“Role of β1,6 branched N-oligosaccharides and associated terminal substitutions on tumour cells and their possible receptors on lungs in organ specific metastasis”

By
Manohar C. Dange
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As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Manohar C. Dange entitled "Role of β1,6 branched N-oligosaccharides and associated terminal substitutions on tumour cells and their possible receptors on lungs in organ specific metastasis" 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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V. Prasanna ----------Date 20.04.2015

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A Karande ----------Date: 20.4.2015

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Date: 20/04/2015

Place: Navi Mumbai

R Kalraiya

Dr. Rajiv D. Kalraiya

Guide

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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.

Manohar C. Dange

Navi Mumbai,

Date: 20/4/2015

List of Publications arising from the thesis

Journal

1. "Galectin-3 expressed on different lung compartments promotes organ specific metastasis by facilitating arrest, extravasation and organ colonization via high affinity ligands on melanoma cells", **Manohar.C. Dange**, N. Srinivasan, S.K. More, S.M. Bane, A. Upadhya, A.D. Ingle, R.P. Gude, R. Mukhopadhyaya, R.D. Kalraiya. Clin Exp Metastasis (2014) 31: 661-673.
2. Extracellular galectin-3 induces MMP9 expression by activating p38 MAPK pathway via Lysosome Associated Membrane Protein-1 (LAMP1)". Manuscript under review**, Manohar.C. Dange**, Akhil Kumar Agarwal and Rajiv D. Kalraiya, Mol Cell Biochem, March 2015 (DOI- 10.1007/s11010-015-2367-5).
3. "Identification of galectin-3 binding proteins carrying Poly-*N*-acetyllactosamine substituted β1,6 branched N-glycans by mass spectrometry to understand their role in metastasis associated processes". Manuscript under preparation, **Manohar.C. Dange**, Hemangi S. Bhonsle, Rashmi K. Godbole, Shyam K. More, Sanjay M. Bane, Mahesh J. Kulkarni and Rajiv D. Kalraiya, Manuscript under preparation.

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Presentations at conferences

1. Presented poster titled *"Galectin-3 induced MMP-9 secretion regulated by LAMP-1 is a critical step for organ specific metastasis of melanoma cells"* at **Society of biological Chemistry, India (SBCI) meeting** organized at University of Hyderabad, **in December, 2013.**

2. Presented poster titled "*Identification of galectin-3 binding proteins carrying Poly-N-acetyllactosamine substituted β1,6 branched N-glycans by mass spectrometry to understand their role in metastasis associated processes"* at International Conference on **"Proteomics from Discovery to Function" organized at IIT Bombay, December 2014.**

Navi Mumbai,

April, 2015

Manohar C. Dange

Dedicated to

my family

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CONTENTS

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Ph. D. PROGRAMME

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2. Name of the Constituent Institution:	Tata Memorial Centre**,** Advanced Centre for Treatment, Research and Education in Cancer
3. Enrolment No. :	LIFE09200804005 (25th August 2008)
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5. Board of Studies:	Life Sciences

SYNOPSIS

Introduction:

Metastasis is a complex multistep process which is the major cause of cancer related deaths. For metastasis to occur, tumors cells released from primary site should gain access into circulation, survive the harsh flow conditions and interact with host organ endothelium to initiate the process of organ colonization (1). Cell surface molecules have been shown to play an important role in mediating these multiple steps involved in metastasis including interaction with organ endothelium at the site of metastasis (2). Further, apart from molecules themselves, modifications on these molecules including their altered glycosylation status has been shown to be a universal feature of metastastic cells. One such consistently observed modification associated with metastasis is the altered expression of β1,6 branched N-oligosaccharides on cell surface glycoproteins (3,4). In case of human tumours, their expression correlates with the disease progression. Further, the expression of these oligosaccharides has also been shown to be strongly associated with the invasive as well as metastastic phenotype of various human and murine tumor cell lines. Also, inhibition of expression of β1,6 branched N-oligosaccharides leads to reduced metastatic potential which firmly establishes their role in regulating metastasis (3).

Interestingly, majority of the cell lines expressing β1,6 branched N-oligosaccharides metastasize to either lungs or to the liver. These oligosaccharides may mediate organ specific metastasis in two ways. Firstly, as a highly substituted branched multi-antennary, bulky carbohydrate structures, they can alter structural and functional properties of their carrier proteins including cadherins, integrins, CD44, growth factor receptors like epidermal growth factor receptor (EGFR) and Lysosome Associated Membrane Proteins (LAMPs). Secondly, β1,6 branched N-glycans expressed on tumor cells can act as a preferred site for further substitutions like Lewis antigens, poly-*N*-acetyl-lactosamine

(PolyLacNAc) and sialic acids which may serve as ligands for several endogenous lectins such as selectins, galectins and siglecs.

In order to study the role of β1,6 branched N-oligosaccharides in lung specific metastasis, we chose B16 murine melanoma model. Using B16 melanoma model, work in our lab has shown that expression of polyLacNAc substituted β1,6 branched N-glycans correlates with metastastic potential of melanoma cells. PolyLacNAc has been shown to serve as high affinity ligand for galectin-3 expressed at highest levels in lungs (5). It is expressed on all the tissue compartments of the lungs and constitutively on its vascular endothelium. Galectin-3 not only aids arrest of melanoma cells on organ endothelium, but also mediates subsequent events of extravasation and organ colonization (6).

PolyLacNAc only on N- and not O-oligosaccharides were shown to participate in these processes and even the role of other galectin-3 ligands such as T/Tn antigens in melanoma metastasis was ruled out. β1 integrin and the lysosomal associated membrane protein-1 (LAMP1) have been identified to be the major carriers of polyLacNAc on melanoma cells.

Key Questions:

- Can polyLacNAc expression on melanoma cells be modulated to understand the effect of inhibition of polyLacNAc on galectin-3 mediated arrest and extravasation processes?
- Which are the galectin-3 interacting proteins through which these processes are mediated?

Through which signalling pathways, galectin-3 binding proteins regulate metastastic associated processes

Objectives:

The following objectives were proposed to answer these questions:

- To understand the role of galectin-3/polyLacNAc pair in mediating metastasis associated events by inhibiting the expression of polyLacNAc on melanoma cells and its effect on galectin-3 mediated processes.
- To identify the galectin-3 binding proteins carrying β1,6 branched N-oligosaccharides and to study their involvement in regulation of galectin-3 mediated processes.
- To elucidate the downstream signalling events of galectin-3 induced MMP-9 secretion and involvement of identified proteins in this process.

Methodology:

Cell culture and experimental metastasis assay

B16F10 (F10) melanoma cells were routinely cultured in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal bovine serum supplemented with 0.03% glutamine, antibiotic-antimycotic solution at 37°C in CO_2 incubator. For experimental metastasis assay, melanoma cells were injected intravenously in female C57BL/6 mice which were sacrificed after 21 days.

Purification of recombinant human galectin-3 and preparation of sepharose conjugated galectin-3 affinity column

The expression of galectin-3 was induced in *Escherichia coli* BL-21 cells containing pET3C plasmid using Isopropyl Thio D-Galactopyranoside (IPTG).The protein was purified from bacterial cell lysate using lactose sepharose affinity column. Purified galectin-3 was incubated with Cynogen Bromide (CNBr) activated sepharose beads for preparation of galectin-3 affinity column.

Lectin affinity chromatography for purification of glycoproteins from F10 melanoma cells

F10 cells were lysed in 10 mM Tris chloride buffer containing 30 mM N-octyl β-D-glucopyranoside, 3 mM Protamine sulphate and protease inhibitor cocktail. Cell lysates

were incubated with L-PHA/galectin-3 agarose beads overnight at 4°C. Proteins specifically bound to the column were eluted with Tris buffer containing either 150 mM lactose (for galectin-3 column) or 300 mM N-acetyl galactosamine (GalNAc, for L-PHA column).

Identification of proteins using Mass Spectrometry

The eluted fractions from galectin-3 and L-PHA affinity chromatography columns were loaded onto 10% SDS PAGE gel and protein profile was visualised by staining with 0.2% coomassie solution. Observed bands were excised, cut into small pieces and destained, alkylated and reduced before trypsin digestion. Tryptic digested peptides were deglycosylated by PNGase F treatment. Deglycosylated peptides were passed through C18 column and then subjected for analysis using nano LC-ESI-Q-TOF MS/MS by Synapt, Waters. Protein Lynx Global Server (PLGS) software was used for post acquisition analysis.

Cloning of shRNA for targeting β1,4 galactosyltransferases-I and -V genes (GalTs) in melanoma cells.

A common 21 nucleotide stretch was chosen for designing shRNA against GalT-I and –V genes according to recommended guidelines. ShRNA cassettes containing shRNA sequence were PCR amplified using specific primers containing EcoRI and XhoI sites, gel purified and ligated into double digested inducible lentiviral pTRIPz vector. The ligated plasmid was then transformed into *E. coli* cells, positive clones were selected and plasmid was purified by maxiprep column for further use. Lentiviral particles were generated by transfecting HEK293FT cells with pTRIPz along with helper constructs pMD2.G and psPAX2. Viral supernatant was collected 24 h post changing of transfection medium and stored at -80ºC. One non targeting (NT) clone and two shRNA clones (Clone I & II) were selected after transduction of F10 cells, using puromycin treatment.

Flow cytometric analysis

Paraformaldehyde fixed melanoma cells were incubated with biotinylated lectins (LEA and Galectin-3) followed by Extravidin FITC. Cells treated with FITC tagged secondary antibody alone served as control. Fluorescent cells were acquired by BD FACS Calibur at 488 nm and analyzed by cell quest software.

Real time PCR analysis

Trizol was used for preparation of RNA from F10 cells. 1 µg of RNA was then used for cDNA preparation. For detecting transcript levels of GalT-I, GalT-V and MMP-9 genes, specific primers were designed. RPL4 was used as a housekeeping gene for relative quantification of transcript levels. Analysis was performed using $2^{-\Delta\Delta Ct}$ method.

Adhesion Assays

For Adhesion assays, F10 cells were labeled with Calcein AM (3 µg/ml), seeded on galectin-3 (50 µg/ml) coated wells in 96 well plate and washed after incubation for one hour in a CO_2 incubator. Fluorescence of cells was measured before and after washing and percentage adhesion was calculated by considering F10 cells bound to galectin-3 as 100%.

Cell spreading assays

Melanoma cells were seeded on galectin-3 coated coverslips, in serum free medium for 45 min at 37°C in a CO_2 incubator. After incubation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Phalloidin FITC or Phalloidin TRITC was used for F-actin staining and DAPI was used for nuclear staining.

Gelatin zymography

F10 cells were grown under serum free conditions on galectin-3 coated culture dishes. The culture medium collected after 24 h was loaded on 10% SDS-PAGE resolving gel containing 0.1% gelatin under non-reducing conditions at 4°C. The proteins were

renatured by soaking the gel in 2.5% Triton X-100, washed and incubated for 24 h in Tris buffer containing 50 mM $CaCl_2$ at 37°C. Gels were stained with 0.2% Coomassie brilliant blue and destained to visualize the zone of lysis.

Wound healing assays

Melanoma cells were cultured on 6 well plates precoated with galectin-3, blocked with BSA and grown for 24 h in DMEM. A uniform straight wound was made using a 2 µl tip. Wound closure was measured under time lapse inverted microscope for 20 h at 37°C in 5% CO_2 chamber.

Preparation of lysates for detection of total and phospho levels of signalling molecules

Cells grown on immobilized galectin-3 were serum starved for 24 h. After starvation, cells were harvested in lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail. Cells were then sonicated, centrifuged and supernatant was collected. The protein concentration was estimated using modified Peterson-Lowry method.

Results:

Objective I: To understand the role of galectin-3/polyLacNAc pair in mediating metastasis associated events by inhibiting the expression of polyLacNAc on melanoma cells and its effect on galectin-3 mediated processes.

Poly-*N*-acetyllactosamine (polyLacNAc) is repeating units of galactose and N-acetylglucosamine. It is synthesized by the concerted action of β1,4 galactosyltransferase (GalTs) and β1,3 N-acetylglucosaminetransferase (GnTs). Targeting either of these enzymes can affect polyLacNAc synthesis. We chose GalTs specifically GalT-I and-V (based on previous observation) for shRNA mediated downregulation because their role in synthesis of polyLacNAc on β1,6 branched N-glycans has been reported (7). A sequence

of 21 nucleotides common to both of the genes was chosen for designing shRNA which was cloned into pTRIPz, an inducible lentiviral vector.

Downregulation of GalT-I and -V enzymes in F10 cells leads to significant reduction in the expression of polyLacNAc and galectin-3 binding

The expression of shRNA for GalT-I and –V genes in melanoma cells (Clone I and II) affected expression of these genes at transcript levels and also lead to reduced expression of polylacNAc at cell surface levels. Further, these cells showed lower galectin-3 binding as compared to cells in which vector containing non targeting shRNA (NT) sequence was transduced.

Downregulation of polyLacNAc results in decreased adhesion, spreading, MMP-9 secretion and motility of F10 cells on galectin-3 together with reduced experimental metastasis:

Reduced expression of polyLacNAc in clone I and II was associated with significantly decreased adhesion and spreading on galectin-3 as compared to NT clone. Similarly induction of shRNA expression affected MMP-9 secretion by these clones on galectin-3 coated plates as compared to NT clone. Also, motility of these cells on galectin-3 coated plates was affected. Inhibition of these galectin-3 mediated processes ultimately affected the ability of clone I and II to metastasize to lungs of C57BL/6 mice(6). These results confirm the involvement of polyLacNAc in galectin-3 mediated extravasation events.

Objective II: To identify the galectin-3 binding proteins carrying polyLacNAc on β1,6 branched N-oligosaccharides and to study their involvement in regulation of galectin-3 mediated processes.

Galectin-3 binds to polylactosamine (polyLacNAc) repeats expressed on both N- and O-linked proteins. Hence by purifying proteins from galectin-3 affinity column, it would not be possible to selectively enrich proteins which carry polyLacNAc only on N-glycans. The

other available option was to purify proteins using L-PHA affinity chromatography. By this method, we would be able to selectively purify proteins which carry β1,6 branched N-glycans. Though β1,6 branch is the preferred site for addition of polyLacNAc repeats, still it cannot be confirmed that these sites are indeed substituted with polyLacNAc. Therefore, we have used a comparative approach by which we independently purified glycoproteins by galectin-3 as well as by L-PHA affinity chromatography and then identified the purified proteins by LC-MS/MS. List of common proteins included the proteins modified by addition of polyLacNAc on β1,6 branched N-glycans. Some of the identified proteins were validated and their role in mediating cell spreading, one of the galectin-3 regulated processes, was investigated.

Identification of proteins carrying polyLacNAc on Beta1,6 branched N-linked oligosaccharides using LC-MS/MS approach

Identification of glycoproteins is difficult due to several reasons including low glycopeptide abundance, overall heterogeneity/glycosylation variants and poor ionisation. Therefore, to avoid interference of glycans, we chose an enzymatic deglycosylation strategy. Peptides generated from trypsin digestion were incubated with PNGase F, an enzyme which removes N-glycans covalently attached to asparagine residue. Also, PNGase F treatment results in shift in molecular mass of the deglycosylated peptide which can be used for annotating N-glycosylation sites (8). Results from proteomic analysis showed that out of total 134 identified glycoproteins, 54 proteins carry β1,6 branched N-glycans and 80 proteins carry polyLacNAc. After comparative analysis, 25 proteins were found to carry both polyLacNAc as well as β1,6 branched glycans. Further, in many cases, even the identified glycosylation sites were the same suggesting that these sites indeed carried polyLacNAc substituted β1,6 branched N-oligosaccharides (For e.g., LAMP1 identified by both the approaches shared 10 common glycosylation sites).

Validation of identified proteins using immunoblotting and role of these proteins in galectin-3 regulated metastastic processes

Identities of some of the proteins including LAMP1, LAMP3, α5, α3 and β1 integrin, Basigin, and Embigin was confirmed by immunoblotting. Also, to understand the role of these proteins in regulating galectin-3 regulated metastastic processes, cell spreading assay was performed. Incubating melanoma cells with antibodies for LAMP1, β1 integrin and Basigin affected spreading of these cells on galectin-3 coated coverslips. These results suggest that galectin-3 binding proteins can regulate galectin-3 mediated processes and probably they may play a crucial role in activating downstream signalling events.

Objective III: To elucidate the downstream signalling events of galectin-3 induced MMP-9 secretion and identification of protein involved in this process.

Matrix metalloproteinases (MMPs) especially MMP-2 and -9 which are members of gelatinase family of enzymes play a crucial in degradation of matrix components. Our results show that extracellular galectin-3 in solubilised or immobilized form can induce secretion of MMP-9. It is a novel finding and has not been reported previously. Therefore, we were interested in understanding the exact signalling mechanism by which galectin-3/polyLacNAc pair is regulating the MMP-9 secretion.

Galectin-3 induces MMP9 expression at mRNA level through p38 MAPK pathway

Extracellular matrix (ECM) proteins such as fibronectin, osteopontin are known to induce expression of MMPs at transcript level (9,10). To understand if galectin-3, as an ECM molecule, induces MMP-9 expression at transcript levels, melanoma cells were grown on galectin-3 coated plates and MMP-9 mRNA levels were analysed by Real time PCR. Results show that cells grown on galectin-3 coated plates express higher levels of MMP-9 transcripts as compared to cells grown on plastic alone.

Downregulation of polyLacNAc or its major carrier, LAMP1 affects galectin-3 induced MMP-9 secretion through p38 MAPK pathway

Reduced levels of polyLacNAc, a high affinity galectin-3 ligand, in melanoma cells affected galectin-3 induced MMP-9 secretion. Activated levels of p38 (phospho p38) were also affected in these cells when grown on galectin-3 clearly suggesting that polyLacNAc expression is essential for MMP-9 induction through p38 MAPK pathway. Further, as one of the major carrier of polyLacNAc, LAMP1 expression was also shown to be important for induction of MMP-9. The mechanism by which LAMP1 regulates MMP-9 expression would be discussed in detail in thesis.

Summary and Conclusions

Host tumor cell interactions are key determinants of organ specific metastasis. The molecular pathways by which these interactions regulate metastasis should be studied in detail for deeper understanding of mechanisms of organ specific metastasis. Galectin-3, a lactose binding lectin, is expressed in all the major compartments of lungs, mediates arrest and extravasation of melanoma cells in the lungs. Inhibition of expression of polyLacNAc, a high affinity ligand for galectin-3, on melanoma cells affected galectin-3 mediated extravasation events along with metastasis. Further, several galectin-3 binding/polyLacNAc carrying proteins were identified including integrins (α5, β1), LAMPs (LAMP1, 2 and 3), immunoglobin superfamily proteins (Basigin and Embigin) in melanoma cells and involvement of some of the proteins in regulating galectin-3 mediated cell spreading was investigated. LAMP1 which was found to be a major carrier of polyLacNAc was shown to be involved in regulating galectin-3 mediated MMP-9 secretion through p38 MAPK pathway.

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Publications in Refereed journals:

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Signature of Student:

Date: 19/8/14

Doctoral Committee:

S. No.	Name	Designation	Signature	Date
1	Dr. Shubhada V. Chiplunkar	Chairperson		22/8/14
2	Dr. Rajiv D. Kalraiya	Convener		19/8/14
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4	Dr. Anita Borges	Invitee		21/8/14

Forwarded through:

Dr. S.V. Chiplunkar
Director, ACTREC
Chairperson,
Academics and Training Program,
ACTREC

Dr. S. V. Chiplunkar
Director
Advanced Centre for Treatment, Research & Education in Cancer (ACTREC)
Tata Memorial Centre
Kharghar, Navi Mumbai 410210.

Prof. K. Sharma
Director, Academics,
Tata Memorial Centre

Prof. K.S. Sharma
DIRECTOR - ACADEMICS, TMC
Mumbai - 400 012

ABBREVIATIONS

AVP : Avidin HRPO

BLAST : Basic Local Alignment Search Tool

BM : Basement Membrane

BSA : Bovine Serum Albumin

cDNA : Complementary DNA

C/N ratio : Cytoplasmic to nuclear area ratio

CNBr : Cynogen Bromide

CRD : Carbohydrate Recognition Domain

Ct : Cycle threshold

DAPI : Diamidino-2-phenylindole dihydrochloride

DEPC : Diethyl Pyrocarbonate

DMEM : Dulbecco's Modified Eagle's Medium

DMSO : Dimethyl Sulphoxide

ECL : Enhanced Chemiluminiscene

EDTA : Ethylene Diamine Tetra Acetate

ECM : Extracellular matrix

ER : Endoplasmic Reticulum

ER : Endoplasmic Reticulum

ERK : Extracellular signal regulated kinase

FITC : Fluorescein isothiocynate

FACS : Fluorescent Activated Cell Sorter

FBS : Fetal Bovine Serum

FN : Fibronectin

Gal3 : Galectin-3

GalNAc : N-acetylgalactosamine

GnT-V : N-acetylglucosaminyltransferase-V

GalT : N-acetylgalactosaminyltransferase

HRPO : Horse Radish Peroxidase

IM Gal-3 : Immobilized galectin-3

JNK : c-Jun N-terminal kinase

kDa : Kilo Dalton

LAMPs : Lysosome Associated Membrane Proteins

LAMP1 : Lysosome Associated Membrane Protein-1

LAMP2 : Lysosome Associated Membrane Protein-2

LAMP3 : Lysosome Associated Membrane Protein-3

LB : Luria Bertani

LC-MS : Liquid chromatography mass spectrometry

LEA : *Lycopersicon esculentum* Agglutinin

LPHA : Leucoagglutinin Phytohemagglutinin

MAPK : Mitogen activated protein kinase

MMP : Matrix Metalloproteinase

NP-40 : Nonidet P-40

NT : Non-targeting shRNA

PAGE : Polyacrylamide Gel Electrophoresis

PBS : Phosphate Buffered Saline

PI3K : Phosphatidylinositol-3-Kinase

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

PMSF : Phenyl Methyl Sulfonyl Fluoride

PCR : Polymerase Chain Reaction

PolyLacNAc : Poly-*N*-acetyllactosamine

PLGS : Protein Lynx Global Server

PVDF : Poly Vinylene DiFlouride

qRT-PCR : Real time quantitative reverse transcriptase PCR

RPL4 : Ribosomal protein L4

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

WM : Wortmanin

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CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Cancer

Cancer is one of the most widely occurring diseases across the globe. It is the second most common disease after cardiovascular disorders for maximum deaths in the world (11). According to International agency for research on cancer, in 2008, 635,000 people died from cancer in India which accounts for 8% of all estimated global cancer deaths (12). It is one of the important cause of adult deaths in India, with 70 % of fatal cases were reported to occur during productive ages of 30-69 years (13). Most frequently observed cancers in Indian population are of oral, lungs, breast, colon, rectum, stomach and liver (13). Recent advancement in science and technology has significantly contributed in understanding many underlining principles which govern this disease. Nevertheless, the complete understanding of molecular mechanisms which mediate transformation of normal cells into neoplastic tumor cells and possible therapeutic targets to inhibit these processes is still a major scientific challenge. The characteristic feature of tumor cells is the ability to undergo uncontrolled cell proliferation by overriding the regulatory circuits which govern the growth and homeostasis of normal cells.

