solely to the carbohydrates on LAMP1, as they would glycosylate several other surface proteins as well. The present study thus clearly demonstrates that although increased surface expression of LAMP1 may aid in mediating interactions with the ECM and BM components, it has no influence on melanoma metastasis to the lungs unless it carries high density of ligands (polyLacNAc) for galectin-3.

Conclusions

Metastasis being a multistep process, only cells proficient in all the steps of metastasis are able to metastasize. Cells deficient in mediating even one of these critical events are unable to metastasize, which is often referred to as metastatic inefficiency (Fidler 2003; Weiss 1990). These studies demonstrate that interaction of polyLacNAc on surface LAMP1 with galectin-3 on organ endothelium may be a critical rate-limiting step in the arrest and metastasis of melanoma cells to the lungs. In spite of gaining additional characteristics of interacting with organ ECM and BM, unless proficient in getting arrested in target organ endothelium, the cells fail to metastasize.

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Conflict of interest We declare that we have no conflict of interest.

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Extracellular galectin-3 induces MMP9 expression by activating p38 MAPK pathway via lysosome-associated membrane protein-1 (LAMP1)

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Abstract Matrix metalloproteinases (MMPs) play a key role in matrix remodelling and thus invasion and metastasis. Extracellular galectin-3 has been shown to induce MMP9 secretion. Here, we demonstrate that galectin-3 induces MMP9 at transcript level and it is dependent on the surface levels of poly-*N*-acetyllactosamine (polyLacNAc). By employing signalling pathway inhibitors, MMP9 expression was shown to be induced via p38 MAP-kinase pathway. Using clones of melanoma cells expressing shRNAs to lysosome-associated membrane protein-1 (LAMP1), a major carrier of polyLacNAc, surface LAMP1 was demonstrated to serve as one of the key mediators of galectin-3-induced MMP9 expression via p38 MAPK pathway.

Keywords Galectin-3 · PolyLacNAc · MMP9 · p38 MAPK · LAMP1

Introduction

Metastasis accounts for majority of cancer-related deaths. It is a complex multistep process which involves multiple host tumour interactions [1]. To metastasize, cancer cells must dissociate from the primary, invade the surrounding normal tissue, intravasate, survive in circulation, get arrested in the target organ vasculature, extravasate and survive in the new growth environment [1, 2]. Molecules on the cell surface play a key role in these processes and tumour cells show several metastasis-associated membrane modifications [3, 4]. Expression of β1,6 branched N-oligosaccharides is one such modification [5, 6]. Their expression has been shown to correlate with disease progression and with metastatic potential in several human and murine cancer cell lines [7, 8].

Previously, we have demonstrated that substitution of these oligosaccharides with polyLacNAc promotes lung metastasis of B16 murine melanoma cells [9]. Lungs were shown to express highest levels of galectin-3 and express it constitutively on its vascular endothelium [9]. Galectin-3 on lungs was shown to not only aid circulating tumour cells to anchor on to organ endothelium, but in all the steps of extravasation. It was shown to promote spreading of cells post adhesion, degradation of vascular basement membrane (BM) and movement into organ parenchyma [10]. PolyLacNAc only on N- and not O-oligosaccharides was shown to participate in these processes and even role of other ligands such as T/Tn antigens in melanoma metastasis was ruled out [11]. β1 integrin and the lysosome-associated membrane protein 1 (LAMP1) have been identified to be the major carriers of polyLacNAc on melanoma cells [9].

Expression of LAMP1 on the melanoma cell surface has been shown to correlate with the metastatic potential, and downregulation of its expression inhibits its surface expression and impacts cellular properties like spreading, movement on immobilized galectin-3 and metastatic potential [9, 12]. We for the first time demonstrated that galectin-3 also induces secretion of MMP9 in these melanoma cells in a metastatic potential-dependent manner. Galectin-3 in both soluble and immobilized/coated form was shown to induce secretion of MMP9 in high metastatic

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melanoma (B16F10) cells in a concentration-dependent manner. Inhibition of polyLacNAc on these cells inhibited secretion of MMP9 [10].

Present investigations reveal that galectin-3-induced secretion of MMP9 is regulated at the transcriptional level by the extent of polyLacNAc on the cell surface. The signalling pathway and the polyLacNAc carrying protein through which galectin-3 transmits these signals have been identified in these studies.