Genomic instability resulting due to mutations at the DNA level are the actual drivers involved in initiating the process of carcinogenesis. The genes which undergo mutations during the process of carcinogenesis are broadly classified as 'oncogenes', which confer 'gain in function phenotype'; e.g. EGFR, MYC, B-RAF, Abl, Cyclin D etc.; 'tumor suppressor genes' which on mutation lead to 'loss in function phenotype' e.g. TP53, BCl2, APC, PI3K etc. and 'stability genes' which function as caretaker of the cell and mutation in these genes affects functions including repair, differentiation, growth and invasion e.g. BRCA1/2, ATM, MLH1 etc. (14). Alterations in these genes can be either quantitative or qualitative including point mutation, genetic deletion, amplification, translocation and

structural including epigenetic modifications such as DNA methylation, chromatin organisation etc. Apart from modifications at genetic level, tumor cell microenvironment can also influence the growth and proliferation of tumor cells.

Depending on the growth of tumor cells they can be classified as "Benign", if they are confined to grow at specific site. These tumors are generally treatable by surgery or radiation therapy. As the disease progresses to advanced stages, tumor cells become "malignant" during which they become more aggressive, invade nearby tissue and migrate to a non contiguous distant site where they proliferate to form secondary tumor. At molecular level, tumor cells progressively undergo certain alterations which ultimately results into acquisition of metastastic phenotype. For tumor cells to acquire malignant phenotype, multiple mutations are necessary. These alterations can be categorised into six distinct events.

1. **Autonomous growth signalling:** For proliferation, normal cells are dependent on extracellular mitogenic signals which activate cell surface growth factor receptors to initiate downstream signalling events which ultimately aid cell division. Tumor cells undergo several mutations which enable them to override these regulatory events and allow unrestrained cell growth. This is achieved by producing growth factors including Platelet derived growth factor (PDGF) and Transforming growth factor-α (TGF- α) (15) or by overexpressing growth factor receptors such Her/Neu or expressing mutant forms of receptors such as truncated versions of EGFR to initiate ligand independent signalling (15,16).
2. **Unresponsiveness to anti growth signalling:** Apart from inducing and sustaining positively acting growth proliferative signals, tumor cells have also evolved mechanisms to restrain anti proliferative signals. Cancer cells undergo mutations in two most important growth suppressors coding for Retinoblastoma (Rb) protein and

TP53 (17). These two molecules act as central control nodes to regulate key complementary cellular signalling circuits that govern the decisions of cells to proliferate or, alternatively, activate senescence and apoptotic programs. Alternatively, tumors cells can bypass growth suppressive signals by inhibiting TGF β mediated anti growth signalling by either downregulating or by expressing mutant forms of its receptor (18,19).

3. **Evading cell death signals:** The growth of tumor is not only affected by rate of proliferation of transformed cells but also by rate of cell attrition. Apoptosis is the major mechanism involved in cell attrition. The expression of death family receptors is reduced in tumor cells, for instance CD95 is downregulated in neuroblastoma and lymphoma, or overexpression of inhibitor of apoptosis proteins like survivin is reported in several cancers (20,21). Recent reports also indicate autophagy is alternative mechanism involved in cell death which can either have pro tumor activity or anti tumor activity.
4. **Limitless replicative potential:** Apart from above discussed mechanisms which regulate cell proliferation; there is another independent mechanism which can determine the overall potential of cellular proliferation. 'Hayflick limit' defines the maximum number of times cells can undergo division (22). After every cell division, chromosome ends called as 'telomeres' loses 50-100 bp of DNA, which ultimately activates cellular senescence pathway which inhibits further division. Tumor cells overexpress the enzyme called as telomerase which prevents loss of telomeric DNA and hence avoid activation senescence programme (23).
5. **Activating angiogenesis:** The process of angiogenesis which involves formation of new blood vessels from pre-existing blood vessels is regulated by stoichiometric levels of pro and anti angiogenesis signals. Tumor cells have evolved mechanisms

which help them shift the balance towards pro-angiogenesis signals. Hypoxia induced at the primary tumor site as a result of its increased size, leads to expression of hypoxia induced factor (HIF), which in turn, upregulates expression of Vascular Endothelial Growth Factor (VEGF) which is involved in endothelial cell proliferation (24).

6. **Invasion and metastasis:** The process by which of tumor cells detach from primary site, invade the surrounding tissue, disseminate to adjacent or distant non contiguous site to form a new lesion is called as 'Metastasis'. It is a highly complex process in which cancer cells acquire multiple cellular properties which determines survival and growth of tumor cells outside primary site. It is the most intriguing phase of the cancer about which though lot has been studied, least is understood.

All these six critical features are hailed as the hallmarks of cancer development (25). In recent times, two more cellular events have also been considered to be important in cancer progression. Cancer cells secrete cytokines such as TGF-β which has immune suppressive function and can inactivate infiltrating Cytotoxic T Lymphocytes (CTLs) and Natural Killer (NK) cells (26). In addition, they can also recruit inflammatory cells that have immuno-suppressive effect such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) which are known to suppress the actions of cytotoxic lymphocytes (27). Such mechanisms allow cancer cells to **evade immune response** which is now considered as additional hallmark of cancer.

Cancer cells have higher proliferation rate which also increases there demands for metabolic energy. Normal cells depend on glycolysis, Krebs cycle and mitochondrial oxidative phosphorylation pathways for glucose dependent metabolism under aerobic conditions. However, cancer cells reprogram the metabolism mainly to glycolysis pathway under aerobic conditions (Warburg effect) (28). Tumor cells have devised several

strategies to fulfil their energy demands. For instance, glucose transporter (Glut) proteins which facilitate the import of glucose into cytoplasm are often found dysregulated or overexpressed in malignant cells (29). 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 macromolecule and organelles required for assembling new cancer cells. **Reprogramming of energy metabolism** is now considered to be an additional hallmark to the existing list**.** Tumor cell microenvironment is now an emerging field of research in cancer. However, metastasis is the major cause of mortality in cancer patients; the molecular mechanisms underlying metastasis is still poorly understood.

1.2 Metastasis:

Tumor progression from benign to metastastic stage involves complex interplay of events which enables tumor cell survival and growth outside primary site. Metastasis of tumor cells is the most devastating aspect of cancer disease. It is the prime reason for therapeutic failures (30). Major advances in surgical procedures along with other treatment regimes have been successful in limiting the growth of primary tumor, if it is detected in early stages. However, in many cases, metastasis has already occurred at the time of diagnosis. Metastasized tumors poorly respond to regular treatment modes and also disease symptoms may reoccur after completion of therapeutic course which is the major hurdle in curing the disease at advanced stages (31,32).

Clinical and pathological studies have successfully categorised the metastatic cells on the basis of extent and severity of the disease. However, these studies are not adequate for deeper understanding of molecular events which play a key role in tumor progression. Using experimental models, it has been possible to decipher some of the molecular events which occur during disease progression. Using such models cancer biologists have divided

the metastasis process into few well defined steps often referred to as metastasis cascade (33,34).

1.2.1 Metastasis cascade:

Multiple biological processes act in sequential manner to aid metastasis of tumor cells. Tumor cells should detach from primary site, invade the surrounding tissue, enter into blood vessels or intravasate, survive in the circulation and arrest and extravasate in target organ (35) **(Illustration 1)**. Inability of tumor cells to undergo any one of these processes can ultimately affects metastasis. The overall metastastic process is inefficient as it has been reported that out of millions of cells shed into circulation from primary site; only a tiny minority (>0.01%) of these cells are capable of colonizing at distant site (35). The most likely explanation for this could be inability of many tumor cells to undergo optimal mutation events required for overcoming all the hurdles during organ colonization. The cascade of events along with molecules involved would be discussed in detail as follows.

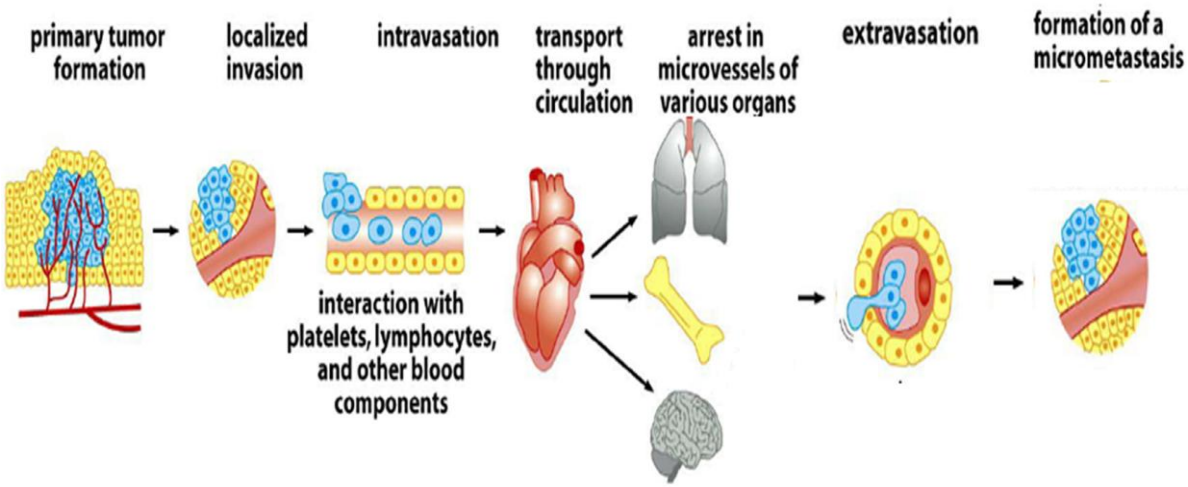


Illustration 1: The Metastasis cascade –modified from (36)

1.2.1.1 Detachment of tumor cells from primary site

Epithelial cells from which most of the solid tumors originate are interconnected via cell-cell adhesion complexes which allow formation of rigid layer of cells. Intercellular adhesive interactions are mediated by tight junctions, adherens junctions and the desmosomes. Claudins, occludins and junctional adhesion molecules (JAMs) are the transmembrane proteins involved in the formation of tight junctions that are connected to the actin cytoskeleton via adaptor proteins like zona occludins (ZO-1, 2 or 3) proteins and several others (37). The classical and non-classical cadherins are involved in the formation of adherens junctions and desmosomes respectively. E-cadherin mediates cell-cell adhesion by mediating homophilic interactions between E-cadherins on neighbouring cells. The adhesive state is maintained by interaction of the cadherins with the actin based cytoskeleton via the adaptor proteins referred to as catenins (38).

During progression of disease from benign to malignant stage, tumor cells undergo alterations which allow them to overcome the intercellular adhesive interactions and aid detachment from the primary site. This process of transition from epithelial to mesenchymal behaviour of cells is termed as Epithelial to Mesenchymal Transition (EMT) which is critical for multiple aspects of normal embryogenesis (39). Cancer cells activate EMT program which involves dissolution of adherens and tight junctions and a loss of cell polarity which aids tumor cell dissemination. In many tumors such as breast, prostrate, colon, skin, kidney, lung and liver cancer, the loss of E-cadherin is associated with gain in expression of Neuronal (N)-cadherin which is typically associated with mesenchymal type phenotype (40). Further, numerous reports demonstrate that tight junction proteins such as ZO-1 and ZO-2 are downregulated in many human carcinomas including breast, pancreatic and colorectal cancers (37). Such mechanisms allow cancer cells to detach from the primary site and initiate the further steps of metastastic cascade.

1.2.1.2 Degradation and invasion into the surrounding tissue

For invasion cancer cells detached from primary tumor create space for movement by degrading the extra cellular matrix (ECM). Cancer cells are surrounded by ECM and each organ is separated from the rest by a specialised form of ECM called as Basement membrane (BM) consisting of dense, uniform, sheet like meshwork. Both ECM and basement membranes are composed of collagenous proteins (like collagen-I, collagen-IV and so on), noncollagenous glycoproteins like fibronectin, laminin, vitronectin proteoglycans and glycosaminoglycans. However, their composition differs with respect to each member, like collagen-IV, laminin and nidogen are present only in BM, whereas collagen-I and fibronectin is seen only in ECM. Similarly they differ in the composition of proteoglycans and glycosamioglycans. BM separates epithelial cells from stroma and interstitial matrix which is rich in lymphatics and blood vessels. The first barrier for tumor cell metastasis is the organ BM. Once they breach the BM they gain access to lymphatics and blood vessels. The endothelial cells in the blood vessels are also lined by the BM (41,42).

In normal tissues, degradation of matrix is highly regulated process as it can affect the tissue integrity (43). Remodelling of matrix occurs only during certain physiological processes such as inflammation, embryogenesis and wound healing by the action of various proteases and hydrolytic enzymes. In tumor cells, the mechanisms by which matrix remodelling is regulated are disrupted and is used for tumor cell invasion.

1.2.1.3 Matrix lysis

The activity of matrix proteases that act on ECM is normally under tight control and is self limiting through mechanisms such as their specific localization, auto-inhibition, generation of inactive zymogenic forms and secreted tissue inhibitors (44). The principal members of the large repertoire of ECM proteases includes (1) plasminogen activators - urokinase

plasminogen activator (uPA) and plasmin. (2) matrix metalloproteinases (MMPs), a family of secreted and membrane-anchored proteases; (3) Cathepsins, the acid lysosomal sulfhydryl proteases (4) the adamalysin-related membrane proteases (ADAMs) (5) glycosidases (44).

Proteolytic enzymes are secreted in zymogenic, latent or inactive form and their activation occurs in a space and time dependent manner. Urokinase plasminogen activator (uPA) that converts plasminogen into plasmin is one of the key enzymes that regulates the activation of MMPs. Plasmin converts most of the proenzymes like Pro-MMPs into active MMPs by "cysteine switch mechanism" which involves cleavage of cysteine based linkage between the pro peptide and catalytic site (45). MMPs are zinc dependent proteinases which are divided in five sub groups depending on substrate specificity including collagenases, gelatinases, stromelysins, miscellaneous group (matrilysins and elastases) and Membrane Tethered (MT) forms of MMPs. As a result of their diverse substrate specificity MMPs are able to degrade almost all the components of ECM (46).

A related family of MMPs are A Disintegrin And Metalloproteinase (ADAMs) and Disintgrin and Metalloproteinase with Thrombospondin type-1 motifs (ADAMTS) which contain both metalloproteinase as well as disintegrin domain which shares homology with integrin binding domain. As ADAMS are involved in proteolytic cleavage of membrane bound proteins they are also called a "sheddases". Elevated level of cathepsins, which are another type of proteases is seen in cancer progression in number of tumors. Moreover, the level of cathespin and ADAMs expression positively correlated with poor prognosis for cancer patients therefore it was suggested to be a prognostic marker (47,48).

Matrix degradation is a highly regulated process, as tumor cells utilize the same matrix to generate traction for forward movement. This is often achieved by limiting matrix degradation near the leading edge of a moving cell. Urokinase Plasminogen Activator

(uPA) which converts plasminogen into plasmin is often restricted to the leading edge via its receptor (uPA-R). The plasmin thus generated activates proMMPs into active enzymes only in this region (49). Similarly MT-MMPs which are activated during their transport to the cell surface via furin have also been shown to be associated with the motile machinery, thus coupling degradation and movement.

1.2.1.4 Tumor cell motility

Degradation of matrix is coupled with cell motility as the cleaved matrix components often serve as chemoattractants for directional movement, whereas the immobilized matrix serves as ligands for motility receptors expressed on cell surface. Cell motility mediated by matrix components which serve to generate traction for forward movement is termed as **"Haptotaxis"** while when degraded matrix components or growth factors serve as chemoattractant for directional movement it is termed as **"Chemotaxis"**. Tumor cells use both these mechanisms for migration through ECM (50).

At mechanistic levels, haptotactic motility on ECM is a dynamic multistep process which involves leading edge protrusion, establishment of focal adhesion contacts, generation of tractional forces, tail retraction and finally detachment and movement of the cell in leading direction. The major players involved in ECM associated adhesion and motility are integrins. They are heterodimeric proteins consisting of alpha and beta subunits with wide range of specificity for different ECM molecules including collagens, and non collagenous glycoproteins like laminin, fibronectin, vitronectin etc. Binding of ECM ligands to integrins initiates transduction of signal in both the directions i.e. inside out (interaction of molecules at cytoplasmic tail of integrins changes their conformation at extracellular domain to facilitate binding to high affinity ligand) and outside in (after ligand binding integrin clustering initiates cyctosketal remodelling) (51). Integrins can bind to same ECM substrates with differential dynamics. For e.g. $\alpha 5\beta 1$ and $\alpha v\beta 3$ both can bind to fibronectin

but adhesive interactions of former are more dynamic than later which allow persistent cell motility. In tumor cells, changes in integrin repertoire correlate with their invasion and migration. For e.g. αvβ3 expression in melanoma cells correlates with their invasive potential (52).

Using either haptotactic or chemotactic mode of motility tumor cells invade through BM/ECM, move through the organ stroma but further they need to surmount another barrier before getting into circulation- intravasation.

1.2.1.5 Intravasation

The entry of invasive tumor cells in lymphatic or blood circulation is called as Intravasation. Though lymphatic spread of tumor cells is often observed and is important prognostic marker for disease progression still dissemination via haematogenous circulation appears to be the major mechanism of dispersion of tumor cells (53). Tumor cells undergo different changes at molecular level to promote their ability to cross the pericyte and endothelia barriers which forms the walls of microvessels. For e.g. Expression of transforming growth factor-β (TGF-β) is enchaned in mammary carcinoma intravasation, alternatively, Epidermal growth factor (EGF) secretion by tumor associated macrophages cells can also facilitate intravasation of breast carcinoma cells (53).

Simultaneously, tumor cells can themselves stimulate the process of angiogenesis which is defined as the process of formation of new blood vessels from the existing ones. 'Angiogenesis switch' is triggered in tumor cells by elevated expression of angiogenic promoters like Vascular Endothelial Growth Factors (VEGF) and angiopoietins in comparison to the angiogenic inhibitors belonging to the statin family. In contrast to blood vessels present in normal tissues, the neovasculature generated by carcinoma cells is leaky, poorly formed and has defective endothelial monolayer which allows tumor cells to cross the endothelial barrier and enter into the circulation (53).

1.2.1.6 Survival in circulation

After intravasation, tumor cells can disseminate through the venous and arterial circulation. Such tumor cells can be termed as 'metastastic intermediates' as they are en route between primary tumors and sites of dissemination (53). Once in circulation, tumor cells should survive a variety of stresses in order to reach to a distant site. First and foremost is survival in non adhesive or anchorage independent environment. In absence of integrin mediated adhesive interactions which are essential for survival, epithelial cells undergo anoikis – a form of apoptosis triggered by loss of anchorage to substratum. To overcome anoikis, tumor cells overexpress Tyrosine receptor Kinase (TrkB receptor) that suppresses anoikis and promotes their survival (54).

Also, tumor cells in the circulation must also overcome the damage caused by hemodynamic shear forces and predation by natural killer cells which is a component of innate immune system. To evade both these threats tumor cells have evolved a mechanism which mimics one of the aspects of blood coagulation- emboli formation. Tumor cells expressing L or P-selectins interact with platelets to form large emboli which not only shield themselves from shear forces but also help in evading immune detection (55).

1.2.1.7 Organ colonization

The ability of tumor cells to colonize at an organ site ultimately determines the fate of metastasis. Once in circulation tumor cells are able to reach almost all organ sites. However, some tumors metastasize only regionally either to the lymph nodes or to the organs in the anatomic vicinity, while several others metastasize specifically to a distant organ site. Depending upon this mechanism of metastasis can be broadly categorised in two distinct types i.e. **Mechanical/Anatomical mode and Organ specific metastasis.**

In 1920s, Dr. James Ewing hypothesized that organ colonization of tumor cells can be explained solely on the basis of pattern of haematogenous or lymphatic circulation.

According to his theory, tumor cells released into the circulation from the primary site are trapped into the vascular bed of first organ encountered. For e.g. In case of metastasis of colon cancer cells to liver, hepatic portal vein connecting these two organs carries all the tumor cells released from colon which get trapped in sinusoidal circulation in liver (56). Similarly, in case of breast, head and neck carcinoma regional metastasis can be explained on the basis of the anatomy of the draining lymphatics (57). At mechanistic level, such mode of metastasis can be explained by formation of multicellular aggregates/ emboli by tumor cells which results in their arrest in microvasculature of nearby organ due to size difference between aggregates and diameter of blood vessels. This restricts tumor cell movement and leads to micrometastasis.

However, several reports suggest that this mechanical theory of metastasis can be explained only in case of regional metastasis. Metastasis at a specific distant site cannot be explained solely on the basis of blood or lymphatic flow. This predisposition or bias of tumor cells to metastasize at a specific organ site is termed as **Organ Specific Metastasis.** The first clinical evidence of organ specific metastasis was reported by Dr. Paget in the year 1889 (58). In his seminal paper titled "Distribution of secondary growths in cancer of the breast", he had analysed the autopsy records of 735 women with fatal breast cancer. In his findings, he reported a marked discrepancy between relative blood supply and the frequency of metastasis in certain organs. According to his observations, there were higher incidences of breast cancer metastasis in liver, ovary, and bones, as compared to spleen. Also, the incidence of metastasis to the ovaries is higher than to the spleen and kidneys combined. In short, it was concluded that metastasis is a non random process and certain organ sites are more prone to metastasis than the others. On these grounds, **Seed and soil hypothesis** of metastasis was established which states, "when a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial

soil". Seed here can be considered as initiator or progenitor tumor cells for secondary metastasis while the term soil can be attributed to various host factors such as organ microenvironment and stroma (59).

Apart from breast cancers there are many other human carcinomas which tend to show organ specific pattern of metastasis. For instance, clear cell carcinoma of the kidney is found to specifically metastasise to the thyroid, uveal melanomas to the liver and sarcomas to the lungs (60-62). Prostate carcinoma is well known for its preference to metastasize to bone. Interestingly, certain tissues including skin, spleen, kidney, muscle and intestine account for majority of arterial output from heart still they are sporadically colonized (61,62).

Concurrently, pioneering work from Hart, Nicolson and Isaiah Fidler in the field of cancer biology not only experimentally proved the Paget's hypothesis but also elucidated the possible mechanism of organ specific metastasis. Their findings were based on diverse and unique patterns of metastatic spread of different murine tumor cell lines including lymphoma derived RAW 117 cells which showed predisposition towards liver, reticulum cell sarcoma and S91 Cloudman melanoma cells towards spleen and lung, respectively (61,62).

Further, Hart and Fidler also performed quantitative analysis of tumor cell arrest and distribution by injecting radiolabelled melanoma cells in syngenic mice. Their results clearly showed that initial distribution of tumor cells was independent of route of injection and the fate of circulating tumor cells depends only on their ability to survive at metastastic site (63). 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 same organ (lungs) of both the mice (64). They

also were successful in the enrichment of highly metastatic cells by repeatedly allowing low metastatic cells to adhere to their target tissue (63,65). Additionally, studies on the metastatic patterns by Kinsey *et al* also showed that lung homing tumor cells colonized both host as well as ectopically implanted lungs, but not to other control organ grafts (66). These pivotal studies in metastasis lead to foundations of current understanding of mechanisms of organ specific metastasis.

1.3 Cellular basis of site specific metastasis

It is important to understand the cellular as well as molecular events which account for the colonization of malignant cells to a particular site. These events require participation of host as well as tumor derived macromolecules. Three major theories have been put forth to explain the mechanism of organ specific homing of tumor cells.

1.3.1 Role of organ microenvironment in mediating organ specific metastasis

Appropriate growth environment not only encourages growth of tumor cells at the metastatic site but also aids invasion of tumor cells. 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 (67). In case of breast cancer cells and prostate cancer cells metastasizing to the bone, molecules such as parathyroid hormone related protein and TGF-β produced in the bone microenvironment stimulate the growth of tumor cells (67).

1.3.2 Effect of organ microenvironment on invasion of tumor cells

Invasive ability of tumor cells is also greatly influenced by the presence of specific ECM molecules at the metastastic microenvironment. There is a strong correlation between expression profile of matrix degrading enzymes secreted by tumor cells with that of ECM

molecules expressed at the metastastic site. For instance, type IV collagenase (MMP2) activity of human colon cancer cells aids metastases in liver due to its favourable ECM composition and cytokines (68)**.** Also, expression of organ specific fibroblasts present in colon and lung directly stimulates secretion of MMPs by human colon cancer cells aids their invasion at the metastatic site (69).

1.3.3 Expression of chemokines and their receptors as a determinant of organ specific metastasis

Chemokines are low molecular weight cytokines that induce directional motility of leucocytes and also mediate a variety of immune reactions including infection, inflammation, and tissue repair. In association with their receptors chemokines play an important role in homing of immune cells to the specific organs. Recent elegant studies have shown that tumor cells can mimic this homing mechanism by expressing receptors for chemokines which are specifically expressed at the target site. To date, involvement of several pairs of chemokines and their receptors in organ selectivity have been reported. For example, human breast cancer cells express high levels of the chemokine receptor CXCR4 and CCR7 while ligands for these receptors- CXCL12 and CCL21 are found to be elevated in lung, liver and bone marrow- organs which are frequent sites of metasasis in breast cancer (70). CXCR4/CXCL12 pair regulates key processes involved in organ homing of breast cancer cells including cell migration, adhesion and extravasation. Chemokines may possibly promote site specific colonization by inducing migration of tumor cells, up-regulation of proteases such as MMP9 expression and by aiding signalling. Furthermore, blocking CXCR4 in tumor cells inhibited their metastasis in experimental animal model (71). Another example chemokines ligand pair involved in organ homing is CCR10/CCL27. CCR10 is expressed by various human melanoma cell lines and it is also

known to be expressed in melanomas. Overexpression of CCR10 in B16F10 cells allowed these cells to metastasize to skin expressing CCL27 (70).

1.3.4 Role of adhesive interactions in organ specific metastasis

Organ specificity of tumor cells can be determined by specific adhesive interactions between the target organ and tumor cells. Several reports have shown that tumor cells can preferentially adhere to histological sections or endothelial monolayers prepared from favoured site of metastasis (72). Vascular endothelium at the secondary site is the first tissue compartment which tumor cells encounter during extravasation. There are multiple experimental evidences which suggest that tumor cells preferentially arrest at organ endothelium which mediates strong adhesive interactions (73). Furthermore, tumor cells selected for their enhanced ability to adhere to a specific tissue have demonstrated enhanced metastasis to the same tissue (61). These studies reveal that cell adhesion at the metastastic site can be a crucial determinate in organ specific metastasis.

Endothelium is an extremely heterogeneous organ which differentiates in an organ specific manner. The cell surface receptor profile of the endothelium shows organ specific variations (74). Thus, adhesion of tumor cells to tissue specific cell surface proteins of the endothelial cells appears to be a major regulatory event in organ specific metastasis. In some cases, the adhesion molecules involved in organ specific homing of tumor cells have also been identified. These molecules can be expressed either constitutively or their expression may be induced in response to inflammatory stimuli. Also, the constitutively expressed adhesion molecules differ quantitatively or qualitatively in their distribution to the various tissue endothelia. For instance, inducible expression of ICAM-1 on pulmonary endothelial cells may dictate the binding of tumor cells such as C6 gliomas during metastatic colonization (75). Cytokine mediated induction of adhesion molecules such as E-Selectin, VCAM-1 and αV integrins in hepatic cells have also been reported to enhance

liver metastasis of colon cancer cells (76). Constitutive expression of E-selectin on vessels of the bones or on inflamed organ endothelium has been shown to facilitate organ homing of cells expressing specific E-selectin ligands (77). Lung colonizing cancer cells have been shown to get redirected to liver upon forced constitutive expression of ligands for E-selectin on hepatic cells (78). Also, in case of lung specific metastasis, receptors/ligand interactions include dipeptidyl peptidase IV (DPP-IV), Lu-ECAM1, VCAM-1, CLCA2, galectin-3 on the lung endothelium and their counter receptors like fibronectin; CXCR4, α4β1 integrin, β4 integrin, polyLacNAc on tumor cells (5,79-83).

Though adhesion to vascular endothelium can have a direct impact on organ specificity of tumor cells, adhesion to the subendothelial basement membrane can also be important factor which can influence site specific metastasis. For instance, potential of 13762NF rat mammary adenocarcinoma cells to metastasise to the lungs is dictated by their adhesion to lung sub-endothelial matrix and not endothelial cells (61). During extravasation, tumor cells interact with components of various components of BM including laminin, fibronectin, vitronectin and type IV collagen. Interaction with ECM not only mediates adhesion but also aids events of extravasation including matrix degradation, cell migration and entry into the organ parenchyma (62).

In all the different mechanisms of organ specific metastasis, the participation of **cell surface proteins** is indispensible as these proteins are in constant interaction with surrounding tumor milieu through which they regulate both extravasation as well as organ colonization.

1.4 Tumor cell surface modifications and metastasis

Extensive 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. The direct evidence for importance of cell surface molecules

in metastasis was shown by experiments in which plasma membranes of cell with low metastastic potential was modified by fusion with plasma membrane vesicles from cells with high metastastic potential. These vesicle modified low metastastic cells showed higher metastastic potential as compared unmodified low metastastic cells thereby underscoring the importance of cell membrane components in metastasis (84).

Changes in expression levels of cell adhesion molecules, together with alterations in their activity and affinity can also facilitate acquisition of the metastatic phenotypes**.** For instance, under expression of E-cadherin on tumor cells, aids detachment by weakening cell-cell interactions**.** Similarly, reduced Neural Cell adhesion Molecule (N-CAM) expression, or its switch to a low adhesive isoform has been associated with poor prognosis for several cancers due to disruption of homotypic adhesion (40). Altered expression of specific integrins including α6β4, α5β1, α4β1 αvβ5, and αvβ3 correlated with disease progression (85). Also, tumor cells from different origins can have different integrin signature. For instance, adenomas are strongly positive for α6-, β1-, and β4-integrins, but show noticeably reduced α3- and α5-integrin expression levels. In contrast, most colonic carcinomas are characterized by diminished or lost expression of α6-, β1-, and β4-integrins, whereas α3- and α5-integrins were completely absent (86). Surface expression of certain proteases such as MT-MMP and uPA and their localization on the invasive front may also aid metastasis and invasion by weakening adhesive interactions and promoting invasion (87,88).

Another important class of molecules modified on metastatic cancer cells are **cell surface carbohydrate structures.** Glycosylation is one of the most abundant posttranslational modifications, and nearly 50% of all known proteins and more than 80% of the cell surface proteins in eukaryotes are glycosylated. In fact, changes in oligosaccharide

structure show association with many physiological and pathological events, including cell adhesion, migration, growth, differentiation and tumor invasion.

1.4.1 Altered glycosylation and metastasis

Alteration of tumor cell surface glycosylation is one of the characteristic traits associated with malignancy. Glycans are oligosaccharide structures that are covalently bound to proteins or lipids. They are expressed as glycolipids, proteoglycans and glycoproteins. Alteration in all these classes of carbohydrates has been associated with metastatic progression

1.4.1.1 Glycolipids

Glycolipids consist of carbohydrate moieties which are covalently attached to ceramide through glycosidic linkage. The first sugars added to ceramide can be either β-linked galactose (GalCer) or glucose (GlcCer). When GlcCer is followed by addition of galactose, lactosyl ceramide (LacCer) is produced, which is at a branch point for formation of the root structure series (globo-,isoglobo-,lacto-, neolacto-,and ganglio-) (89).

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 adenocarcinoma. Similarly, increased expression of gangliosides such as GD_2 and GM_2 is reported in neuroblastoma and melanoma (90).

Glycolipids play an important role in formation of membrane microdomains called as rafts which modulate transmembrane signaling responsible for growth, motility, invasion and metastasis. Raft associated receptors including integrins, protein kinase C, tyrosine kinase-linked growth factor receptors and G-protein-linked receptor affecting protein kinase A, are directly modulated by gangliosides and sphingolipids. Glycosphingolipids such as ceramide and sphingosine have also been reported to induce tumor cell differentiation and

subsequent apoptosis. Furthermore, gangliosides released by tumour cells may suppress host immune response aiding tumour cell escape (91,92).

1.4.1.2 Proteoglycans

Proteoglycans (PG) are formed by the covalent addition of linear anionic polysaccharides called as glycosaminoglycans (GAGs) to core proteins. There are five major types of GAGs including chondroitin suphate (CS), dermatan sulphate (DS), keratin sulphate (KS), heparan sulphate (HS) and hyaluronic acid (HA). They form a hydrated gel by occupying a large volume and provide stability to the ECM. PGs are also present on the cell membrane, where they function as co receptors for proteases and integrins, to help cells bind to the matrix and respond to growth factors (93).

The content and distribution of many PGs are altered during cancer progression. For instance, elevated levels of hyaluronan (HA) have been reported in various epithelial cancers. Similarly, overexpression of alternately spliced variants of CD-44, a membrane associated HA receptor belonging to the CS-proteoglycan family has been found to correlate with poor prognosis in colon carcinomas (94). The expression of Heparan Sulphate Proteoglycans (HSPGs) including syndecans and glypicans has been associated in pancreatic and breast cancer progression. Glypican-3 expression and function correlate with tumor type and thus its analysis can aid prognosis and diagnosis in some cancers. Syndecan-2 plays important role regulating metastasis associated processes in tumor cells such cell adhesion and migration (95).

1.4.1.3 Glycoprotein glycosylation

Glycoproteins are the third group of glycoconjugates which carry oligosaccharide chains covalently attached to proteins. Carbohydrates attached to proteins can be classified into three major groups Glycosylphosphophatidylinositol (GPI) - linked proteins, O-glycans, and N-glycans (96).

1.4.1.4 GPI- linked proteins consist of a glycosylphosphophatidylinositol moiety which is covalently attached to the carboxy terminal of the protein, thereby acting as an anchor for linking proteins to the plasma membrane. Their importance can be understood by the fact that, proteases (MT-MMPs) and protease receptors (uPAR) which are directly implicated in cancer cell invasion belong to GPI anchored proteins.

1.4.1.5 O-linked glycoproteins are synthesized by addition of of N-acetylgalactosamine (GalNAc) to the –OH group of serine or threonine residues. Further extension of O-linked glycans occurs by sequential addition of sugars where the product of one glycosyl transferase becomes the acceptor substrate for the next glycosyl transferase (97).

Mucins are the major carriers of O-glycans. They are characterised by their "rod-like" conformation owing to tandem repeats of glycosylated serines and threonines. Overexpression of mucins such as Muc-1 has been widely reported in carcinomas. Their rod like structure and negative charge repels intercellular interactions and stearically prevent adhesion molecules such as E-cadherin and integrins from carrying out their function. In this way, mucins can act as "antiadhesins" and thus promote the displacement of tumor cells from the primary mass during the initiation of metastasis (94).

Mucins also known to terminate in unique structures such as Sialyl Lewis 'x' or 'a' antigen which correlate with poor prognosis in patients with colon carcinoma (98)**.** Another abnormal feature of mucins is there incomplete glycosylation. Frequently observed hypoglycosylated structures include T/Tn (Thomson-Friedenreich antigen) and Sialyl Le (Lewis x or a antigen), which are indicative of poor prognosis in cancers of breast, ovary, stomach and prostrate (99)**.** These structures can act as progonostic markers and can serve as as ligands for endogenous lectins. Muc-1 expressing DF3 antigen is being used as a prognostic marker in breast cancer (100). T/Tn antigens on tumor cells in can mediate both homophillic, as well as heterophillic adhesive interactions with endothelial

cells by binding to lectin receptors of the galectin family. Furthermore, terminal O-linked sugars of highly metastatic colon cancer cell lines have been implicated in liver specific adhesion and metastasis (101).

1.4.1.6 N-glycosylation and metastasis

One of the universally observed alterations in cancer is aberrant expression of N-glycans on tumor cell surface proteins. Several evidences reported over the past two decades have contributed to establish the association of expression of N-glycans with invasion and metastasis. N-glycan profile of tumor cells differs from their normal counterparts with respect to their increased size, branching and sialyation. This was experimentally demonstrated by Warren and Glick by using comparative analysis of surface glycoproteins of variety of transformed and non transformed cells, thus it is called as "Warren and Glick phenomenon" (102,103). Detailed structural studies of oligosacchides from virus transformed cell lines have convincingly concluded that increase in β1,6 branching of N-glycans, which results from enhanced expression of N-acetylglucosaminyltransferases-V (GnTV), is the structural basis of Warren and Glick phenomenon (104).

In order to understand the involvement of these structures in regulation of tumor cell invasion and metastasis, it is essential to understand the biosynthesis of N-glycans.

1.4.1.6.1 Biosynthesis of N- linked oligosaccharides:

N-glycan synthesis is a co-translational event which takes place 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. The synthesis of N-glycans can be divided in five distinct phases, each associated with different compartments of the secretory pathway –

(1) Synthesis of fourteen sugar Glc3Man9GlcNAc2 N-glycan precursor on Dolicol phosphate, a lipid like precursor at the cytoplasmic face of the ER.

(2) transfer of Glc3Man9GlcNAc2 en bloc from a Dolichol linked donor to nascent Asn-residues of glycoproteins in the lumen of the rough ER;

(3) glycosidase-mediated trimming in the rough ER and Golgi;

(4) substitution by GlcNAc-Transferases (GnTs) in the medial-Golgi; and

(5) 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 general types of N-oligosaccharides, namely, oligomannose, hybrid, or complex type, having a common pentasaccharide core ($Man_3GlcNAc_2$) may be formed. Formation of these structures depends on availability of oligosaccharide substrates, presence of remodelling enzymes and retention time of the protein in the ER and golgi. While the oligomannose structure ($Man_8GlcNAc_2$) 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, followed by the addition of sialic acid. Some chains may also terminate with repeats of Gal and GlcNAc, termed as poly-*N*-acetyllactosamine (polyLacNAc) which may bear additional sugars like sialic acid and fucose. These numerous terminal substitutions on complex N-glycans provide tremendous heterogeneity to oligosaccharides (105). **Illustration 2** shows antennary structures of N-glycans which share a common pentasaccharide core.

While the bi-antennary structures are commonly seen on normal cells, the tri and tetra antennary complex N-glycans are characteristic of highly metastatic cells. GnT-V is the key enzyme involved in the formation of highly branched N-oligosaccharides. It catalyzes the formation of β1,6 branch by transferring GlcNAc to α1,6 side of mannose of N-glycan core. The plant lectin-Leuco Phyto Heam agglutinin lectin (L-PHA) binds with high specificity to the Galβ1,4 GlcNAc β1,6 branch of tri and tetra antennary N-glycans, and is hence used as a probe for the analysis of the β1,6 branch.

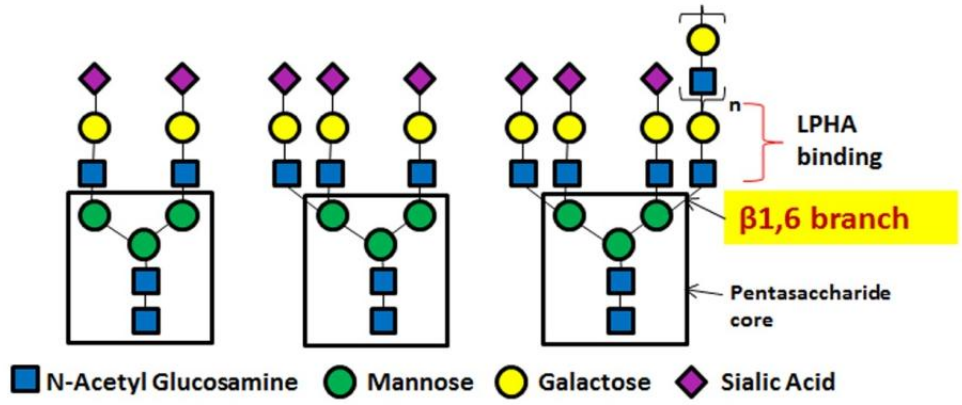


Illustration 2: Typical bi, tri and tetra antennary structures of N-linked oligosaccharides modified from (105)

1.5 β1, 6 branched N-glycans and cancer

The importance of the increased expression of the β1,6 branched N-glycans in metastatic progression has emerged from several clinical evidences reported in human tumours. For instance, clinical specimens from several human cancers including breast, endometrium liver, colorectal and melanoma show increased staining for L-PHA which is associated with metastatic disease progression and poor prognosis (106-111). Several experimental findings using cell lines and animal models have corroborated with clinical evidences and thus a strong association between expression of β1,6 branched N-glycans and metastasis was established (4,112).

Expression of these structures was blocked by either using chemical inhibitors or by genetic manipulations. Chemical inhibitors such as tunicamycin, castanospermine and deoxynojirimycin which block N-glycosylation synthesis at early steps lead to a dramatic inhibition of metastatic potential of tumour cells (113). In addition, inhibition using swainsonine (which blocks α-mannosidase II, thereby preventing the formation of complex N-glycans including β1,6 branched N-glycans) affects metastastic potential of tumor cells (114). Further, studies using L-PHA resistant mutants also lead to similar observations (4).

Cell lines with upregulated expression of GnT-V showed an increased frequency of metastasis in animal models, and spontaneous revertants for loss of GnT-V activity lose this metastatic phenotype. Transformation of non-metastatic cells with oncogenes such as T24 H-Ras, V-K-ras, or tyrosine kinase oncogene v-fps were found to induce GnT-V activity as well as metastatic potential (115). However, ectopic expression of GnT-III enzyme, a competitive inhibitor of GnT-V enzyme was found to suppress metastasis in a B16 murine melanoma cell line (116). Transfection of Mgat-V (gene which codes for GnT-V) cDNA into cultured cells has been shown to be associated with colony formation in soft agar, increased cell spreading, enhanced invasiveness through membranes, and tumorigenic behaviour by previously nontumorigenic cells (117). Thus in a conventional sense, Mgat-V can be considered as an oncogene. Studies using anti-sense and si-RNA to GnT-V have confirmed that loss of β 1,6 branch leads to a significant reduction in invasive and metastatic potential (118,119). Finally, the most important evidence came from studies with Mgat-V deficient mice. Polyomavirus middle T oncogene induced mammary tumor metastasis was considerably affected in Mgat5 (-/-) mice as compared to Mgat5 (+/+) counterparts (120).

All these evidences, explicitly 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 structures appears to act as a positive modulator of metastasis by regulating two key processes i.e invasion and organ specific metastasis.

1.5.1 Association of β1,6 branched N-glycans with invasion:

Expression of β1,6 branched N-linked oligosaccharides have been shown to be strongly associated with invasion of normal as well as metastatic cancer cells. Endothelial cells express these oligosaccharides when they need to be invasive during angiogenesis (121). During inflammation activated granulocytes and macrophages express them to extravasate and reach the inflamed site. Trophoblast cells express these oligosaccharides during implantation of embryo into uterus (122). Increased expressions of these oligosaccharides are reported in several invasive cancers such as gliomas and towards invading front in esophageal carcinoma (110,123).

Though a clear association between β1,6 branching and invasive potential of cancer cells has been established, their precise mechanisms are still being investigated. Expression of these oligosaccharides on cell adhesion molecules like integrins, cadherins, CD44 can regulate cell-cell and cell-ECM interaction. For instance, recently it has been demonstrated that β1,6 branched N-glycans can regulate matrix degradation by influencing the association of cell adhesion molecule such as integrin β1 with MT1-MMP (124). Alternatively, these structures can modulate degradation of ECM by transcriptional repression of TIMPs- the tissue inhibitors of matrix–metallo proteases, and thus facilitate invasion (125). β 1,6 branched N-glycans terminally substituted with α2,6 linked sialic acids can regulate the adhesive as well as invasive potential of melanoma cells (126). Furthermore, downregulation of GnT-V activity by siRNA mediated approach reduced the

invasion of breast cancer cells by inhibiting EGF- mediated dephosphorylation of FAK and thus signalling (118). Thus on the basis of these multiple evidences it can be conclusively said that expression of β 1,6 branched N-glycans affects invasive potential of cancer cells.

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

Another intriguing observation associated with expression of β1,6 branched N-glycans with cancer is that majority of the tumor cell lines expressing these structures primarily metastasize to either liver or to the lungs (4,112,114). For instance, the murine lymph reticular cell line MDAY-D2 over expressing β1,6 branching selectively metastasizes to liver (127). On the contrary, oncogenic Ras mediated overexpression of β1,6 branch 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 (106).

1.5.3 Emerging mechanisms by which β1,6 branched N-glycans aid organ specific metastasis:

The role of β1,6 branched N-linked oligosaccharides in organ specific metastasis is hypothesized for two main reasons. Bulky substitutions on these oligosaccharides may alter structural and functional characteristics of proteins that carry them. The small subset of proteins that carry these oligosaccharides include, cell adhesion molecules like integrins, cadherins, CD44, ECM components like laminin, proteolytic enzymes like matriptase; growth factor receptors like EGF-R (epidermal growth factor receptor) and others like Lysosome associated membrane proteins (LAMPs) (3,99,117,128-132).

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. In case of integrin subunits which have more than ten N-glycosylation sites, presence of β1,6

branched substitutions can strongly affect integrin interactions with extracellular ligands and thus ultimately influence cell behaviour. For instance, aberrant N-glycosylation of β1 integrin by transfecting GnT-V in human fibrosarcoma cells caused reduced α5β1 integrin clustering and increased cell migration (133). On the other hand, reduced β1,6 branching of E-cadherin on melanoma cells by GnT-III transfection, suppressed metastasis by increasing cell to cell adhesion (131). Recent studies have shown that altered glycosylation of EGFR on tumour cells affects receptor retention on the cell surface and subsequent signalling (134**).** Also, proteins carrying β1,6 branched structures can form membrane microdomains such as lipid raft through which cellular processes such as motility can be regulated (135).