Materials and methods

Reagents

Escherichia Coli BL 21 with pET3C plasmid containing a full-length recombinant human galectin-3 was a kind gift from Prof. Hakon Leffler, Lund University, Sweden. TRIzol was from Invitrogen, USA. Power SYBR Green PCR Master Mix and high capacity cDNA reverse transcriptase kit were from Applied Biosystems, Life technologies, USA. Primers for real-time PCR and shRNA cloning, polybrene, anti-mouse antibody for MMP9 raised in rat were purchased from Sigma Chemical Company, USA. Anti LAMP1 antibody (clone 1D4B) raised in rat was from BD Biosciences, USA. Anti-goat HRPO was from Santa Cruz Biotechnology, USA. Inhibitors for PI3K (Wortmannin), ERK (PD169316), p38 MAPK (SB203580) and JNK pathways, protease inhibitor cocktail and phosphatase inhibitor cocktail were from Calbiochem, USA. pTRIPz vector and packaging vectors (pMD2.G and psPAX2) were from Open Biosystems, USA. Dulbecco's Modified Eagle's Medium (DMEM) and Foetal Bovine Serum (FBS) were purchased from Gibco, Invitrogen, USA. All other chemicals were purchased locally and were of analytical grade.

Cell lines

B16F10 (F10) murine melanoma cell line obtained from the National Centre for Cell Science, Pune, India was cultured, stored and maintained as described in [9]. PolyLacNAc downregulated clones and LAMP1 downregulated clones were validated as described in [10] and in [12]. For induction of shRNAs, cells were grown in complete medium containing doxycycline (4 μg/ml) for 96 h. The inducible lentiviral vector system (pTRIPz) allowed tight temporal control of target gene knock down with minimal off-target and deleterious effects on cells.

Purification of recombinant human galectin-3

Galectin-3 was purified as described in [11].

Detection of MMPs by gelatin zymography and Western blotting

Gelatin zymography was performed as described in [13]. 60-mm plates were coated overnight at 4 °C with 2 ml either of 10, 25 or 50 μg/ml galectin-3 or of 10 μg/ml fibronectin. For blocking signalling pathways, equal number of cells were seeded on galectin-3-coated plates and grown till 50 % confluency for 24 h in the presence of serum-containing DMEM medium. Cells were then gently washed thrice with plain DMEM and were incubated in serum-free DMEM containing required concentrations of signalling inhibitors. DMSO was used as vehicle control. Culture supernatant collected after 24 h (conditioned medium) was then subjected to either zymography or Anti-MMP9 immunoblotting as described in [10]. Also, to assess if inhibitor treatment had any effect on cell viability, cells remaining in the plates were lysed in 1X reduced sample buffer and equal volumes of lysates from each sample were resolved by SDS-PAGE, proteins were transferred onto PVDF membrane and probed with β actin antibody or stained with coomassie brilliant blue. All the experiments were repeated in triplicates, and quantitation by densitometric analysis (for zymography experiments) was performed as described in [14].

Real-time PCR

The cells remaining in culture plates after collection of conditioned medium were subjected for RNA preparation by TRIzol solution. 1 μg of RNA was then used for preparation of cDNA using high capacity cDNA reverse transcriptase kit. The real-time PCR reaction was performed as described in [10]. RPL4 was used as housekeeping gene for relative quantification of MMP9 transcript levels. The data represent mean of three independent experiments carried out in duplicates with different batches of cDNA. Analysis was performed using $2^{-\Delta\Delta Ct}$ method [15].

The sequence of primers (left to right in 5′ to 3′ direction) used for amplification is as follows:

MMP9 forward primer-TCATTCGCGTGGATAAGGAG

MMP9 reverse primer-AGGCTTTGTCTTGGTACTGG

RPL4 forward primer- GACAGCCCTATGCCGTCAGTG

RPL4 reverse primer- GCCACAGCTCTGCCAGTACC

Preparation of cell lysates for detection of phosphorylated signalling proteins by immunoblotting

Cells were grown on galectin-3-coated plates as described in previous method. After growing under serum-free conditions for 24 h, cells were harvested in lysis buffer

containing protease and phosphatase inhibitor cocktail. Cells were then sonicated and centrifuged at 16,000 rpm for 30 min at 4 °C. The protein concentration was estimated as described in [9]. 100 μg of protein was mixed with Laemmli buffer, boiled for 5 min and loaded on SDS-PAGE under reducing conditions. The proteins were transferred on PVDF membrane and probed with phospho-specific p38 MAPK antibody and blots probed with p38 MAPK antibody served as loading control.