Secondly, the β1,6 branch serves as the most preferred site for further substitutions, resulting in significant heterogeneity. Terminal substitutions on β1,6 branched N-glycans may serve as ligands for endogenous lectins **(Illustration 3)**. The elevated expression of **sialic acids** as terminal substitution with different types of linkages has been reported to affect malignancy. The ST6Gal I sialyltransferase, which adds α2,6-linked sialic acids to glycoproteins is often upregulated in a number of tumors including colon adenocarcinoma, and its expression positively correlates with tumor metastasis and poor patient survival (3). Hypersialylation of surface receptors such as β1 integrin may contribute to the invasive phenotype by modulating cell/matrix interactions. Recently, it has been shown that downregulation of STGal6-I affects invasive properties of murine melanoma cells (98,136). Additionally, the terminal sialic acids may be recognized by the siglec family of endogenous lectins. The possibility of binding of carcinoma cells having high levels of α2,3 and α2,6 linked sialic acids with vascular siglecs on target organ during metastasis is under speculation (98).

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 carbohydrates structures called as **Lewis antigens**. These structures, in turn, may facilitate adhesive interactions by binding to receptors of the selectin family (137). For example, binding of Lewis antigens on tumour cells to P-selectin receptors on platelets, has been shown to facilitate heterotypic adhesion and formation of multicellular emboli (98). Lewis antigens substituted with sialic acid residues are termed as **Sialyl-Lewis antigens (138)**. Sialyl $Lewis_x$ and $Lewis_a$ antigens are often over-expressed in human carcinomas and have been shown to mediate attachment of colon tumor cells to selectins in vitro (139). Inhibition of Sialyl Lewis antigen using antisense to human α1,3 Fucosyl transferase (FUT) gene inhibited selectin mediated adhesion and liver metastasis of colon carcinoma cells. Forced expression of E-selectin in the liver of transgenic mice enhanced metastasis of Sialyl $Lewis_X$ expressing B16F10 melanoma cells to the liver, rather than their usual destination of the lung (78). Therefore, these selectin ligands seem to play a role in liver specific-metastasis of tumor cells.

Poly N-acetyllactosamine (polyLacNAc) are major terminal substitutions observed on the β1,6 branch of N-glycans as well as O-glycans. PolyLacNAc are composed of repeating units of N-acetylglucosamine and galactose i.e (Gal β1,4 - GlcNAc β1,3)n (105). It is synthesised by the concerted action of two glycosyltransferases, the β-1,3-*N*-acetyl glucosaminyltransferase (β3GnT) and the β-1,4-galactosyltransferase (β4GalTs), each having multiple isoforms (140). The polylacNAc chains on the β1,6 branched N-glycans are primarily of the branched (I type) as compared to non-branched and linear (i type) seen on embryonic cells. PolylacNAc chains represent a backbone on which additional modifications by other glycosyltransferases including fucosylation, sialylation and sulfation take place (105).

The levels of poly-*N*-acetyllactosamine (polylacNAc) on N-glycans is reported to increase during malignant transformation (3). Furthermore, MDAY-D2 mutants, deficient in UDP-Gal transport, lack polyLacNAc on both O- and N-glycans and show the most extreme attenuation of tumor growth and metastasis. Revertants of the UDP-Gal transporter mutation selected in-vitro regain the malignant phenotype confirming that suppression of the mutation restored malignant potential (141). PolyLacNAc have been found to serve as high affinity binding ligands for the endogenous lectins – galectins (3). Dennis *et al* have reported that galectin-3 may bind to growth factor receptors such as EGF-R, expressing high levels of polylacNAc and alter their signalling (142).

To summarize, these terminal substitutions not only generate heterogeneity on cell surface glycoproteins but may also influence cancer progression by altering the functions of carrier proteins. It is clear from multiple evidences that β1,6 branched N-glycans can aid organ specific metastasis of tumor cell lines. The exact mechanism by which these oligosaccharides influence the steps of organ colonization is still unknown. How does endogenous lectin after recognizing these glycan structures mediate the downstream signalling events which modulate organ colonization steps needs to be understood.

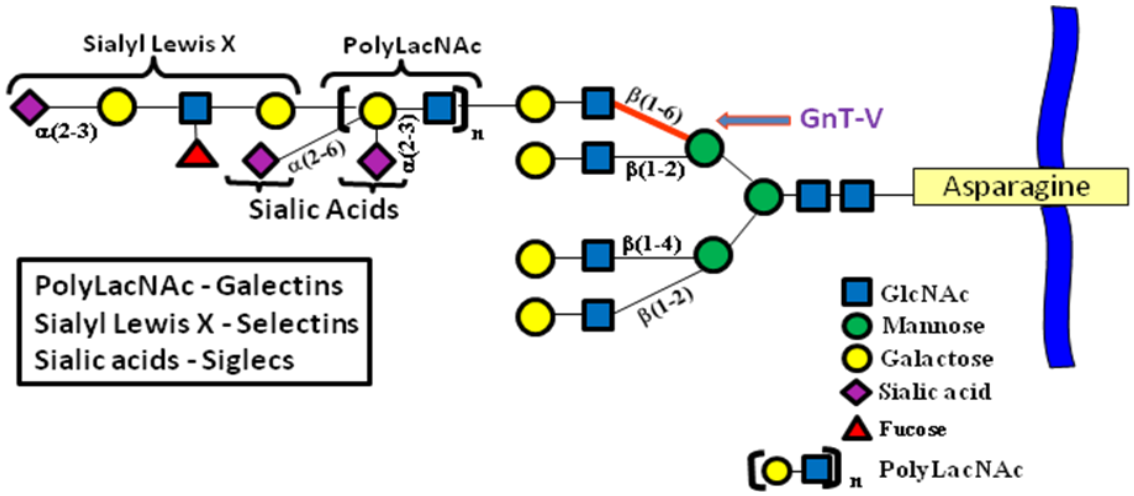


Illustration 3: β1,6 branched N-linked oligosaccharides and its terminal substitutions modified from (105)

1.6 Lectins

Glycans can mediate a wide variety of biological functions by virtue of their mass, shape, charge, or other physical properties. However, their specific biological functions are mediated via recognition by glycan binding proteins called as lectins. Initial discoveries in the field of glycobiology were confined to identification and characterisation of lectins isolated from plant sources. These plant derived lectins were termed as 'haemagglutinins' depending on their ability to agglutinate erythrocytes. Agglutination activity of these lectins could be inhibited in presence of specific sugars which for the first time attributed specificity to function of lectins. The first reported lectin in mammalian cells was galactose specific, hepatic asialo-glycoprotein receptor. Following this, several glycan binding endogenous lectins were discovered, which regulate multiple cellular processes including cell proliferation, homing and disease progression. The carbohydrate binding region of a lectin is designated as its Carbohydrate Recognition Domain (CRD). Several types of CRDs have been identified, each of which shares a pattern of unique and highly conserved amino acid residues at a characteristic spacing. On this basis the majority of the animal lectins were divided into structurally related families and superfamilies (143).

1.6.1 C-type lectins are calcium dependent glycan binding proteins which share homology in CRD. Selectins are the best characterized members of the C type lectin family that specifically recognize fucosylated and sialylated structures called Lewis/ Sialyl Lewis antigens**.** Expression of E- and P-selectins is often induced on activated vascular endothelial cells, which helps to mediate the rolling of cells which express of Sialyl Lewis x antigen on their cell surface proteins. P-selectins interact with their ligands on tumor cells to aid formation of emboli which facilitates heterotypic adhesion between tumor cells and platelets (144). Finally, L-selectins on lymphocytes and tumor cells, mediates their lymph node homing suggesting association of C-type lectins with metastasis (138).

1.6.2 P-type lectins consist of mannose 6-phosphate receptors, which recognize phosphorylated high-mannose-type glycans that are selectively expressed on lysosomal enzymes. These lectins play a important role in trafficking of newly synthesized lysosomal enzymes to lysosomes (145).

1.6.3 I-type lectins are glycan-binding proteins that belong to the immunoglobulin superfamily and mediate glycan recognition via immunoglobulin (Ig) like domains. The Siglec family of sialic acid–binding lectins is the only well-characterized group of I-type lectins. Accumulating evidence suggests that tumor specific glycan structures interact with siglecs expressed on immune cells to regulate immune cell response in tumor microenvironment. The direct role of siglecs in cancer progression is currently being investigated (146).

1.6.4 S-type lectins or Galectins have emerged as major players of both normal cellular physiology as well as malignant progression. Galectins are referred to as S-type lectins to denote their sulfhydryl dependency, the presence of cysteine residues, their solubility, and their shared primary sequence. A large number of galectins, have now been identified based on the conserved galectin CRD. The canonical CRD of galectins has approximately 130 amino acids, although only a small number of residues within the CRD directly contact glycan ligands. A total of 15 galectins have now been found in mammals, which have been classified into three major groups:

1. The prototypical galectins (galectin-1, 2, 5, 7, 10, 11, 13, 14 and 15), which contain a single CRD that may associate to form homodimers.
2. The tandem-repeat galectins (galectins 4, 6, 8, 9 and 12), in which at least two CRDs occur within a single polypeptide. They are bridged or linked by a small peptide domain. These link domains can range from 5 to more than 50 amino acids in length.

3. The chimeric galectin - galectin-3, is characterized by having a single CRD and a large amino-terminal domain, which is rich in proline, glycine, and tyrosine residues and contribute to its self-aggregation. The COOH-terminal domain contains the carbohydrate recognition domain consisting of 140 amino acids, which define the molecule as a galectin (147,148).

In addition, many of the galectin transcripts may be differentially spliced to generate different isoforms. For instance, at least seven different mRNAs have been identified for human galectin-8, some encoding a tandem-repeat form and others a prototypical form which may be differentially expressed in different tissues**.** Similarly, isoforms of galectin-9 differing in the length of the linker domains have also been identified (137).

Each galectin CRD differs in its affinity towards different types of β-galactosides structures. For example, galectin-3 binds tightly to glycans with repeating [-3Galβ1-4GlcNAcβ1-]$_n$ or polylacNAc sequences containing three to four repeating units, regardless of the presence of a terminal β-galactose residue. In contrast, human galectin-1 also binds well to long polyLacNAc chains, but it requires a terminal β-galactose residue (149).

Members of galectin family proteins are expressed in a tissue-specific and developmentally regulated fashion. For instance, galectin-1 is found in a wide variety of cells, especially of the mesothelial origin whereas galectin-3 and galectin-4 are expressed on epithelial cells, macrophages, cartilage, and, gastrointestinal tract, respectively (150)**.**

Galectins are probably unique among all types of animal lectins, in that, they can be found in the nucleus, cytoplasm, outer plasma membrane, and extracellular matrix. They lack a signal peptide, and are secreted to the extracellular milieu through a non-classical endoplasmic reticulum (ER)/Golgi-independent pathway. Depending upon their cellular

localization, they are involved in a wide variety of functions. For instance, galectins such as galectin-1, 3, 7, 10 and 11 are known to be expressed in the nucleus. Among these, nuclear galectin-1 and 3 are reported to be involved in pre-mRNA splicing by binding to Gemin-4, a component of the spliceosome complex (151).

Whereas, the galectins present in the cytoplasm are involved in regulation of cell growth and proliferation. For instance, galectin-3 in the cytoplasm is known to inhibit apoptosis, possibly by translocating to the perinuclear membranes and inhibiting cytochrome *c* release from the mitochondria, however, galectin-1,7 and 12 have pro-apoptotic activities (152). Galectins such as galectin-1, 3 and 12 have also been shown to regulate the cell cycle. For instance, overexpression of galectin-3 leads to G1 arrest by down regulating Cyclin E and A (151).

Altered expression of galectins such as galectin-1, 3 and 9 has been clearly associated with tumor progression. Many types of tumors, including thyroid carcinomas, melanomas, astrocytomas, and bladder and ovarian tumors overexpress various galectins, and their elevated expression usually correlates with clinical aggressiveness of the tumor and the progression to a metastatic phenotype. For instance, galectin-3 is absent in the cytoplasm of normal thyroid tissue but is aberrantly expressed in follicular carcinomas (153,154). Similarly, studies with galectin-transfected tumour cell lines show that they may specifically facilitate invasion, angiogenesis and metastasis. For instance, transfection of galectin-3 increases α6β1 integrin expression altering adhesion and thereby facilitating invasion of tumour cells (154).

Galectins are also known to be secreted by cells, by a yet unknown non-classical secretory pathway, and get associated with the cell surface and extracellular matrix (150). These galectins appear to bind selectively to extracellular matrix ligands including basement membrane proteins (such as laminin and fibronectin), membrane receptors (such as

integrins α7β1 and α1β1, CD43, CD7, and CD45), lysosome-associated membrane proteins (LAMP1 and LAMP-2) (155). Binding to these cell surface proteins involves interaction of CRD of galectins with precise glycan structures on these molecules**,** as reviewed in (156).

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, galectins deliver signals intracellularly, as well as mediate cell–cell and cell–extracellular matrix adhesion (157). For instance, carbohydrate dependent binding of galectin-3 to β1 integrins leads to their rapid endocytosis, thereby modulating cell adhesion (158). Similarly, cross linking of cell surface growth factor receptors, such as EGF-R by galectin-3 results in increased retention time on the cell surface, delays removal by constitutive endocytosis, thereby altering signalling**.** Galectins such as galectin-3 secreted by cells, is 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).

Extracellular galectin-3 has also been reported to be associated with malignant progression. For instance, large amounts of secreted galectin have been found in the sera of patients, which seem to have diagnostic value (159). Galectin-3 on the surface of tumour cell lines has been reported to facilitate homotypic aggregation (160). Furthermore, extracellular galectin-3 also aids angiogenesis by stimulating capillary tube formation and neovasularization in vitro (161). Galectins are also known to regulate immune and inflammatory responses. For instance, galectin-1 is generally associated with attenuating inflammatory responses while, galectin-3 has a proinflammatory role. Therefore, intracellular and extracellular galectin-3 expressed on tumour cells has been found to affect cancer progression and metastasis.

1.7 Model system for the study

To understand the mechanism by which β1,6 branched N-glycans promote homing we chose low (B16F1) and high (B16F10) metastatic variants of B16 melanoma as the model **(Illustration 4)**. There are three major reasons for choosing this particular model. First, 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) (63). Second, this model offers cell lines with vastly different metastatic potential. Third, quantitation of metastatic colonies in the lungs is relatively simple as the melanoma cells express the black pigment- melanin.

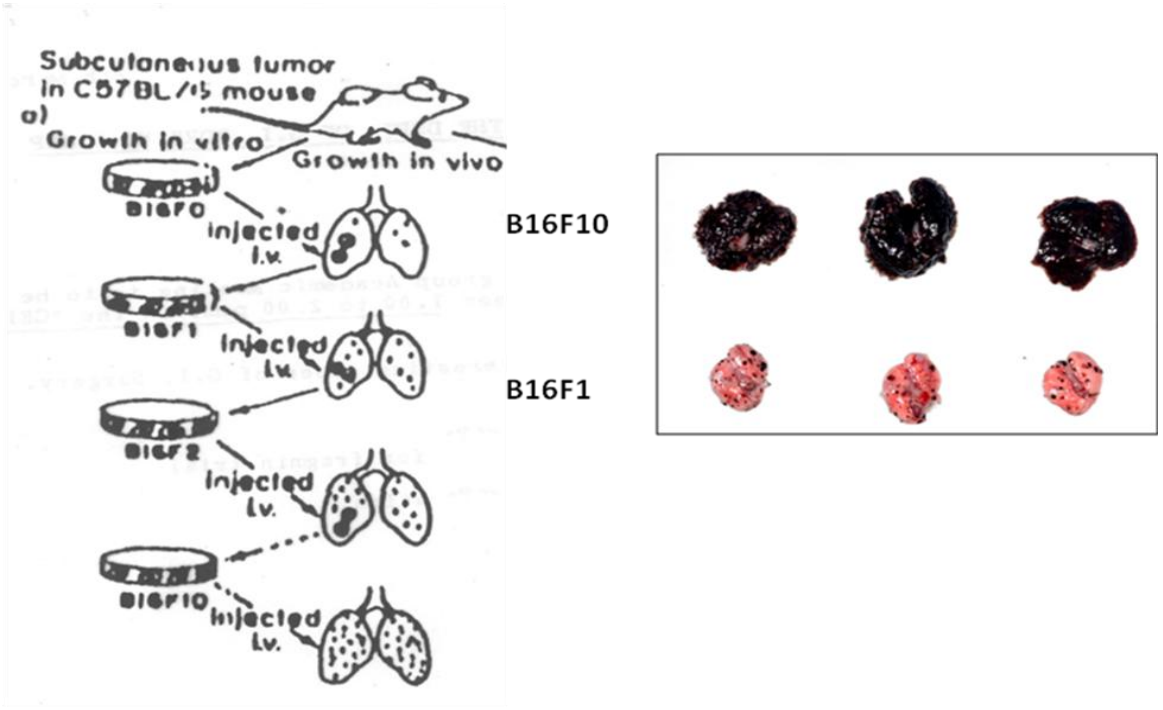


Illustration 4: The B16 murine melanoma model. Scheme of generation of B16 F1 and B16F10 cells by repeated growth in vivo (in lungs) followed by in vitro culturing of melanoma cells to select metastatic phenotype. Box shows metastatic melanoma colonies on lungs of mice injected with B16F10 (high metastatic) and B16F1 (low metastatic) cells.

1.8 Rational of the study

Metastasis, the dissemination of tumor cells to distant organs, is often associated with fatal outcome in cancer patients**.** However, the molecular mechanisms which determine the fate of colonization of tumor cells to specific distant organs are still poorly understood. Tumor cell surface molecules would be the major participant in regulating organ homing of tumor cells as these molecules regulate the crosstalk between host and tumor cells at the site of metastasis. As majority of the cell surface proteins are glycosylated and metastastic cells show aberrant glycosylation pattern, therefore it becomes evident that glycans on surface proteins can have indispensible role in metastasis. It would be important to explore how organ specific metastasis is regulated by tumor cell surface glycoproteins and their constituent receptors on host organ tissue which can modulate the molecular signalling events during process of colonization.

Using B16 melanoma model, previous work done in the lab has shown that the expression of polyLacNAc substituted β1,6 branched N-oligosaccharides correlates with the metastatic potential of melanoma cells. PolyLacNAc is the preferred ligand for endogenous lectin, galectin-3 which is expressed in highest amounts in lungs and constitutively on its organ endothelium. Further, expression of galectin-3 in lungs was not only restricted to endothelium, but it was shown to be expressed on all the other major compartments of lungs. On these grounds, the role of cell surface polyLacNAc and galectin-3 on the different lung compartments in facilitating cell adhesion, spreading, matrix degradation and motility was investigated. It was shown that galectin-3 can participate in all the events of intial arrest and extravasation, thus facilitate metastasis of melanoma cells. LAMP1 and β1 integrin were identified as the major galectin-3 binding proteins/polyLacNAc carrying proteins on melanoma cells.

Galectin-3 binds to polyLacNAc on both N- as well O-oligosaccharides. Using inhibitors which target N- or O-glycosylation synthesis (Swainsonine, SW Benzyl-α-N-acetylgalactosamine, BG), it was shown that only N-glycans play an important role in metastasis of melanoma cells. Further, participation of T/Tn antigen, a weak affinity ligand for galectin-3 in these processes, was also ruled out (162). Although such results underscore the importance of involvement of N-glycans in metastasis of melanoma cells still they do not emphatically demonstrate that polyLacNAc on β1,6 branch of N-glycans are indeed the determining factor in metastasis. Further, apart from LAMP1 and β1 integrin which other proteins carry these structures and can participate in organ colonization of melanoma cells.

On the basis of these results several key questions were raised

- How important is the expression of polyLacNAc on melanoma cells in mediating arrest and extravasation of melanoma cells?
- Apart from LAMP1 and β1 integrins which other proteins carry polyLacNAc substituted β1,6 branched of N-glycans through which galectin-3 associated metastastic processes might be regulated?
- Which molecular pathways are activated downstream of galectin-3/polyLacNAc interaction and does inhibition of these pathways affect the galectin-3 associated metastastic processes?

1.9.1 Objectives:

The following objectives were proposed to address these questions:

1. **To understand the role of galectin-3/polyLacNAc pair in mediating metastasis associated events by inhibiting the expression of polyLacNAc on melanoma cells and its effect on galectin-3 mediated processes.**
2. **To identify the galectin-3 binding proteins carrying β1,6 branched N-oligosaccharides and to study their involvement in regulation of galectin-3 mediated processes.**
3. **To elucidate the downstream signalling events of galectin-3 induced MMP9 secretion and involvement of identified proteins in this process.**

1.9.2 Plan of work

1. **To understand the role of galectin-3/polyLacNAc pair in mediating metastasis associated events by inhibiting the expression of polyLacNAc on melanoma cells and its effect on galectin-3 mediated processes**

   Participation of galectin-3 in organ specific metastasis of melanoma cells has been confirmed using multiple approaches; however the specific role of polylacNAc which is the major ligand for galectin-3 has not been confirmed. In the present study, the genes coding for enzymes involved in synthesis of polyLacNAc were targeted using shRNA mediated strategy. The effect of downregulation of polyLacNAc on metastastic properties of melanoma cells was studied.
2. **To identify the galectin-3 binding proteins carrying β1,6 branched N-oligosaccharides and to study their involvement in regulation of galectin-3 mediated processes.**

Glycoproteomic studies generally involve two important steps, first glycoprotein enrichment and second identification of enriched proteins using mass spectrometry. Lectins which recognise specific carbohydrate structures are preferably used for enrichment of glycoproteins. For identification of galectin-3 binding proteins carrying β1,6 branched N-oligosaccharides, galectin-3 and L-PHA affinity chromatography was used. Enriched proteins were resolved by 1-D SDS PAGE and proteins bands were subjected for sample preparation using mass spectrometry. Enzymatic deglycosylation was performed post trypsin digestion and deglycosylated peptides were then subjected for identification using nano LC-MS^E. The role of identified proteins in participation of metastasis associated events was studied.

3. **To elucidate the downstream signalling events of galectin-3 induced MMP9 secretion and involvement of identified proteins in this process.**

   MMPs are the key players involved in degradation of extracellular matrix. Extracellular galectin-3 has been shown to induce secretion of MMP9 in melanoma cells. The molecular mechanism behind galectin-3 induced MMP9 expression was studied and effect of downregulation of either polylacNAc or polyLacNAc carrying protein on cellular signalling involved in regulation of MMP9 expression was addressed.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines and Reagents

Murine melanoma cell line B16F10 was obtained from National Centre for Cell Science Pune, India (Ref). Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum, L-glutamine, Antibiotic–Antimycotic solution, agarose, PCR buffer, TRIzol, Calcein AM were obtained from GIBCO, Invitrogen Corporation, USA. Trypsin (cell culture and proteomics grade), Iodoacetic acid, Dithioreitol, Formic acid, Dextrose, Aprotinin, Leupeptin, Pepstatin, PMSF, Tween-20, BSA, TEMED, Beta mercaptoethanol, Glycine, Coomassie Brilliant Blue, formic acid, Ponceau-S, Lactose, Sucrose, Ampicillin, Divinyl Sulphone, Dialysis Tubing, N-hydroxysuccinimido biotin, Gluteraldehyde, Paraformaldehyde, Poly L-Lysine, Diaminobenzidine, Glass Fibre filters, POPOP, PPO, Lysolecithin, Phalloidin TRITC and FITC, Trypan Blue, Gelatin, N-octyl β-glucopyranoside, Protamine sulphate, N-acetylgalactosamine, PIPES, Lysozyme, Ethidium Bromide, DEPC, Primers for Real time PCR were obtained from Sigma Chemical Co, St. Luis, U.S.A. Tris, NP-40, Sodium deoxycholate, Sodium Dodecyl Sulphate (SDS), Bisacrylamide, Isopropyl Thio d-galactopyranoside (IPTG), Triton X-100 were obtained from USB, Cleveland, Ohio, USA. Taq Polymerase, ATP, dNTPs, T4 DNA ligase enzyme, Protoscript first strand cDNA synthesis kit, restriction enzymes and deglycosylation enzyme (PNGaseF) were obtained from New England Bio labs, USA. DNA gel extraction kit was obtained from Qiagen, Netherlands. PVDF, Protein concentrators (30 MW and 10 MW), western blot developing reagent were from Merck-Milipore, USA. Fibronectin was purchased from BD, USA. Biotinylated lectins, mounting medium with DAPI were obtained from Vector Labs, USA were employed for all studies. Inhibitors for PI3K (Wortmannin), ERK (PD169316), p38 MAPK (SB 203580) and JNK pathways, protease inhibitor cocktail and phosphatase inhibitor cocktail were from

Calbiochem, USA. Power SyBR green was purchased from Applied biosystems (ABI), Life technologies, UK.

Tissue culture plastic ware was obtained from BD Falcon or Nunc, USA. Cryovials were either from Corning, USA. Sepharose 4B beads and Cyanogen bromide activated Sepharose 4B beads, were procured from Pharmacia Fine Chemicals, Sweden.

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 reagents was of Milli-Q grade.

2.1.2 Antibodies

Rat anti-mouse β1 integrin monoclonal antibody (clone 9EG7) was obtained from BD Pharmingen, USA. Goat anti-mouse MMP9, mouse anti-β actin antibody (clone AC-74), anti-rat HRPO, anti-mouse HRPO, Extra-Avidin FITC conjugate, Avidin Peroxidase were obtained from Sigma Chemical Co, USA. Rabbit anti-human α5 Integrin polyclonal antibody (clone H104), mouse anti-mouse LAMP3, donkey anti-goat HRPO and goat anti-rabbit HRPO were purchased from Santacruz Biotechnology, Inc., USA. Rabbit p38 MAPK (D13E1) was from Cell signalling technology, USA. Rabbit anti-Basigin, anti-Embigin, anti-Prosaposin were from Abcam, USA.

2.2 METHODS

2.2.1 Maintenance of cell lines in-vitro

B16F10 (F10) murine melanoma cell line (63) was obtained from National Centre for Cell Sciences, Pune, India. Melanoma cells were routinely cultured in DMEM supplemented with 0.03% glutamine, antibiotic-antimycotic solution (10 units/ml of Penicillin G-sodium, 10µg/ml of Streptomycin sulphate and 25µg/ml of Amphotericin B) and 10% fetal bovine serum (complete medium). Cell suspension (1 X 10^6 cells/ml) in 8 ml of

complete medium was cultured in a 90 cm^2 tissue culture flask and incubated in a humidified atmosphere containing 5% CO_2 at 37ºC to achieve 90% confluency.

2.2.2 Cell harvesting, passaging and cryopreservation

Confluent monolayer was washed with Phosphate Buffered Saline (PBS – 150 mM Sodium Phosphate and 150mM NaCl, pH 7.4), and trypsin solution (0.25% trypsin, 0.02% EDTA and 0.05% glucose in PBS) was added in plate and mixed throughout the surface of the plate by repeated pipetting. Trypsin was removed and plate was incubated for 30 seconds at 37ºC and cells were washed twice with complete medium. Cell number and viability was determined by dye exclusion with 0.04% trypan blue in Tris Buffered Solution (TBS-20mM Tris chloride and 150mM NaCl). Cells with greater than 95% viability were used for all the assays. For preservation of cells, early passage cells (2.5 X 10^6 cells/ml) were resuspended in cryopreservant medium (FBS containing 10% DMSO), added to cryotubes stored in liquid Nitrogen.

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

A cryovial containing frozen cells was quickly thawed in water bath at 37°C followed by two washes with complete medium by centrifuging the cells at 1500 g for 10 min at 37°C. After centrifugation cell pellet was resuspended into 5ml of complete medium and seeded into 35 mm cell culture plate and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. On the next day, medium was changed to remove nonviable, non-adherent cells.

2.2.4 Experimental Metastasis assay:

Experimental metastasis assay was performed according to the method described in (63). Briefly, cells were harvested, washed and resuspended in serum free DMEM. C57BL6 mice (6-8 weeks old female) were injected with 100µls of a single cell suspension of melanoma cells (1.5 X 10^6 cells/ml) in DMEM via the lateral tail vein. Mice were

sacrificed after 21 days, dissected and their lungs were excised. To quantitate the metastastic potential of cells colonies on the lungs were counted using a dissecting microscope.

2.2.5 Preparation of total cell lysates

Melanoma cells were harvested, washed thrice with chilled PBS (pH 7.4) and solubilised in chilled lysis buffer containing 10 mM Tris chloride, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM magnesium chloride and 1mM calcium chloride and protease inhibitors (1µg/ml of each of pepstatin, leupeptin, aprotinin and 0.3 mM PMSF). About 15 X 10^6 cells were lysed in 1 ml of lysis buffer by three cycles of sonication each cycle is of 30 sec with 30 sec intervals at 50% output on ice. The clarified supernatant (lysate) obtained by centrifugation at 20,000 rpm for 1 h at 4°C was aliquoted and stored at -20°C.

2.2.6 Preparation of lysate for detecting phosphorylated proteins

After harvesting cells were lysed in buffer containing 10 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1% NP40, 1%, Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, Protease inhibitor cocktail (containing Aprotinin-0.4 µM, Bestatin- 0.025 mM, E-64-0.0075 mM, Leupeptin- 0.01 mM, Pepstatin A- 0.005 mM), Phosphatase inhibitor cocktail (containing Sodium Fluoride- 5 mM, Sodium Orthovanadate- 1 mM, Sodium Pyrophosphate Decahydrate- 1 mM, β-Glycerophosphate- 1 mM). Cells were then sonicated and were centrifuged at 16000 rpm for 30 min at 4^0c. Supernatant was collected, aliquoted and stored at -20°C.

2.2.7 Protein estimation

Protein was estimated according to Peterson's modification of Lowry method (163). Samples (5µls) were taken in 1 ml of distilled water and mixed with 1 ml each of Copper-Tartarate-Carbonate (CTC) reagent [Mixture of equal volumes of solution A (10% Sodium

Carbonate) and Solution B (0.1% Copper Sulphate containing 0.2% Sodium Potassium Tartrate)], 10% Sodium Dodecyl Sulphate (SDS), 0.8 N NaOH, and distilled water and incubated for 10 min. 0.5 ml of six times diluted Folin and Ciocalteau's reagent was mixed and incubated for 30 min in dark. Absorbance of solution was measured at 750 nm using a UV Spectrophotometer. Bovine Serum Albumin was used as the standard.

2.2.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as per the method described by (164). 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 min 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 (0.025 M Tris-chloride, 0.2% SDS (w/v) and 0.192 M glycine) at 100 V (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% mili Q water), or by Silver staining or proteins was transferred to polyvinylidene diflouride (PVDF) membrane for blotting.

2.2.9 Western Blotting

The transfer of proteins from the gel to a polyvinylidene diflouride (PVDF) membrane was done as per (165). The resolving gel was equilibrated in chilled transfer buffer (0.025 M Tris, 0.192 M Glycine, and 20% Methanol) for 15 min. 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 90 Volts, for 3 h. Amount of protein transferred to the membrane was checked by soaking the membrane in 0.2% (v/v) Ponceau–S stain in 3% trichloroacetic and 3% sulphosalicylic acid, for 2 min. The stain was later washed off with Tris Buffered Solution (TBS-20 mM Tris and 500 mM NaCl).

2.2.10 Probing of Western blots with lectins or specific antibodies

Cell surface glycans on glycoproteins were detected by using carbohydrate specific lectins as described in (166). Biotinylated lectin such as L-PHA (*Leukoagglutinin phytohemagglutinin*) is used as a probe for β1,6 branched N-linked oligosaccharides and LEA *(Lycopersicon esculentum)* for Poly-N-acetyllactoasmine. Lectin blots were subsequently developed using Avidin peroxidase (AVP) using chemiluminiscent reagent. Concentration of lectins and AVP used for lectin blotting is listed in the Table I.

Table 1: List of the biotinylated lectins used for the study, their concentration and incubation time

Lectin	Blocking (1hour)	Lectin incubation (1hour)	Avidin peroxidase (AVP) incubation (1hour)
Biotinylated L-PHA	T-TBS*	2µg/ml	1:30,000
Biotinylated LEA	T-TBS*	0.5µg/ml	1:30,000

***T-TBS-Tris Buffered saline containing 0.1% Tween 20, 20 mM Tris pH 7.4 and 500 mM NaCl**

2.2.11 Probing of Western blots with specific antibodies

The PVDF membranes blotted with protein were blocked with either 3% BSA or 5% milk and probed with their specific primary antibodies and secondary HRPO conjugated antibody. All the solutions including blocking and antibody dilutions were prepared in T-TBS. Concentration of primary antibodies and their respective secondary HRPO conjugated antibody is listed in **Table II**. The blots were developed using Chemiluminiscent reagent.

Table 2: List of the primary antibodies, their respective secondary antibodies used for the study, their concentration and incubation conditions.

Antibody	Blocking (1 hour)	Primary antibody	Secondary antibody (1 hour)
LAMP1	3% BSA	1:1000 in 1% BSA for 1 hour	1µg/ml Anti-Rat HRPO in 1% BSA
LAMP3	3% BSA	1:500 in 1% BSA for 1 hour	2 µg/ml Anti- goat HRPO in 1%BSA
Basigin	3% BSA	1:3000 in 1% BSA for 1 hour	1 µg/ml Anti- Rabbit HRPO in 1%BSA
Embigin	5% BSA	1:500 in 1% BSA for 1 hour	2 µg/ml Anti- Rabbit HRPO in 1%BSA
α5 Integrin	5% skimmed milk	1:1000 in 1% BSA overnight at 4ºC	1 µg/ml Anti-Rabbit HRPO in 1% BSA
β1 Integrin	5% skimmed milk	1:1000 in 1% BSA overnight at 4ºC	1 µg/ml Anti-Rat HRPO in 1% BSA
Total p38 MAPK	5% BSA	1:1000 in 1% in BSA for 1 hour	1 µg/ml Anti- Rabbit HRPO in 1%BSA
Phospho p38	5% BSA	1:500 in 1% BSA, overnight at 4ºC	1 µg/ml Anti- Rabbit HRPO in 1%BSA

MAPK			
MMP9	3% BSA	0.3µg/ml in 1%BSA, overnight at 4ºC	1 µg/ml Anti- goat HRPO in 1%BSA
β-actin	3% BSA	0.1µg/ml in 1%BSA, for 1 hour	0.5 µg/ml Anti-mouse HRPO in 1%BSA

2.2.12 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 50 mg/litre of Isopropyl Thio D-Galactopyranoside (IPTG) as described (167). The culture was grown in Luria Bertani (LB) medium (prepared by dissolving tryptone (1.0%), yeast extract (0.5%), NaCl (1.0%), pH 7.5). The bacterial cell lysate was obtained by sonication followed by centrifugation and was loaded onto Lactose-Sepharose affinity Column, prepared as per (168). 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.

2.2.12 Lectin affinity chromatography and identification of glycoproteins

2.2.12.1 Preparation of cell lysate for lectin affinity chromatography

B16F10 cells grown in 180 mm flasks were harvested washed free of serum with chilled PBS and number of cells were counted. Cell lysate was prepared as described in (130). 15 X 10^6 cells were resuspended in 1 ml of lysis buffer containing 10 mM Tris–HCl (pH 7.5), 150mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and protease inhibitor cocktail. Sonication was performed for 30 cycles with interval of 30 seconds at 50% output and protein extraction was allowed to occur for 1 hour on ice with the addition of 30 mM N-octyl-ß-D-glucopyranoside (a mild detergent required for solubilisation of membrane proteins) and

0.3% Protamine sulphate (a salt which precipitates DNA and RNA) prepared in the same lysis buffer. The cell extract was clarified by centrifugation at 16,000 rpm for 1hour at 4ºC and protein concentration was determined as described earlier.

2.2.12.2 Preparation of lectin Sepharose affinity column

For purification of proteins carrying β1,6 branched N-glycans L-PHA affinity chromatography was performed. L-PHA agarose beads used for purification were purchased from Vector labs. For purification of proteins carrying polyLacNAc, galectin-3 affinity chromatography was performed. Galectin-3 Sepharose affinity column was prepared by conjugating purified galectin-3 to Cynogen Bromide (CNBr) activated sepharose beads as describe in (169). Dried galectin-3 aliquots (10 mg) were resuspended in coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl and were incubated with CNBr activated beads overnight at 4^0C on rocker. Unbound fraction was collected and beads were first washed extensively with 0.1 M $NaHCO_3$ buffer, pH-8.5 containing 0.5 M NaCl followed by washing with acetate buffer containing 0.1 M NaOAc, 0.5 M NaCl, pH-4.5. Amount of bound protein was calculated based on absorbance at 280 nm of unbound fraction. Excess of active groups on CNBr beads were blocked by incubating with 0.2 M glycine. The column was washed with excess of coupling buffer to remove blocking agent and it was stored at 4^0C**.**

2.2.12.3 Enrichment of glycoproteins by lectin affinity chromatography

Cell lysates containing 25 mg of proteins were incubated with L-PHA/galectin-3 agarose beads (1 ml bead volume) overnight at 4°C. Beads were washed thrice with Tris chloride buffer containing 1 M NaCl. Proteins bound to the column were eluted with Tris buffer with 0.5 M NaCl containing either 150 mM Lactose (for galectin-3 column) or 300 mM N-acetyl galactosamine (GalNAc, for L-PHA column). The eluted fractions were resolved

by SDS-PAGE, proteins were transferred to PVDF membrane and were lectin blotted using biotinylated Lycopersicon esculentum agglutinin (LEA) or L-PHA for bound proteins eluted from galectin-3- and L-PHA-agarose, respectively. Percentage yield obtained was approximately calculated by comparing the intensity of signal in lane loaded with known amount of cell lysate with corresponding equivalent amount of protein in purified fraction.

2.2.12.4 Sample preparation for LC-MS analysis

Enriched glycoproteins were resolved by one dimensional SDS-PAGE as described by Lamelli (164) and gel was stained with coomassie brilliant blue (0.2% in destainer containing methanol, acetic acid and distilled water in 5:1:4 ratio). Destaining was performed gradually by increasing the strength of destaining solution (10%, 50% and finally 100%). Bands were individually cut and chopped into small pieces. The gel pieces were reduced using 10 mM dithiothreitol (DTT) and alkylated using 55 mM Iodoacetamide, both prepared in 50mM Ammonium bicarbonate. The protocol used for trypsin digestion is as described by (170). Briefly, the proteins in the gel pieces were trypsinized overnight with 10 ng/µl trypsin in 25 mM ammonium bicarbonate (10 µl/sample). Tryptic peptides were extracted using 5 % formic acid in 50% ACN and were concentrated by vacuum drying. Peptides were reconstituted in 10µl reaction buffer containing 50 mM Sodium Phosphate and subjected to deglycosylation using PNGase F (500 units) digestion for 1 hour at 37^0C.

2.2.13.1 LC-MS^E analysis:

Sample was prepared by using one part of deglycosylated peptides solution with 4 parts of 0.1% formic acid in 3% ACN. 2µl of diluted deglycosylated peptides were analyzed by nano LC-MS^E (MS at elevated energy) using a Nano Acquity UPLC system (Waters Corporation, Milford, MA) coupled to a Q-TOF, SYNAPT-HDMS (Waters Corporation).

The nano-LC separation was performed using a BEH-C18 reversed phase column (1.7µm particle size) with an internal diameter of 75µm and length of 150 mm (Waters Corporation). The binary solvent system used comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). Peptides were initially concentrated and desalted online at a flow rate of 5 µl/min using a Symmetry C18 trapping column (internal diameter 180µm, length 20 mm) (Waters Corporation) with a 0.1% B. After each injection, peptides were eluted into the NanoLockSpray ion source at a flow rate of 300 nL/min. All mass spectrometric analysis was performed in a positive V-mode at a resolution of about 9000 full width half maximum (FWHM). The instrument was calibrated with a MS/MS spectra of glufibrinopeptide B (600 fmol/µl), and the lock mass correction was done every 30s by the same peptide delivered through the reference sprayer of the NanoLockSpray source. MSE was performed by acquiring the spectra at a constant low collision energy (4 eV) to generate intact peptide masses, and the collision energy was elevated (20 to 40 eV) to get product ions at an alternative 1s scan. The radio frequency voltage applied to the quadrupole mass analyzer was adjusted such that ions from *m*/*z* 50 to 2000 were efficiently transmitted.

2.2.13.2 Analysis of MS/MS results

Protein Lynx Global Server 2.4 (PLGS, Waters Corporation) software was used for data analysis. The ion accounting search parameters included precursor and product ion tolerance 100 ppm and 250 ppm respectively, minimum number of peptide matches-1, minimum number of product ion matches per peptide-3, minimum number of product ion matches per protein-5 and the number of missed tryptic cleavage sites were-1. The false positive rate was 4%. Ion intensity threshold was set at 1000 counts. A preliminary search was performed for protein identification using UniProt mouse database containing 16,676

reviewed protein entries. LC–MS^E data was searched with a fixed carbamidomethyl modification for Cys residues, along with a variable modification for oxidized Met residues and deamidation of asparagines residues. An enriched variable modification of deamidation along with N-and O-glycosylation modifications were also set in the workflow design.

2.2.14 Cell adhesion assays

For Adhesion assays, F10 cells were labeled with Calcein AM (3µg/ml). Calcein AM is a non fluorescent dye which on cleavage by cellular esterases emits fluorescence. Assay was carried out in 96 well plates. Required numbers of wells were coated overnight at 4ºC with galectin-3 (50 µg/ml). Labeled cells were harvested, washed free of serum, counted and 40,000 cells (resuspended in 100 µl) were seeded in triplicates in uncoated and galectin-3 coated wells. Wells were gently washed three times with 100 µl of PBS to remove the unbound cells. A separate plate (control plate) was also seeded in triplicate with 40,000 cells from each cell type/treatment group, which was not washed. Fluorescence was measured in 96 well plate reader from Berthold Mithras LB-940 machine (Excitation filter-485 nm and Emission filter- 535 nm). Fluorescence units from control plate served as a control for calculating fluorescence/per cell. The percent adhesion was calculated as percentage of bound cells with respect to adhesion of untreated cells which was taken as 100%.

2.2.15 Cell spreading assays

Cell spreading assays were done as described by (171). Briefly, melanoma cells were harvested, washed thrice with DMEM without serum medium to negate any effect from FBS and seeded at a cell density of 0.5 x 10^6 cells/ml in plain medium on the coverslips coated overnight with 50 µg/ml galectin-3 in serum free DMEM at 4°C. The coverslips

were blocked with 2% BSA for 1 hour at 37°C. Coverslips coated with BSA only, served as control. Bound cells were fixed in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 for 15 min and stained with 2 µg/ml Phalloidin FITC or TRITC staining solution made in PBS (containing 1 µg/ml of Lysolecithin, 10% Methanol, 0.5% BSA in PBS) for 15 min at 37°C. The stained cells were mounted using Vectashied with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, for nuclear staining) and images were acquired using a Carl Zeiss Laser confocal Microscope (LSM 510) at 63x magnification.

2.2.16 Gelatin gel Zymography:

Matrix metallo proteinase (MMP9) were detected by gelatine zymography as per (172). One million B16F10 cells were seeded in 60 mm plate coated overnight with galectin-3 (50 µg/ml) or with fibronectin (10 µg/ml) in serum free DMEM at 4ºC. Cells seeded on uncoated plates served as control. The cells were incubated for 24 hours at 37 ºC in a CO_2 incubator. On attaining 50% confluency, the cells were washed free of serum and incubated for 24 hours in serum free medium, which was collected (conditioned medium), dried by speed vac and stored at -80 ºC.

To detect gelatinase activity, the conditioned medium was loaded on 10% SDS-PAGE containing 0.1% gelatin and run under non-reduced conditions at 4ºC. After electrophoresis, the gel was rinsed in renaturing buffer containing 2.5% Triton-X 100 for 1 hour under shaking conditions. The gel was washed thrice with buffer containing 0.1M Tris-HCl, pH 7.5 to remove excess of Triton-X 100 and then it was and incubated in a buffer containing 50mM $CaCl_2$ for 24 hours at 37ºC in a water bath. The gel was stained in 0.2% Coomassie Blue for 1 hour, and destained with 50% destaining solution to visualize the cleared zone of lysis. All the experiments were repeated in triplicates and quantitation by densitometric analysis was performed as described in (173).

2.2.17 Wound healing assays:

For wound healing assays, each well in 6 well plate was coated overnight with 50µg/ml of galectin-3 in serum free DMEM at 4ºC followed by blocking of non-specific sites with 2% BSA for 1 hour. B16F10 cells were harvested and seeded at a cell density of 0.5 X 10^6 cells and incubated at 37ºC for 24 hours in a CO_2 incubator. The cells were subsequently washed free of serum and serum starved for 24 hours for cell synchronization. A straight, uniform wound (approx. 400 µm in width) was made using a micropipette tip on the plate upon reaching 90% confluency. The dislodged cells were washed off and the plate placed in 37 ºC CO_2 chamber focussed at 10 X magnification on Carl Zeiss Inverted Microscope. Wound closure of cells was measured for 20 hours by time lapse video imaging at least three different positions across the length of the wound. Uncoated culture dishes, blocked only with BSA served as control. Area of wound closure was calculated by Image J software. For calculating percent wound closure, area at zero time was considered as 100% and accordingly, percentage wound closure was determined at 5 hour interval.

2.2.18 Real time PCR

For detecting the transcript levels of GalT-I, GalT-V and MMP9, specific primers were designed. Ribosomal protein L4 (RPL4) was used as housekeeping gene for relative quantification of transcript levels (174). The primers sequences are given in **Table 3**. The real time PCR reaction was carried out in 7900HT system (ABI Prism) and for detecting amplicons Power SYBR green was used. The Cycle threshold (Ct) values obtained were normalized to RPL4 values. Analysis was performed using $2^{-\Delta\Delta Ct}$ method (175).

Table 3: List of the primer sequences used in Real time PCR.

1	**RPL4** Forward: GACAGCCCTATGCCGTGACTG Reverse: GCCACAGCTCTGCCAGTACC
2	**GalT-I** Forward: CTTGGAGAGACTTCTTGGTT Reverse: TAAAGGGACAGAGCACGA
3	**GalT-V** Forward: CTAGGAAGGAAGTGGATG Reverse: TGTGTGTGTGAGGAGGTGTA
4	**MMP9** Forward: TCATTCGCGTGGATAAGGAG Reverse: AGGCTTTGTCTTGGTACTGG

2.2.19 Strategy for downregulation of β1,4 galactosyltransferases-I and –V (GalT-I and –V) transcripts which code for enzymes involved in polyLacNAc synthesis in melanoma cells.