Statistical analysis

All the data are represented as mean ± SE unless stated. For comparison of two groups, student's *t* test was employed. All the statistical analysis was performed using GraphPad Prism 5. $P < 0.05$ was considered significant.

Results and Discussion

Matrix metalloproteinases (MMPs) are the key molecules involved in cancer cell invasion and tissue remodelling [16]. MMPs are tightly regulated at multiple levels as it may impact tissue integrity [17]. Multiple mechanisms have evolved to regulate the action of MMPs [18]. It can be at the level of its transcription, expression, secretion, activation of pro form to active form, localization at the invading front and interaction with its tissue inhibitors [19]. The primary regulation is at the mRNA level and under normal physiological conditions; only basal levels of MMP transcripts are produced. In response to external stimuli such as growth factors, cytokines and extracellular matrix (ECM) components, the expression of MMPs is upregulated [20].

Galectin-3 is a nucleo-cytoplasmic protein that also gets secreted in a non-classical manner. The secreted galectin-3 can get immobilized on the cell surface or can become a part of the ECM and BM [21, 22]. Extracellular galectin-3 has been shown to regulate processes associated with invasion and metastasis [23]. Recently, we have shown that both soluble and immobilized form of extracellular galectin-3 induce MMP9 secretion, which appear to depend on the metastatic potential of the cells and the levels of polyLacNAc expression on their surface [10]. Does galectin-3 induce secretion of MMP9 by inducing the transcription of MMP9 and what are the signalling pathways induced.

Extracellular galectin-3 induces MMP9 expression at mRNA level via p38 MAPK pathway

Using zymography it was shown that extracellular galectin-3 in immobilized form induces secretion of MMP9 in culture supernatants of F10 cells in a dose-dependent manner. The maximum induction occurred in cells grown on plates coated with 50 μg/ml of galectin-3 (Fig. 1a). Fibronectin, a well-studied ECM protein and a known inducer of MMP9 secretion [24, 25], served as a positive control (Fig. 1a). The levels of MMP-9 mRNA in cells grown on uncoated plastic plates were compared to those grown on fibronectin or galectin-3-coated plates by real-time PCR to determine if induction occurs at the transcript level. Cells grown on galectin-3-coated plates expressed significantly increased levels of MMP9 transcripts as compared to cells grown on uncoated plates (Fig. 1b). These results suggest that galectin-3 in extracellular form can indeed induce the transcription of MMP9. Our results are also corroborated by the recent findings which highlight the role of extracellular galectin-3 in induction of MMP9 at transcript level in migrating epithelial cells [26].

ECM proteins regulate the cellular signalling pathways involved in matrix remodelling by interacting with their receptors on the cell surface like integrins to initiate outside in signalling and vice versa [19]. In response to external stimuli, cellular signalling pathways are activated which then modulate the transcription of MMPs. ECM proteins such as fibronectin and osteopontin are known to activate MMP9 expression through ERK, PI3K or NF-κB pathways [25, 27]. To understand which downstream pathways are activated by extracellular galectin-3, inhibitors of PI3K and MAPK (ERK, p38 MAPK and JNK) pathways were used.

Comparison of transcript levels of MMP9 in cells grown on galectin-3-coated plates in the absence or presence of different signalling inhibitors showed statistically significant reduction in transcript levels of MMP9 only in the presence of ERK and p38 MAPK inhibitor (Fig. 1c); however, the maximum inhibition was seen in the presence of the latter. Transcript levels of MMP9 in cells grown on galectin-3-coated plates treated with vehicle alone served as control.

These results were further corroborated upon evaluation of MMP9 levels in the conditioned medium collected from cells grown on galectin-3-coated plates in the absence or presence of different signalling inhibitors by zymography and immunoblotting. Both, confirmed that in comparison to cells grown in the presence of ERK, PI3K and JNK inhibitors, cells treated with p38 MAPK inhibitor (SB) showed maximum reduction in MMP9 levels (Fig. 1d–f). Western-blotted lysates of untreated and inhibitor-treated cells were probed with β actin antibody or stained with coomassie brilliant blue which served as control for equal number of cells (Supplementary Fig. 1a, b).

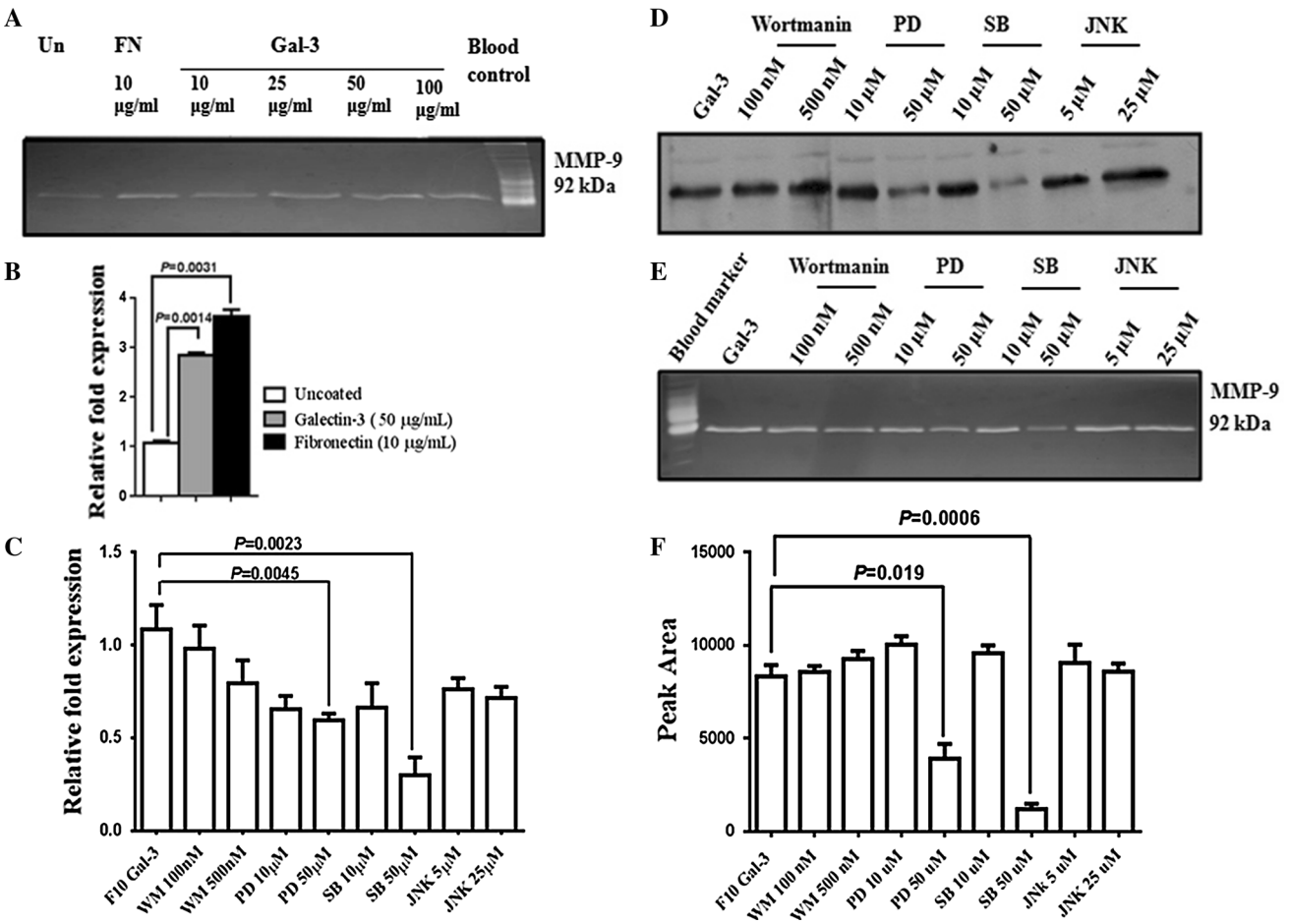

Fig. 1 Immobilized galectin-3 induces expression of MMP9 through activation of p38 MAPK pathway **a** MMP-9 levels in culture supernatant (conditioned media) of F10 cells grown under serum-free conditions for 24 h on uncoated wells were compared with those grown similarly on plates coated with fibronectin or different amounts of galectin-3 (as described in Materials and methods). **b** *Bar graph* represents comparison of levels of MMP9 transcripts by real-time PCR in F10 cells grown on uncoated plates or those coated with galectin-3 or fibronectin. Cells grown on fibronectin served as positive control for both a and b. **c** Comparison of the levels of MMP9 transcript by real-time PCR in F10 cells grown on galectin-3-coated plates in the presence of signalling inhibitors, PI3K (Wortmannin-WM, 100 and 500 nM), ERK (PD, 10 and 50 µM), p38 MAPK (SB, 10 and 50 µM) and JNK (5 and 25 µM). Cells grown on galectin-3 in the presence of vehicle served as control. **d** Western blotting using anti MMP9 antibody for culture supernatants of F10 cells grown on galectin-3 in the presence of signalling inhibitors. **e** Zymography of culture supernatants collected from F10 cells grown on galectin-3 in the presence of signalling inhibitors. **f** Densitometric analysis of bands in **e** by Image J software

Downregulation of polyLacNAc, the high affinity galectin-3 ligand, inhibits induction of MMP9 expression in melanoma cells

Repeating units of galactose and *N*-acetylglucosamine, Poly-*N*-acetyllactosamine (polyLacNAc), is synthesized by the concerted action of β1,4 galactosyltransferase and β1,3 *N*-acetylglucosaminetransferase [28]. PolyLacNAc is the preferred ligand for galectin-3 and the strength of its binding