2.2.19.1 Designing and cloning of shRNA constructs

Earlier results from our group has shown that out of six GalTs expressed in B16 F1 and F10 cells, were found to be expressed in metastastic potential dependent manner. transcripts GalT-I and –V genes of these genes were targeted using short hairpin RNA (shRNA) for downregulating expression of polyLacNAc. The shRNAs were designed against as per the guidelines outlined by (176). A 21 nucleotide common sequence from the open reading frames of murine GalT-I and –V was selected by using sequence alignment tool, Basic Local Alignment Search Tool (BLAST). It was also confirmed that

this shRNA sequence is only specific for GalT-I and –V and would not target any other transcripts. Murine GalT-I and –V sequences used for BLAST were from mouse sequence database available at National Centre of Biotechnology Information (NCBI) website. ShRNA cassette was PCR amplified using forward primer having XhoI site followed by sense sequence and 15 nucleotide loop sequence. The reverse primer contained EcoRI site, sense sequence and 15 nucleotide loop sequence. 11 nucleotide loop sequences are complementary to each other. Primer sequences used for shRNA amplification are mentioned below. (Colour codes: Yellow for dummy sequences, blue for restriction enzyme sites, red for mir sequence, black for sense sequence and green for loop sequence)

GalT-I and-V shRNA

Forward primer

5'GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCGTGGGGCGGAGAAGATGACGACT AGTGAAGCCACAGA3'

Reverse primer

5'GTTGAATTCCGAGGCAGTAGGCATGGGGCGGAGAAGATGACGACTACATCTGTGGCTT C3 **Table 4: List of the PCR conditions for shRNA amplification (100 µl reaction, for 30 cycles)**

Temperature (°C)	**Time (Minutes)**	**Reagents**	**Volume (µl)**
95 (Initial Denaturation)	5	Forward primer	4
95 (Denaturation)	5	Reverse primer	4
55 (Annealing)	1.30	10X buffer	10
72 (Extension)	1	dNTPs (10 mM)	2
72 (Final extension)	10	Taq polymerase	2
		MiliQ H_2O	68

PCR products were run on agarose gel cut purified and digested with XhoI and EcoRI. Digested products were again run on agarose gel and DNA was purified from the cut bands using gel extraction kits. Doxycycline inducible lentiviral vector pTRIPz (**Illustration 6**) was linearised by XhoI and EcoRI digestion. The linearised vector and XhoI and EcoRI digested PCR product was ligated in 1:9 ratios (vector: insert) using T4 DNA ligase Enzyme, overnight at 22°C.

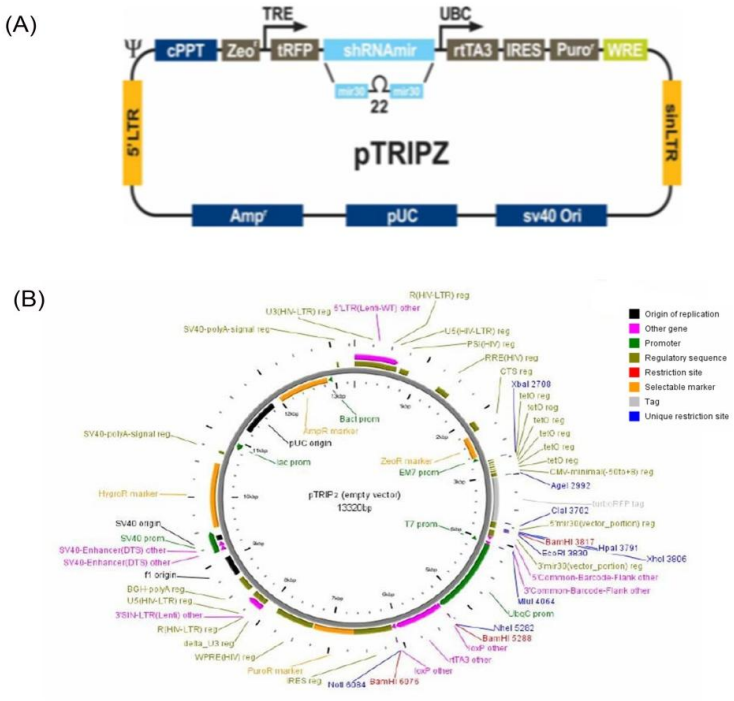


Illustration 5: Features (A) and map (B) of pTRIPZ vector

The ligated plasmid was transformed into ultra-competent DH5α cells. Competent cells transformed with linearized vector alone, served as negative control. Briefly ultra-competent cells were thawed and ligation mixture was added to it and incubated on ice for 30 min. Competent cells mixed with plasmids were given heat shock at 42°C for 90 sec and the mixture were subjected to cold shock on ice for 5 min. The cells were then mixed with 1ml sterile LB broth and incubated with at 37°C for 1 h in

orbital shaker incubator. Cells were spun at 5000 rpm for 1 min cell pellet was resuspended in 200 µl of LB broth and spread on low salt LB agar plate containing 100 µg/ml, ampicillin and incubated overnight at 30°C

2.2.19.2 Screening of the recombinant colonies:

About 15 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 clone were screened by restriction digestion.

2.2.19.3 Isolation of plasmids by alkaline lysis method

Plasmid isolation from the overnight grown culture was carried by the alkaline lysis method. The bacterial culture of 1.5 ml was distributed in sterile microcentrifuge tubes and centrifuged at 5000 rpm, at 4°C for 5 min the medium was removed and the pellets were dried as possible. To the dried bacterial pellets 100 µl of alkaline lysis solution I (GTE buffer: 50 mM Glucose, 25 mM Tris pH8.0 and 10 mM EDTA) was added and vortexed till the pellets were completely dissolved. Then 200 µl of alkaline lysis solution II (0.2 NaOH and 1%SDS) was added, mixed gently by inverting and kept for 2 min 150 µl of ice-cold alkaline lysis solution III (3M potassium acetate pH 4.8 in glacial acetic acid) was then added and kept on ice for 10 min the tubes were then centrifuged at 13000 rpm, 4°C for 15 min and the supernatant containing the renatured plasmid was transferred to a fresh tube. Then equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added 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 for precipitation of plasmid DNA and mixed well. 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 was removed. The pellets were completely dried at 37°C for 30 min. The dried pellets was reconstituted in 20 µl of autoclaved distilled water and 0.3 µl of RNase (1µg/ml) was added to each tube and incubated at 37°C for 45 min to degrade RNA molecules.

Positive colonies containing the shRNA were screened or checked by XhoI and EcoRI digestion, positive clones gave an insert release of 120 bp. Presence of positive clones was also confirmed by EcoRI and AgeI digestion positive clones gave an insert release of 958 bp whereas empty vector without shRNA gave an insert release of 838 bp.

2.2.19.4 Plasmid DNA purification for transfection

For transfection, plasmid DNA was purified in bulk quantities using maxi prep columns. The plasmid isolation was performed exactly as described in GenElute Maxi prep kit (Sigma Aldrich) manual.

2.2.19.5 Preparation of ultracompetent Escherichia coli Dh5α cells

Reagents required

SOB (300 ml)

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 1N NaOH, followed by the addition of 4 ml 1 M glucose and 1ml of 2 mM $MgCl_2$, just prior to inoculation of

bacteria.

Transformation Buffer (200ml)

PIPES- 0.6 gm

$CaCl_2$ 0.4 gm

KCl- 3.7 gm

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

Ultra competent *E. coli* Dh5α cells were prepared as described (177). 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 reaches between0.3 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 pellets were 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 nearly 200 µl into 1.5 ml micro centrifuge tubes, snap freezed into liquid nitrogen and immediately stored at -80°C until further use.

2.2.19.6 Preparation of lentiviral particle and transduction

For the generation of lentivirus particle, empty vector or non-target shRNA or the cloned shRNA constructs in inducible vector pTRIPz along with helper constructs pPAX2 and pMD2.G which codes for viral capsid and envelop protein respectively

were co-transfected into HEK293FT cells using Calcium Phosphate method of transfection, viral supernatant were collected 24 h post changing of transfection medium, it was spun at 5000 rpm for 20 min at 4°C.

CHAPTER 3

RESULTS

3. Results

Earlier results from our lab has shown that expression of β1,6 branched N-oligosaccharides correlates with metastastic potential of B16 murine melanoma cells (low metastastic, B16F1 and high metastastic, B16F10). β1,6 branched N-oligosaccharides is the preferred site for terminal substitutions such as sialic acids, Lewis antigens and poly-*N*-acetyllactosamine (polyLacNAc) which may serve as ligands for several endogenous lectins. It was found that levels of polyLacNAc substituted β1,6 branched N-glycans in these cells correlate with their metastastic potential. PolyLacNAc is a high affinity ligand for endogenous lectin, galectin-3 which is expressed in highest amounts in lungs and constitutively on its vascular endothelium. LAMP1 and β1 Integrin which were identified as polyLacNAc carriers could mediate adhesion of melanoma cells on lungs via galectin-3 and thus facilitate their homing (5).

Adhesion of tumor cells to target organ endothelium is the primary step of organ homing which is followed by extravasation events to establish a metastastic colony. It is reported that galectin-3 is secreted out in a non classical pathway and interact with cell membrane or become a part of BM/ECM. In lung tissues, expression of galectin-3 was not only restricted to vascular endothelium, but it was shown to be expressed on all the other major compartments. On these grounds, participation of galectin-3 in post adhesion events was investigated. It was shown that apart from mediating arrest of melanoma cells onto lung endothelium, galectin-3 could also facilitate cell spreading, matrix degradation and cell motility. The role of galectin-3 in mediating these post adhesion events clearly suggests that it can play an important role in extravasation of melanoma cells (6).

Swainsonine (SW), N-glycosylation inhibitor, inhibits α-mannosidase II, an enzyme involved in initiating the formation of complex type N-glycans. Treatment with SW resulted in inhibition of expression of complex type, including β1,6 branched N-glycans,

and significant loss in the metastatic potential of melanoma cells. Although, β1,6 branched of N-oligosaccharides is one of the most preferred sites for polyLacNAc substitutions, even O-oligosaccharides often carry them. However, inhibition of O-glycan synthesis in F10 cells using Benzyl-α-N-acetylgalactosamine (BG) did not affect the galectin-3 mediated processes including their lung metastatic potential. Further, participation of T/Tn antigen, a weak affinity ligand for galectin-3 in these processes, was also ruled out (162). Although, these results highlighted the importance of expression of β1,6 branched N-glycans on melanoma cells in metastasis associated processes, they did not conclusively establish the specific involvement of polyLacNAc in metastasis. Thus, their role was confirmed by specifically targeting the genes which code for enzymes involved in the synthesis of polyLacNAc.

Objective I: To understand the role of galectin-3/polyLacNAc pair in mediating metastasis associated events by inhibiting the expression of polyLacNAc on melanoma cells and its effect on galectin-3 mediated processes.

Poly-N-acetyllactosamine (polyLacNAc) consists of repeating units of N-acetylglucosamine and galactose. The synthesis of polyLacNAc is mediated by concerted action of β1,3 N-acetylglucosaminyltransferases (GnTs) and β1,4 galactosyltransferases (GalTs). Inhibition of either of GnTs or GalTs can affect polyLacNAc synthesis. We chose GalTs for shRNA mediated downregulation as their participation in synthesis of polyLacNAc on β1,6 branched N-glycans has been reported. Further, expression of some of the GalTs including GalT-V has been shown to correlate with metastastic potential of tumor cell lines (178).

Previous work in the lab has shown that out of six GalTs, GalT-I and –V are expressed in melanoma cells in a metastatic potential dependent manner (6). The levels of GalT-VII

was not analysed as it is reported to be involved in polyLacNAc synthesis on proteoglycans. We chose a unique 22 nucleotide sequence for targeting transcripts of GalT-I and –V. The shRNA was cloned in an inducible lentiviral vector, pTRIPZ. The first major advantage of using inducible system is that it allows tight temporal control of expression for shRNA and second, the shRNA mediated effects can be rescued in absence of inducer.

3.1 Cloning of GalTshRNAmir in pTRIPZ vector, transduction and selection of positive clones

The primers containing GalTshRNA sequences along with mir, loop and restriction enzyme recognition sequences were amplified by PCR. The amplified PCR product of 120 base pairs (bp) and empty pTRIPZ vector were double digested with XhoI and EcoRI restriction enzymes, ligated and transformed into *E.Coli* DH5α cells (Figure 1A). Positive clones were confirmed by EcoRI and AgeI digestion, shRNA containing pTRIPZ vector showed insert release of 958 bp, whereas empty vector without shRNA gave release of 838 bp of vector DNA (Figure 1B).

Lentiviral particles were then generated in HEK293FT cells by co-transfecting GalT pTRIPZ or Non targeting pTRIPZ (NT) with packaging vectors (pMD2.G and psPAX2) which were further transduced in B16F10 cells (Figure 1C). One NT and two GalT pTRIPZ clones I and II were selected after puromycin treatment. Expression of NTshRNA and GalTshRNA was confirmed by observing for RFP fluorescence after induction by doxycycline for 96 hours (Figure 1D).

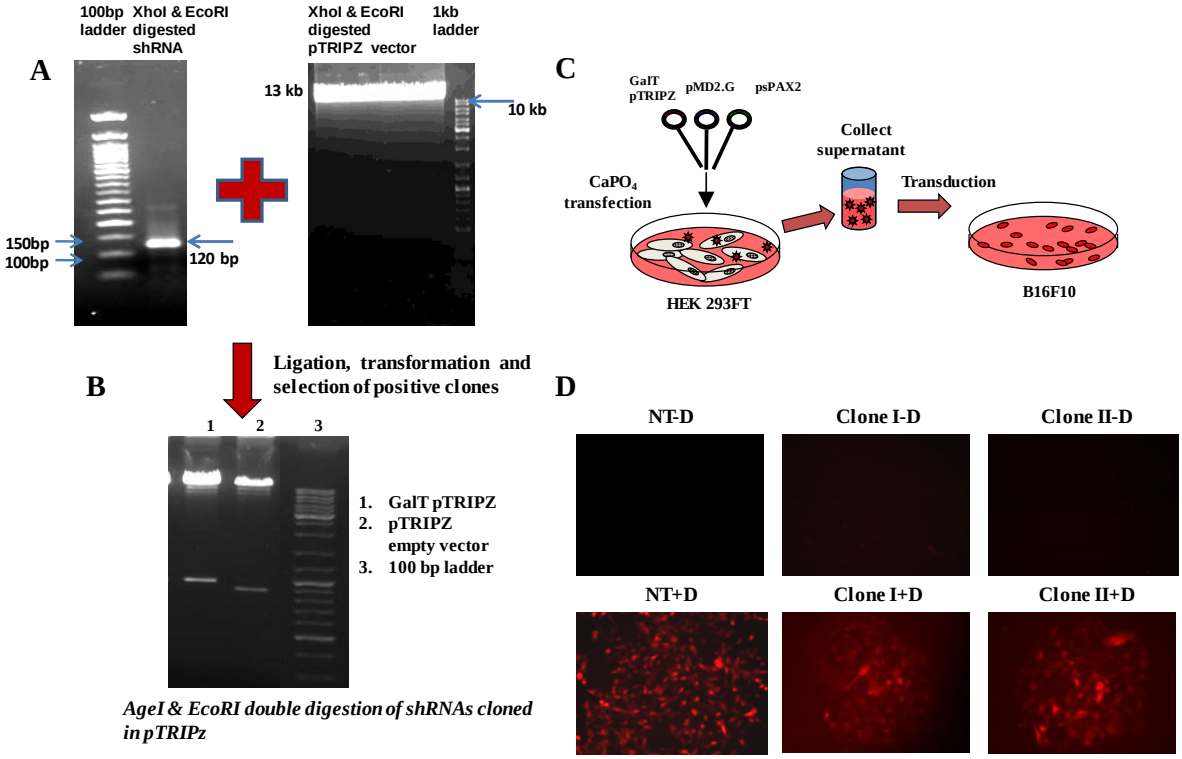


Figure 1: Cloning of GalTshRNA in pTRIPZ lentiviral vector. (A**)** Agarose gel electrophoresis for XhoI and EcoRI double digested GalTshRNA and pTRIPZ empty vector**.** (B) Screening of positive clones using double digestion with AgeI and EcoRI. Empty pTRIPZ vector served as control. (C) Illustration describing the procedure for preparation of lentiviral particles and transduction of F10 melanoma cells. (D) Red fluorescent protein (RFP) expressed in pTRIPZ clones on treatment with doxycycline.

3.2 Downregulation of GalT-I and -V genes in F10 cells leads to significant reduction in the expression of polyLacNAc and galectin-3 binding

Since a common shRNA was used for targeting two genes, it was necessary to analyze if mRNA levels of both GalT-I and –V genes were indeed affected in clones I and II. Using real time quantitative reverse transcriptase PCR (qRT-PCR), it was observed that upon

doxycycline induction, GalT downregulated clones I and II express reduced levels of both GalT-I and V transcripts as compared to NT cells (Figure 2A).

Western blotting of total cell lysates of the B16F10 and clones expressing NT and GalT shRNAs showed reduction in expression of polyLacNAc only in cells expressing shRNA specific for GalT-I and –V and not in cells expressing NTshRNA (Figure 2B). Flow cytometry experiments using LEA showed that GalT shRNA expression also resulted in reduced levels of polyLacNAc on the cell surface and thus possibly resulting in reduced galectin-3 binding, as evident in Figure 2C.

Reduction in binding of galectin-3 to GalT downregulated clones should inhibit their participation in galectin-3 mediated processes such as cell adhesion, spreading, matrix degradation and motility which aid organ colonization.

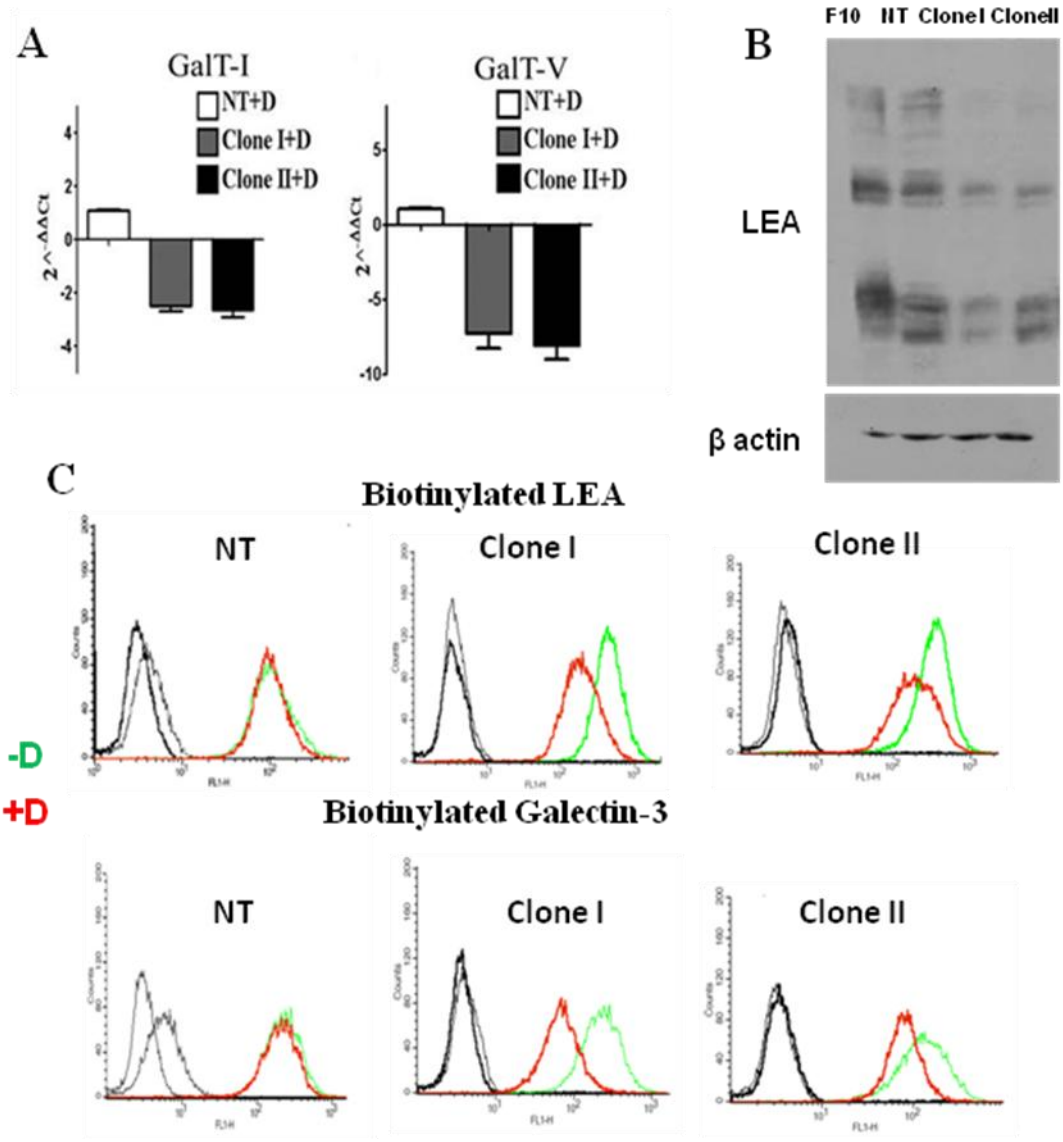


Figure 2: Validation of down regulation of GalT-I and –V transcripts, its effect on polyLacNAc expression and galectin-3 binding. (A) Analysis of transcript levels of beta 1,4 GalT-I and GalT-V in clones I and II, after doxycycline induction by real time PCR. NT (Non Targeting) shRNA served as the vector control. (B**)** Comparison of expression of polyLacNAc in untransduced F10 cells, doxycycline treated NT, Clones I and II by western blotting using biotinylated LEA. β actin served as loading control. (C) and (D) Surface levels of polyLacNAc and galectin-3 binding of NT and GalT downregulated clones was compared under doxycycline treated and non treated conditions by flow

cytometry using biotinylated LEA and galectin-3. Cells treated with only Extravidin FITC served as control.

3.3 Galectin-3 mediated cell adhesion and spreading is affected in polyLacNAc downregulated clones

Adhesion to vascular endothelium is the first major event involved in organ colonization. Cell surface molecules on tumor cells interact with their receptors expressed on organ endothelium to regulate host-tumor adhesive interactions. Our earlier results clearly suggest that galectin-3 expressed on lungs can act as a receptor for polyLacNAc expressed on melanoma cells in regulating adhesion on lung endothelium. If participation of polyLacNAc is crucial in this process then downregulation of GalTs which resulted in reduced polyLacNAc and galectin-3 binding (Figure 2C), should in turn, affect the adhesion of melanoma cells on immobilized galectin-3.

Initially, we confirmed the adhesive potential of untransduced F10 melanoma cells on immobilized galectin-3 (IM Gal-3). In comparison to cells incubated in uncoated wells, there was twofold increase in adhesion of cells on coated galectin-3 wells, confirming that galectin-3 promotes adhesion of melanoma cells (Figure 3A).