is dependent on the number of LacNAc repeats [29]. The expression of polyLacNAc on N-glycans on melanoma cells correlates with their metastatic potential. Inhibition of expression of N-glycans by Swainsonine, or the β1,6 branch by inhibiting the expression of the enzyme GnT-V inhibits experimental metastasis [11, 13]. Beta 1,6 branched N-oligosaccharides are often substituted with polyLacNAc. Downregulating the enzymes involved in polyLacNAc synthesis not only affected binding of galectin-3 to melanoma cells but also galectin-3-mediated processes, including cellular spreading, motility and matrix degradation. Inhibition of these cellular processes ultimately affected metastatic potential of melanoma cells [10]. In light of these findings, it was important to understand if galectin-3-induced transcription of MMP9 is indeed through polyLacNAc. We have used inducible lentiviral shRNA clones of F10 cells in which genes for the enzymes involved in polyLacNAc synthesis (GalT-I and -V) were downregulated [10]. Reduced surface polyLacNAc in these clones not only

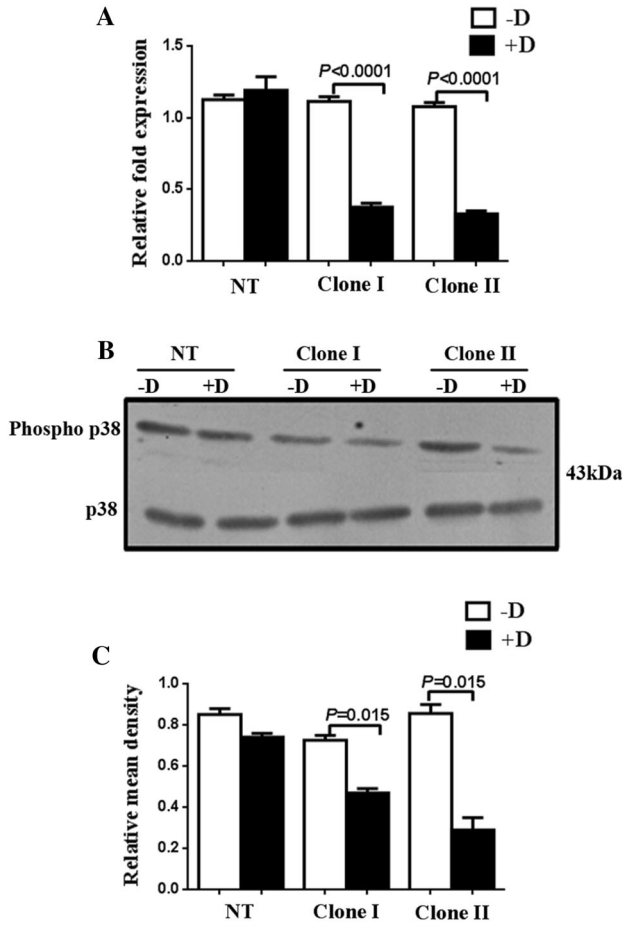

Fig. 2 Downregulation of polyLacNAc affects MMP9 induction via activation of p38 MAPK pathway. **a** *Bar graph* represents analysis of MMP9 transcript levels in polyLacNAc downregulated clones I and II grown on galectin-3-coated plates in doxycycline-treated and -untreated conditions (+D and −D, respectively). Non-targeting clones (NT−D and NT+D) served as vector controls. **b** Western blotting for detection of phospho-specific forms of p38 in NT, clone I and clone II (−D and +D) grown on galectin-3-coated plates. Blots probed with p38 MAPK antibody served as loading control. **c** Densitometric analysis of bands in (**b**) by Image J software