In case of polyLacNAc downregulated clones, upon induction with doxycycline, adhesion of clones I and II was affected as compared to uninduced cells. However, percent adhesion of NT cells remained unaltered in induced or uninduced state (Figure 3B). These results confirm that polyLacNAc plays a vital role in galectin-3 mediated adhesion of melanoma cells.

Cell spreading is the subsequent post adhesion event which facilitates firm anchorage of tumor cells on vascular endothelium. It not only helps in counteracting the hemodynamic forces, but also aids extravasation of tumor cells. At molecular level, in cell spreading process, cytoskeletal network is reorganized leading to formation of membrane protrusions

like lamellopodia and filopodia which initiate assembly of adhesion complexes involving integrins and associated proteins (179,180).

Cell spreading assay was performed using Phalloidin FITC staining. Phalloidin binds to F-actin which is the key player involved in regulation of cytoskeletal reorganization. F10 melanoma cells incubated on galectin-3 coated coverslips typically have flattened morphology and they show lamellipodial like projections (Figure 4A, IM gal-3). Cells incubated on uncoated coverslips do not show such membrane protrusions and these cells have rounded morphology (Figure 4A, Un). Changes in cellular morphology were quantitated by calculating the ratio of cytoplasmic area to nuclear area (C/N ratio) of spreaded cells. C/N ratio of F10 cells spread on galectin-3 was significantly higher (approximately three fold) than that of cells spread on uncoated coverslips (Figure 4B).

Effect of downregulation of polyLacNAc on galectin-3 mediated cell spreading was analyzed in GalT downregulated clones. Results demonstrate that on doxycycline induction, clones I and II show absence of typical galectin-3 induced membrane protrusions, which are clearly visible in uninduced cells. NT cells did not show any change in morphology in induced or uninduced state (Figure 4C). Comparison of C/N ratio between NT and GalT downregulated clones spread on galectin-3 confirm that reduction of polyLacNAc levels affects galectin-3 mediated spreading of melanoma cells (Figure 4D).

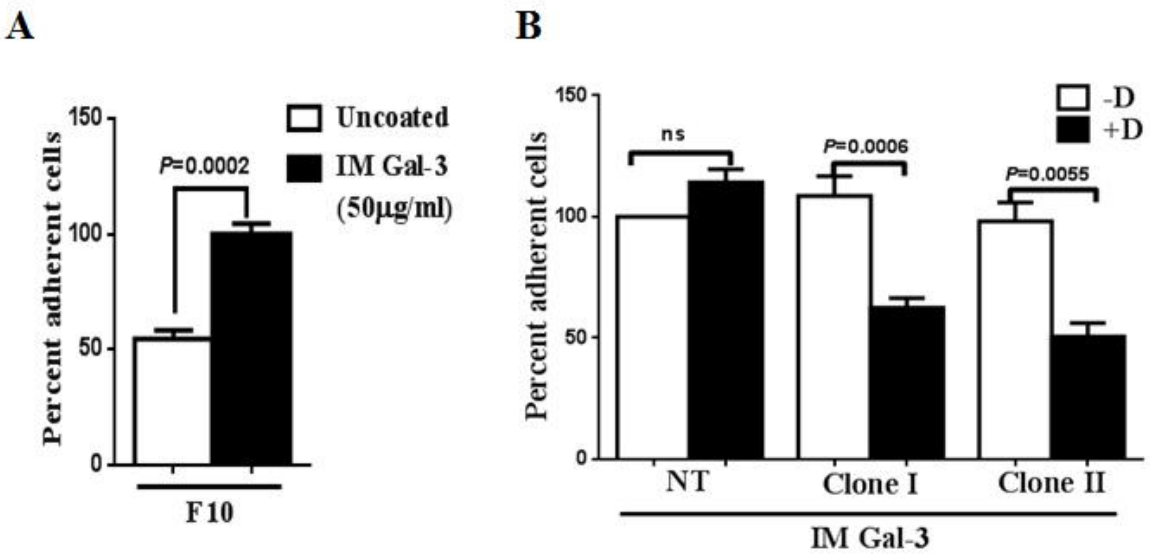


Figure 3: Reduced polyLacNAc levels in GalT downregulated clones affect galectin-3 mediated adhesion and spreading. (A) Bar graph representing adhesion of F10 cells on 96-well plates coated with 50 µg/ml of galectin-3 (Immobilized galectin-3- IM Gal-3). Adhesion on galectin-3 was taken as 100%. Cells incubated in uncoated wells served as control. (B) Adhesion of clones I, II and NT in presence or absence of doxycycline on galectin-3 coated wells. Values are mean ± standard error of three independent experiments. Unpaired student's t-test was applied for statistical analysis and p value > 0.05 was considered significant.

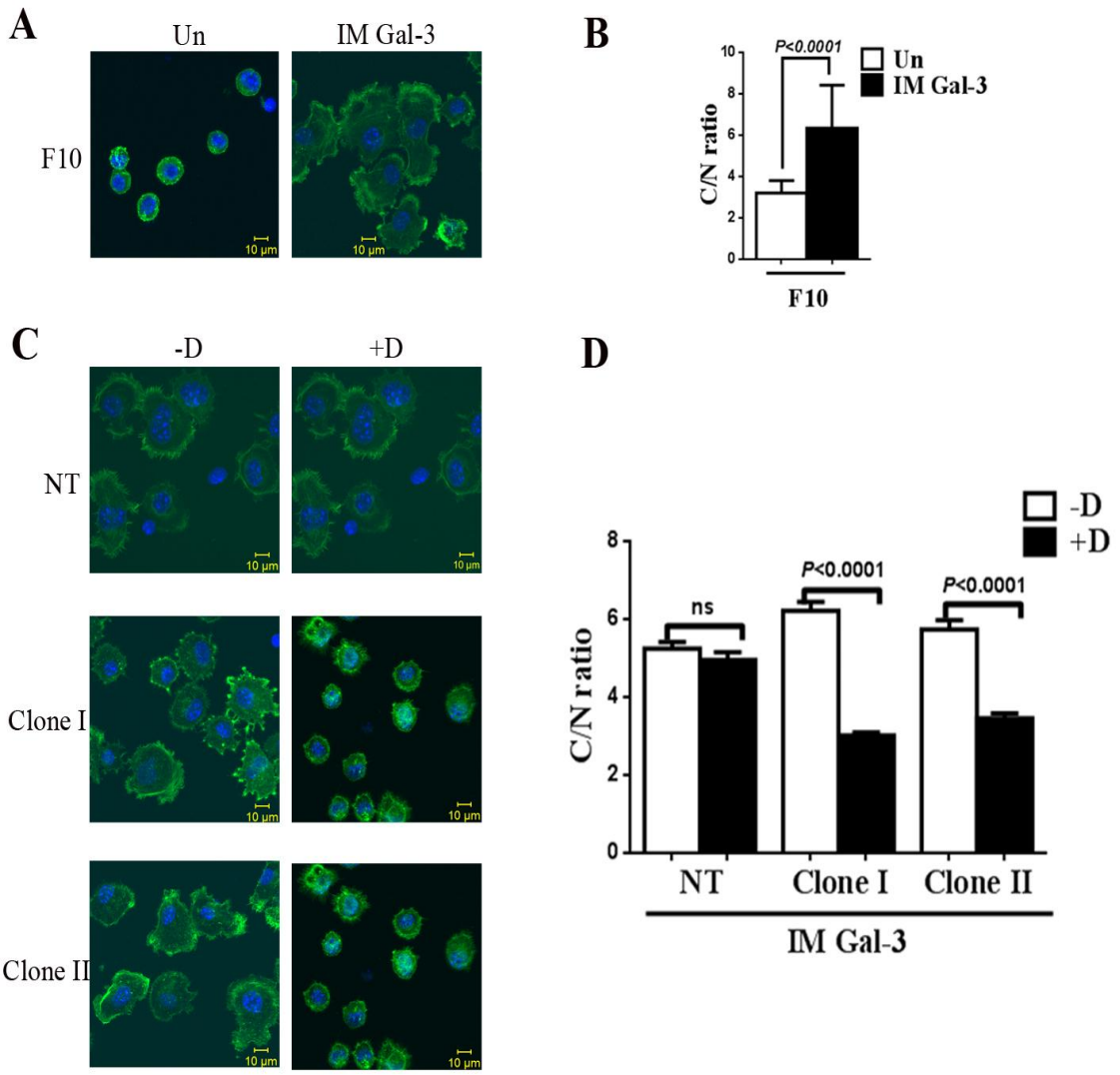


Figure 4: Downregulation of polyLacNAc affected spreading of F10 cells on immobilized galectin-3.

(A) Comparison of cell spreading of F10 cells on uncoated or galectin-3 coated coverslips by F actin staining (green) with Phalloidin FITC using laser confocal microscopy at 63x magnification. DAPI was used to stain nuclei (blue). Scale bar = 10 µm. (B) Bar graph represents quantitation of cell spreading by determining the mean ratio of cytoplasmic to nuclear area of around 100 cells from three different experiments. (C) Spreading of NT, Clones I and II on galectin-3 coated coverslips in presence or absence of doxycycline. (D) Bar graph represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells of

NT, Clone and II from three independent experiments. Unpaired students t-test was employed for statistical analysis and p value < 0.05 was considered significant.

3.4 Reduced levels of polyLacNAc in GalT downregulated clones affect galectin-3 induced matrix degradation, cell motility as well as the metastatic potential

For gaining access into the organ parenchyma, tumor cells need to displace the organ endothelium, degrade the underlying basement membrane (BM) and create space for movement. The key players involved in matrix degradation are matrix metalloproteinases (MMPs) especially members of gelatinases family, MMP2 and MMP9. These MMPs are involved in degradation of different types of collagens (I, IV, V, VII, etc.), non collagenous proteins including fibronectin, vitronectin, laminin and protein part of proteoglycans (43,46). Previous work in the lab has shown that extracellular galectin-3 in both immobilized as well as soluble form induces secretion of MMP9 in melanoma cells. Further, the levels of secretion of MMP9 correlated with metastatic potential of melanoma cells which also differ in expression of polyLacNAc on β1,6 branched N-oligosaccharides (6). The role of polyLacNAc in induction of galectin-3 mediated MMP9 secretion can be confirmed using polyLacNAc downregulated melanoma cells.

Before analyzing the levels of MMP9 in polyLacNAc downregulated cells, we first confirmed the induction of MMP9 in culture supernatant of F10 cells by both zymography as well as Western blotting. As compared to cells grown on uncoated plates, higher levels of MMP9 were secreted in culture supernatant collected from cells grown on coated galectin-3 plates (Figure 5A and B, F10). Densitometric analysis further confirmed this observation. Gelatin zymography was performed to compare the levels of MMP9 in culture supernatants of induced and uninduced clones grown on immobilized galectin-3. The levels of MMP9 in NT clone was unaltered in either doxycycline treated or untreated

condition. In case of GalT downregulated clones I and II, expression of shRNA significantly affected the secretion of MMP9 (Figure 5A). This was also confirmed by immunoblotting using anti MMP9 antibody (Figure 5B). These results emphasize that polyLacNAc expression on melanoma cells is crucial for galectin-3 induced MMP9 expression.

After degradation of vascular basement membrane and the matrix for extravasation, cells move into the organ parenchyma where they can proliferate and form metastatic colony. Tumor cells can use matrix proteins as traction for motility. Galectin-3 has been known to be incorporated in ECM/BM (155,181). Immobilized galectin-3, as a component of ECM, can facilitate movement of tumor cells.

Wound healing assays using time lapse video microscopy showed that motility of F10 cells on immobilized galectin-3 was significantly higher than that on BSA (Figure 6A). Reduced expression of polyLacNAc in GalT downregulated clones affected the percent wound closure after induction with doxycycline (Figure 6C and D). The motility of NT cells showed no alteration either in presence or absence of doxycycline (Figure 6B).

The effect of downregulation of polyLacNAc on metastasis associated events including cell adhesion, spreading, degradation of matrix and motility should ultimately affect the metastatic potential. Experimental metastasis assay was performed to compare the metastastic ability of untreated F10 cells and doxycycline treated NT, Clones I and II. The number of colonies on lungs of mice injected with F10 cells or NT cells were comparable while those injected with GalT downregulated clones showed significantly reduced number of metastatic colonies (Figure 7A and B).

These results altogether conclude that polyLacNAc substituted β1,6 branched N-glycans on melanoma cells not only aid arrest, but also mediate other events of extravasation including spreading, matrix degradation, motility and ultimately metastasis.

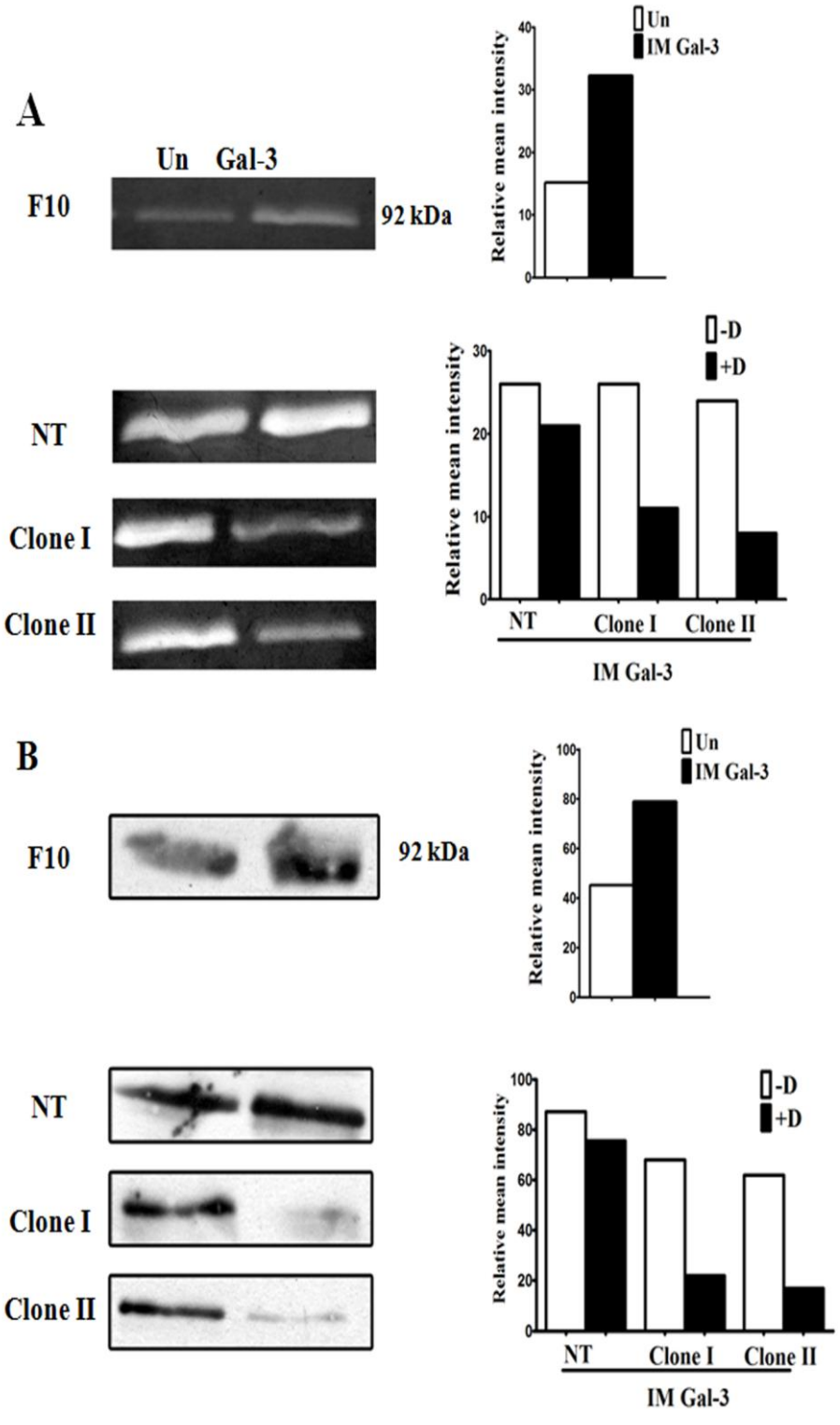


Figure 5: PolyLacNAc downregulation affects galectin-3 mediated secretion of MMP9 (A) Levels of MMP9 in culture supernatants of F10 cells grown on uncoated or

coated galectin-3 plates, NT, Clone I and II grown in presence and absence of doxycycline on coated galectin-3 as detected by zymography and (B) Western blotting. The adjacent right panel of (A) and (B) represent densitometry analysis for bands observed in zymography and western blotting.

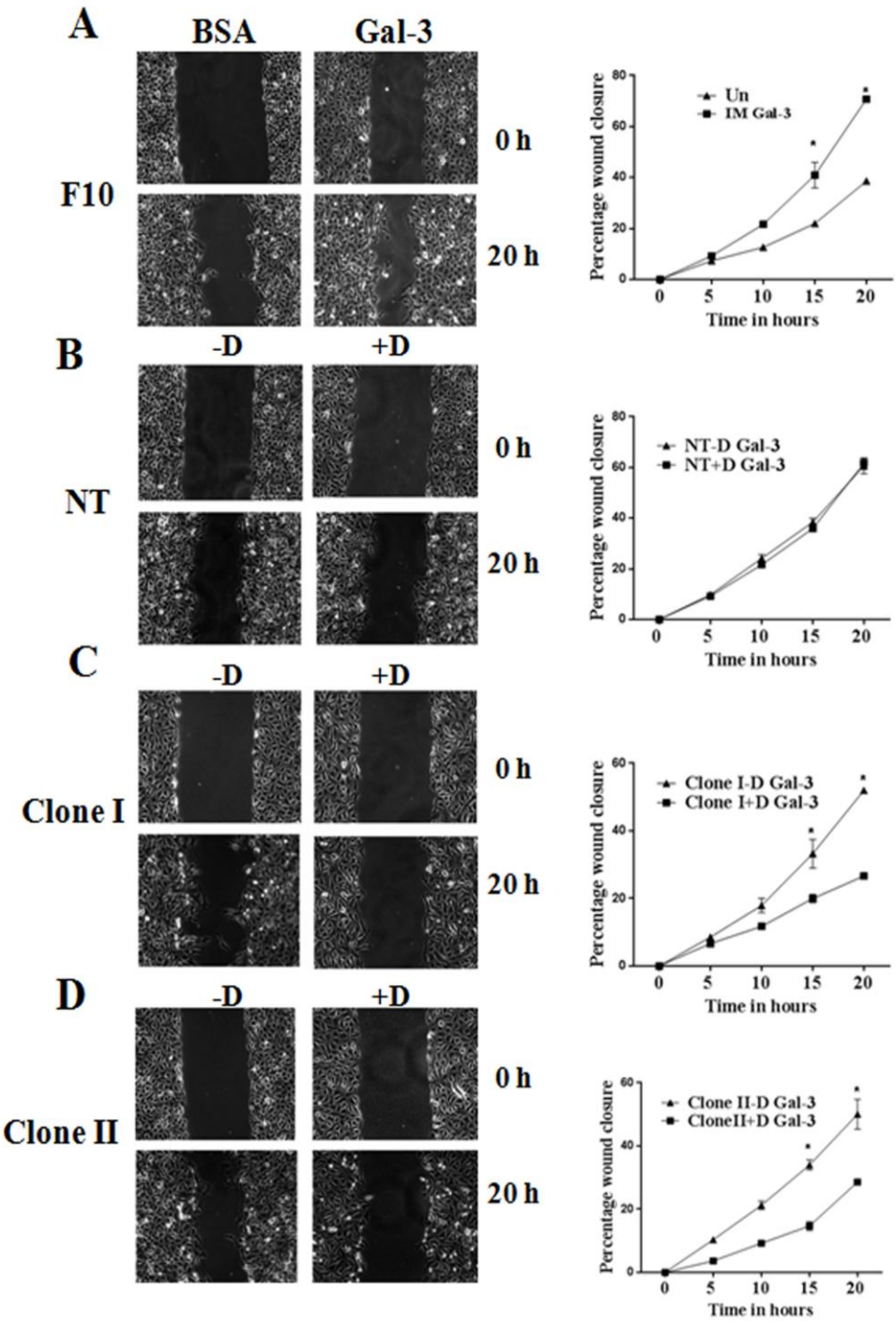


Figure 6: Reduction in expression of polyLacNAc results in reduced motility of GalT downregulated clones on galectin-3. (A) Represent time lapse video microscopy images at 0 and 20 h of wound closure of F10 cells grown on BSA or galectin-3 coated plates. (B) Represent time lapse images of NT, Clone I and II under doxycycline treated and untreated conditions. Adjacent right panel represents mean percent wound closure at 5 h interval. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. For comparison of wound closure between the groups two way ANOVA test with the Bonferroni posttest was conducted * indicates p value < 0.05 which was considered significant.

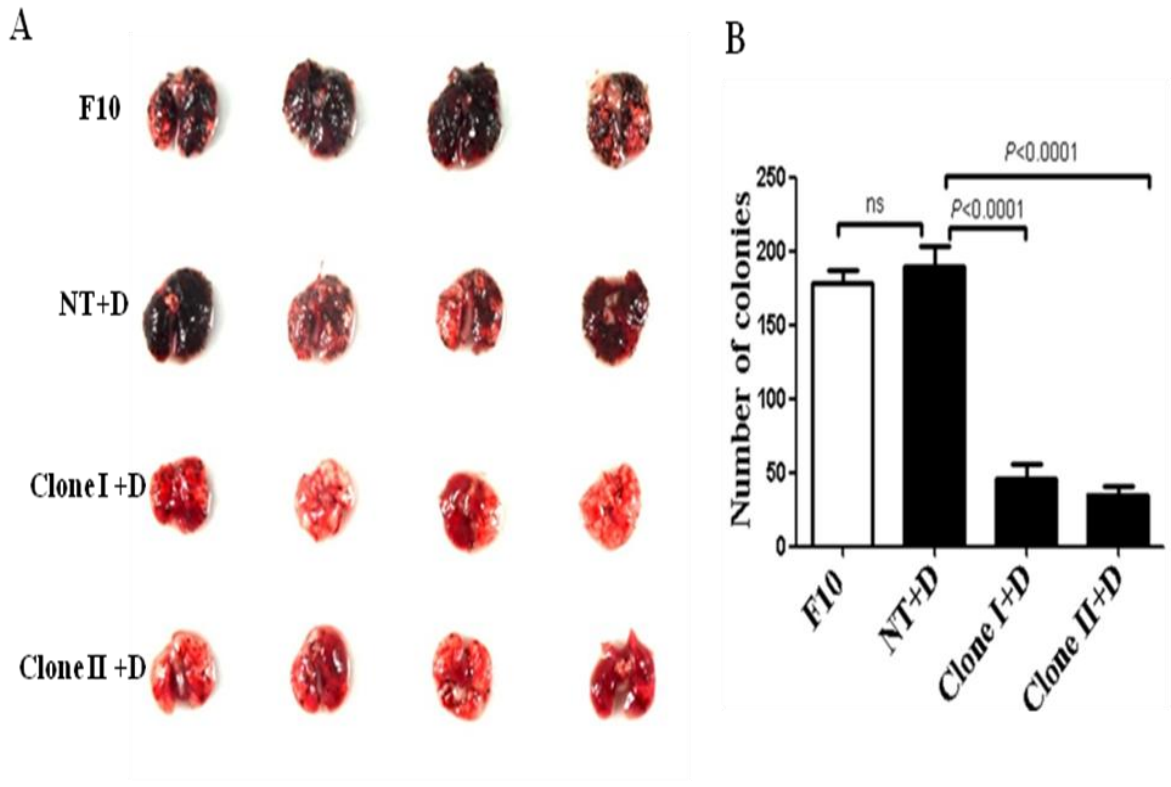


Figure 7: Reduced levels of polyLacNAc in GalT downregulated clones affect their metastastic potential. Experimental metastasis assay for NT and GalT downregulated clones I and II. Untreated F10 cells served as control. The left panel shows the lungs images while right panel is the graphical representation of the mean +/- standard error of mean number of lung colonies from three independent experiments. For statistical

analysis, unpaired students t-test was employed, mean numbers of colonies were compared between two groups and p value < 0.05 was considered significant.