affected MMP9 induction at protein level [10] but also at the mRNA level (Fig. 2a). Downregulation of GalT-I and V did not affect the fibronectin-induced MMP9 expression as seen at transcript (Supplementary Fig. 2a) as well as at protein levels (Supplementary Fig. 2b, c) which clearly highlights the specific role of polyLacNAc in galectin-3-induced MMP9 secretion.

If inhibition of p38 MAPK pathway affects MMP9 induction (Fig. 1c–f), then it would be interesting to investigate if the activation of the same pathway is hampered in polyLacNAc downregulated clones. Both the clones upon doxycycline induction showed significantly reduced levels of phospho-p38 MAPK, suggesting that polyLacNAc also signals galectin-3-mediated processes via this pathway (Fig. 2b, c).

LAMP1 and β1 integrin are among the major proteins identified to carry polyLacNAc substituted β1,6 branched N-oligosaccharides on these melanoma cells [9]. LAMP1 is a highly glycosylated protein that lines the lysosomes [30]. More than 60 % of its weight is contributed by carbohydrates and each molecule carries about 17–20 N-glycans that are highly substituted [30, 31]. In metastatic cells, LAMP1 is known to get translocated to the cell surface [9, 32]. The extent of its surface expression and the levels of polyLacNAc on N-glycans have been shown to correlate with the metastatic potential of melanoma cells [9]. In addition, glycosylation in these cells has been shown to modulate the surface expression of LAMP1 [33]. LAMP1 is reportedly a known ligand for galectin-3 [34, 35]. Recently, downregulation of LAMP1 expression has been shown to significantly affect its surface expression, as well as the spreading and motility on galectin-3 and experimental metastasis of F10 cells [12]. It is thus possible that LAMP1 is one of the molecules involved in galectin-3-mediated signalling that induces MMP9 transcription and secretion.

LAMP1 participates in galectin-3-mediated induction of MMP9 expression via p38 MAPK pathway

To investigate the role of LAMP1 in MMP9 expression, melanoma cells expressing shRNAs for LAMP1 were used [12]. Real-time PCR results show that downregulation of LAMP1 in F10 cells (clones Sh1 and Sh2) considerably affects the galectin-3-induced transcription of MMP9 (Fig. 3a). This is further reflected by significantly reduced secretion of MMP9 in LAMP1 downregulated clones grown on galectin-3, as analysed by zymography (Fig. 3b, c) and immunoblotting (data not shown) suggesting that LAMP1 can be one of the upstream regulators in MMP9 induction. This was also corroborated by decreased levels of activated (phospho) p38 MAPK in the lysates of LAMP1 downregulated clones (Fig. 3d, e). However, these cells did not show any significant change in the levels of the MMP9 or its transcript when grown on fibronectin (Supplementary Fig. 2d–f), suggesting that the signalling event on galectin-3 is specifically via carbohydrate/lectin interaction (Fig. 4).

LAMP1 has a very short cytoplasmic tail (consisting of only eleven amino acids) [30] and has few known binding partners. Ezrin, a member of ERM family of proteins, is one such protein that has been shown to interact with LAMP1 at the cytoplasmic end [36]. Ezrin can function as a linker between membrane proteins and cytoskeletal proteins to modulate cellular adhesion and motility [36–38]. The loss of spreading and motility on galectin-3 in LAMP1 downregulated clones [12] is possibly via some such mechanism. It is possible that induction of MMP9 by surface LAMP1 is also mediated by similar protein(s) that interact with LAMP1 and activate the downstream

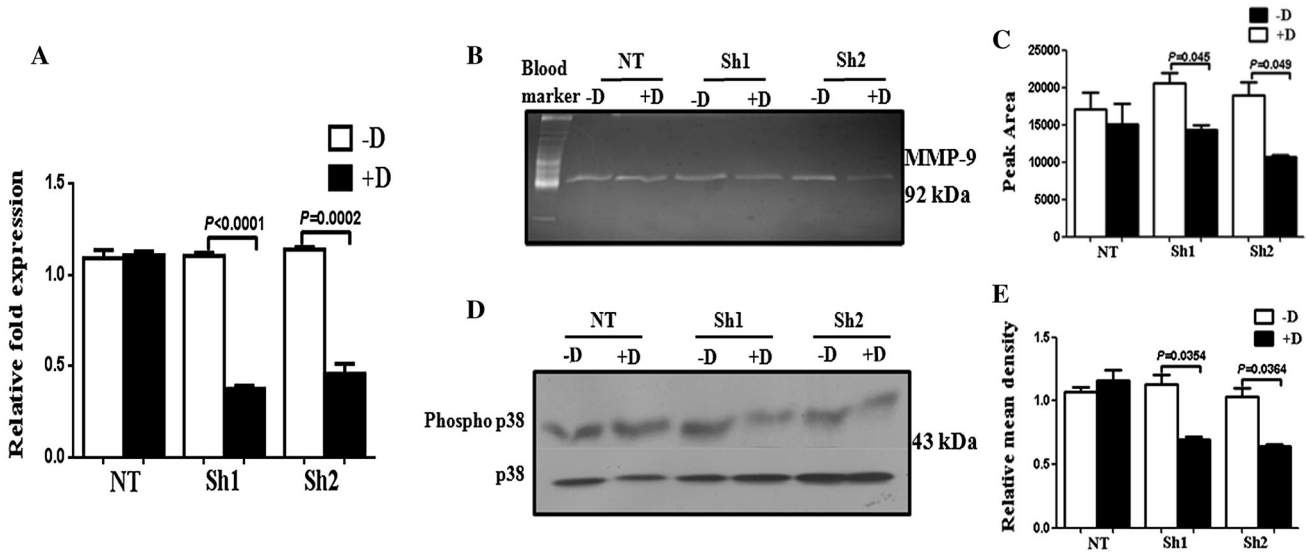

Fig. 3 Downregulation of LAMP1 affects galectin-3-induced MMP9 expression via p38 MAPK pathway. **a** *Bar graph* represents analysis of levels of MMP9 transcripts by real-time PCR for NT and LAMP1 downregulated clones Sh1 and Sh2, grown on galectin-3-coated plates in the absence (−D) and presence of (+D) doxycycline. **b** Levels of MMP9 in culture supernatants of NT, Sh1 and Sh2 cells grown in the presence (+D) and absence (−D) of doxycycline on galectin-3 as detected by zymography. **c** Densitometric analysis of bands in (**b**) by Image J software. **d** Western blotting for detection of phospho-specific forms of p38 in NT, Sh1 and Sh2 clones (−D and +D) grown on galectin-3-coated plates. Blots probed with p38 MAPK antibody served as loading control. **e** Densitometric analysis of bands in (**d**) by Image J software

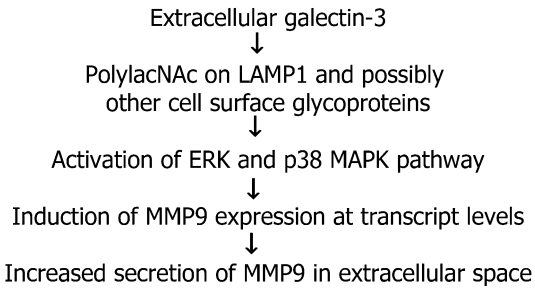

Fig. 4 Schematic representation summarizing the overall events involved in galectin-3 induced MMP9 expression in melanoma cells

signalling pathways leading to induction of MMP9 transcription and secretion.

Alternatively, as a glycosylated cell surface protein, high levels of polyLacNAc on LAMP1 may initiate/contribute to formation of galectin-3-mediated lattices/membrane microdomains. These microdomains may also include other receptors like integrins (α5, α3, αV and β1), cadherins, growth factor receptors such as epidermal growth factor receptor (EGFR), TGF β, etc., [39–43]. The signalling may thus be indirectly mediated through such components of the lattice. LAMP1 may thus be possibly controlling the signalling by regulating the formation of the lattice. It would be interesting to explore such mechanisms which can play an important role in regulating key cellular processes such as matrix degradation.

Our study describes the novel function of extracellular galectin-3 and also explores the downstream signalling mechanisms of galectin-3/polyLacNAc pair which are not yet completely elucidated. In a broader sense, such studies in tumour cell biology can throw light on some of the most intricate mechanisms by which invasion and metastasis is regulated at the molecular level.

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