The importance of polyLacNAc galectin-3 interaction in metastasis of melanoma cells has also been underscored by other experiments in the lab. For e.g. competitive inhibition of endogenous galectin-3 by incubating melanoma cells with truncated galectin-3 (containing only carbohydrate recognition domain of galectin-3) or feeding mice with modified citrus pectin (MCP) not only affected the galectin-3 mediated cellular processes but also affected metastasis (6).

Overall these findings strongly point towards the role of both galectin-3 on the target organ and polyLacNAc expressed on melanoma cells in organ homing. Further, it is plausible that galectin-3 binding/polyLacNAc carrying proteins would play a major role in mediating events associated with metastasis of melanoma cells. These proteins would act as upstream regulators to initiate the downstream signalling events which would then modulate metastasis associated processes. It is thus important to identify the galectin-3 binding proteins that carry polyLacNAc on N-glycans. Previously, only LAMP1 and β1 integrin on melanoma cells have been identified as polyLacNAc carriers, but other proteins carrying such modifications should be identified and their role in metastasis associated events should be addressed

Objective II: To identify the galectin-3 binding proteins carrying polyLacNAc on β1,6 branched N-oligosaccharides and to study their involvement in regulation of galectin-3 mediated processes.

For identification of glycoproteins, lectin affinity enrichment is the primary step. Lectins not only recognize carbohydrate structures but also differentiate them with respect to subtle variations in their composition. Sepharose conjugated lectins are generally used for enrichment of glycoproteins from cell or tissue lysates. Enriched proteins can then be identified using mass spectrometry (182,183).

Galectin-3 is a member of family of β-galactoside binding proteins. Through carbohydrate recognition domain (CRD), it recognizes galactose containing glycans with differential affinity. PolyLacNAc consisting of repeats of galactose and N-acetylglucosamine residues is the preferred ligand for galectin-3. Depending on the number of polyLacNAc units, its affinity for galectin-3 varies, longer units bind more strongly as compared to shorter ones (149) (Illustration 6A). PolyLacNAc expressed on both N- as well as O-glycans can serve as ligands for galectin-3 (184). Apart from recognizing polyLacNAc, galectin-3 also binds to ligands known as Thomsen Friedenreich (T/Tn) antigens with weak affinity (185).

Galectin-3 affinity chromatography can be used for purification of polyLacNAc carrying proteins, but considering the broader affinity of galectin-3, it would be practically difficult to selectively purify proteins carrying polyLacNAc only on β1,6 branched N-glycans using this approach. Alternatively, Leuco-Phyto haemagglutinin (L-PHA), a lectin which specifically recognizes β1,6 branched N-glycans (Illustration 6 B), also can be used for such purification. β1,6 branch on N-glycans is the preferred site for addition of terminal substitutions such as polyLacNAc, sialic acids, Lewis antigens and fucose residues. Since purification by this approach would also lead to copurification of other proteins which carry terminal substitutions apart from polyLacNAc, this method would also be

insufficient to selectively enrich proteins carrying polyLacNAc on β1,6 branched N-glycans. Therefore using the above two approaches in tandem appears to be the best possible strategy to purify such proteins.

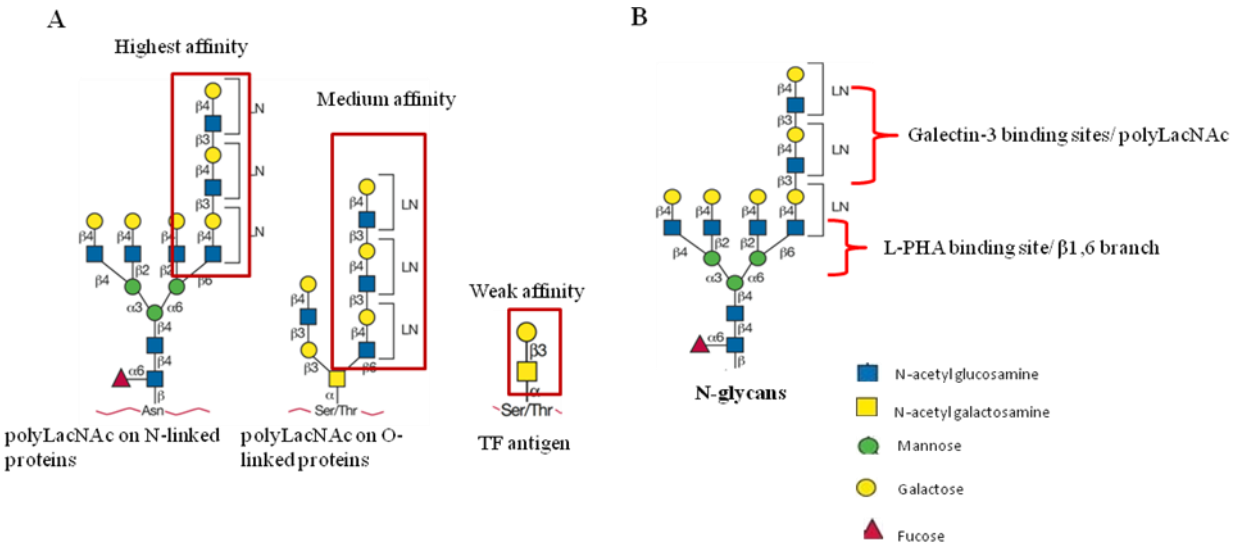


Illustration 6: (A) Galectin-3 specific ligands expressed on N- and O- glycans and (B) L-PHA and galectin-3 binding sites on β1,6 branched N- glycans modified from (186).

3.5 Strategy to purify and identify galectin-3 binding proteins carrying polyLacNAc substitutions on β1,6 branched N-oligosaccharides

Considering the above facts, a serial lectin affinity chromatography approach was employed. In the first step, galectin-3 binding proteins were purified and in the subsequent step, proteins carrying β1,6 branched N-glycans were enriched. Though this strategy had advantage of enriching proteins with glycan modifications of our interest, the total yield of proteins obtained after purification was insufficient for analysis by mass spectrometry. Hence, galectin-3 and L-PHA binding proteins were independently purified and subjected to mass spectrometry.

To enrich membrane associated glycoproteins, F10 cell lysates were prepared in buffer containing N-octyl β-D galactopyranoside (30 mM) which as a non ionic mild detergent

not only aided extraction of membrane proteins but was also compatible with lectin affinity purification. For glycoprotein enrichment, F10 melanoma cell lysate (25 mg) was incubated overnight with galectin-3 or L-PHA sepharose beads. Unbound protein fraction was collected and non specifically bound proteins were removed by repeated washes with Tris buffer containing 1 M NaCl. Proteins bound to galectin-3 and L-PHA sepharose beads were eluted with buffer containing either lactose (150 mM) or N-acetylgalactosamine (300 mM), respectively. After volume normalisation with input/original, 200 µg equivalent protein from eluted fractions was resolved by 1-D gel electrophoresis and protein profile was visualised by coomassie blue staining (Figure 9). Observed bands were cut and processed for sample preparation required for mass spectrometry analysis. The workflow in Figure 8 briefly summarises the overall methodology used for purification and identification of glycoproteins.

3.6 Deglycosylation of glycopeptides and identification using nano LC-ESI-Q-TOF MS/MS

Identification of glycoproteins is difficult due to several reasons including low abundance, high heterogeneity and poor ionisation of glycopeptides. Therefore, to avoid interference of glycans in glycoprotein identification, deglycosylation strategies are preferred. Enzymatic deglycosylation methods are widely used as compared to chemical deglycosylation as former are more selective and can preserve peptide backbone more efficiently than the later.

N-glycosidase F also called as PNGase F deglycosylates proteins or peptides by cleaving the glycosidic bond between Asparagine and innermost N-acetylglucosamine on hybrid, oligomannose and complex type N-glycans. However, it cannot cleave a subset of N-glycans modified with α1,3 fucose at the innermost N-acetylglucosamine (187). This modification is majorly found in plants and insects and therefore this would not serve as a

barrier in deglycosylation of proteins from mammalian sources (105). After PNGase F treatment, N-glycans are released and asparagine is converted to aspartic acid (Figure 8B) with shift in monoisotopic mass by +1 Da. This shift in the molecular mass is readily detected by mass spectrometry and is used for annotating N-glycosylation sites (8).

Earlier, an attempt was made to standardise deglycosylation conditions at protein level by in-gel as well as in-solution PNGase F treatment. We found that deglycosylation by in-solution PNGase F treatment was incomplete, probably due to the heterogeneity as well as complexity of the sample; also steric hindrance by large glycoproteins can restrict the access of PNGase F to N-glycosylation sites. In-gel deglycosylation methods also did not help in identification of proteins.

Therefore, protocol for deglycosylation at peptide level was standardised. Peptides generated after trypsin digestion were subjected to PNGase F treatment. Deglycosylated peptides were passed through C-18 columns and then subjected for analysis using nano LC-ESI-Q-TOF MS/MS by Synapt, Waters. Protein Lynx Global Server (PLGS) software was used for post acquisition analysis.

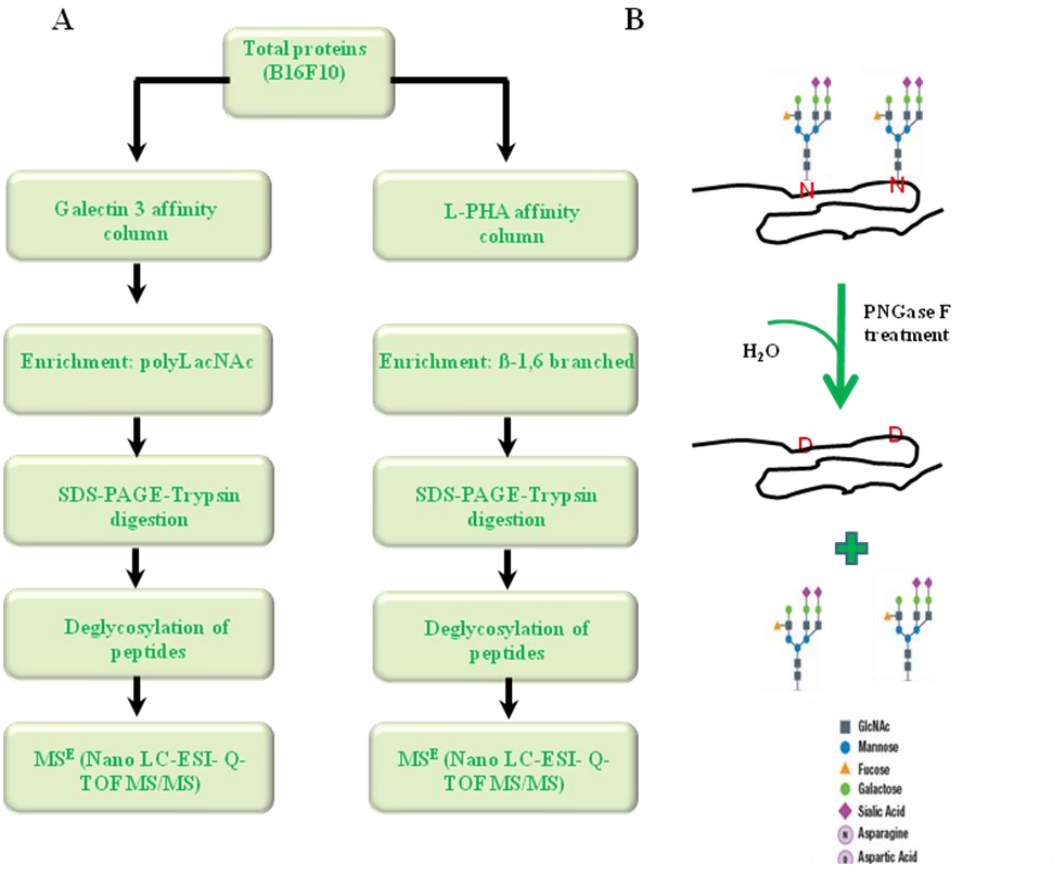


Figure 8: Workflow for purification of galectin-3 binding proteins and deglycosylation of peptides by PNGase F treatment. (A) Summary of steps involved in purification of galectin-3 and L-PHA binding proteins. (B) Illustration representing action of PNGase F on glycopeptides carrying N-glycans.

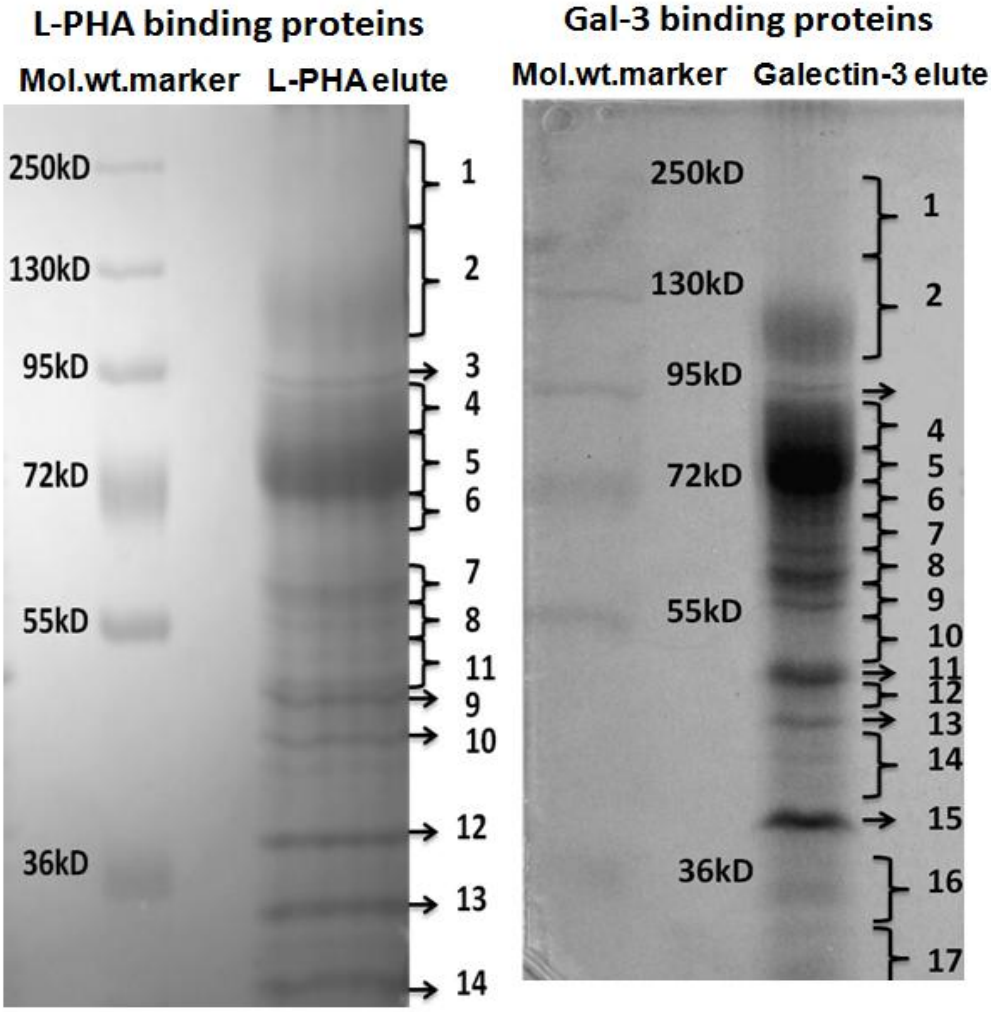


Figure 9: Coomassie blue staining of galectin-3 and L-PHA binding proteins resolved by SDS-PAGE.

Table 5 and 6 briefly summarises the results obtained by mass spectrometry analysis. Protein identities for all the processed bands were obtained with p value > 0.05. In total, 97 glycoproteins were identified, out of which 51 proteins were identified as polyLacNAc carrying proteins (galectin-3 binding proteins) and 79 proteins were identified as β1,6 branched N-glycans carrying proteins (L-PHA binding proteins). Representative fragmentation data for some of the annotated glycopeptides in Table 5 and 6 is shown in Figure 11 and 12.

After comparative analysis, 33 proteins were found to have affinity for both galectin-3 as well as L-PHA. Therefore, it is possible that these proteins might carry both polyLacNAc as well as β1,6 branched N-glycans. Some of the identified proteins such as integrins (α5 and β1) and LAMPs (LAMP1, -2 and -3) are reported to be substituted with β1,6 branched N-glycans.

Further, in many cases as shown in Table 7, even the annotated N-glycosylation sites for individual proteins were common which strongly point towards the possibility that these sites most likely carry polyLacNAc substituted β1,6 branched N-glycans (For e.g. LAMP1 identified by both the approaches shared 9 modified N-glycosylation sites). Basigin, a protein belonging to immunoglobin family, has been identified as L-PHA binding protein. Further, it has been recently reported that addition of β1,6 branch on basigin modulates its cell surface localization and function (188,189). Thus, Asn-160 on basigin which is reported to carry by N-glycans (190,191) and identified as a common glycosylation site in our study (Table 7) is highly likely to be modified with polyLacNAc substituted β1,6 branched N-glycans. Embigin, another member of immunoglobin family which has been reported to be N-glycosylated, was also identified and the reported glycosylation sites (N-55, 216 and 221) also matched with our data (190,191). We believe that even for rest of the identified proteins, it is highly likely that detected glycosylation sites could be modified with polyLacNAc on β1,6 branched N-glycans.

Table 5: Summary of identified galectin-3 binding proteins

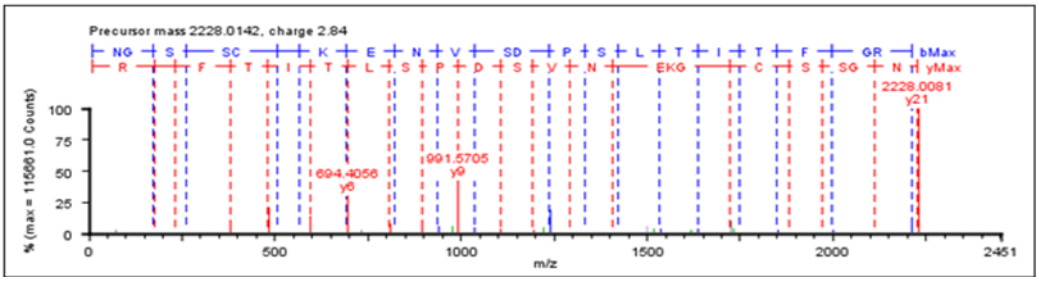
Protein name	Accession number	Molecular weight	Sequence Coverage (%)	Number of glycosylated peptides	Amino acids modified
Integrin alpha 5	P43406	114971	15.57	3	472,581,715, 727, 185, 678
Integrin beta 1	P09055	88173	19.17	3	212, 571, 669
Integrin alpha V	P43406	115287	22.50	4	615, 447, 362, 74
LAMP1	P11438	43837	47	20	70, 78, 97, 101, 115, 159, 177, 252, 296, 311
LAMP2	P17047	45652	47	9	115, 322, 156, 175, 265, 312, 317, 361
LAMP3	P41731	25749	29.83	1	130
Embigin	P21995	37040	33	6	55, 216, 221, 253
Basigin	P18572	42418	33.41	1	160,297,309
5,6 dihydroxy indole -2 carboxylic acid	P07147	60722	59	5	132, 256, 280, 304, 350, 419, 508
L-dopachrome tautomerase	P29812	58473	50	6	230, 300, 342,
MUC 18	Q8R2Y2	71500	41.2	6	167,583,304
Nicastrin	P57716	78442	36.58	2	44, 434, 529,530
Transmembrane NMB	Q99P91	63635	18	9	94, 134, 197, 200, 246, 249, 275
Cell adhesion molecule 1	Q8R5M8	49757	27.85	2	104,116

Figure 10: Representation of glycopeptide fragmentation pattern of identified galectin-3 binding proteins

Lysosome associated membrane protein-1 (LAMP1)

70 78

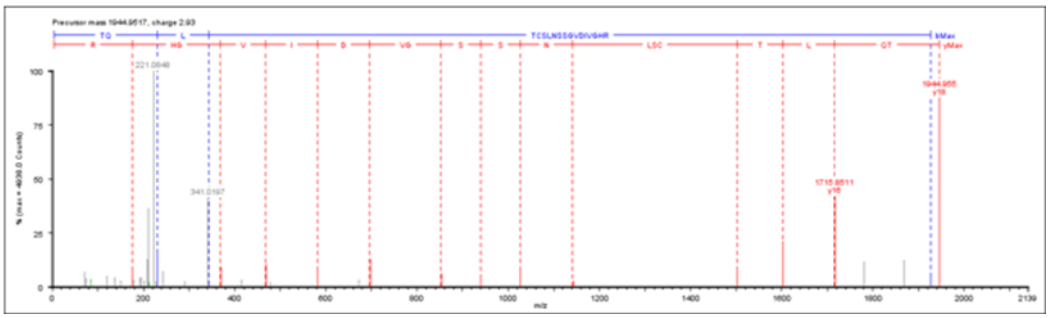
NGSSCGKENVSDPSLTITFGR



Basigin

153 160 170

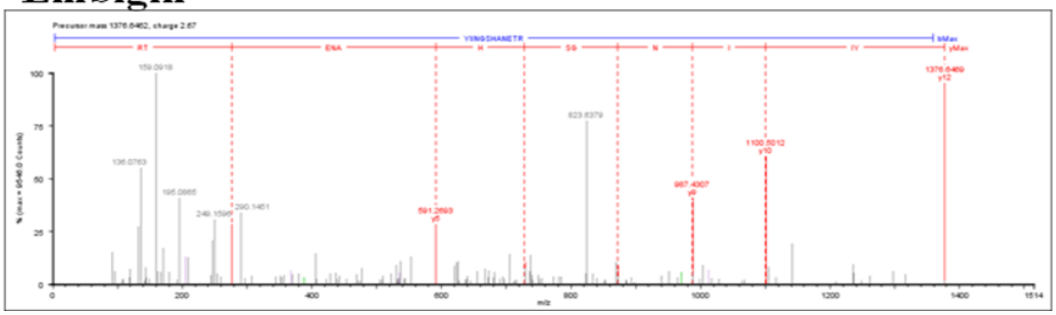
TQLTCSLNSSGVDIVGHR



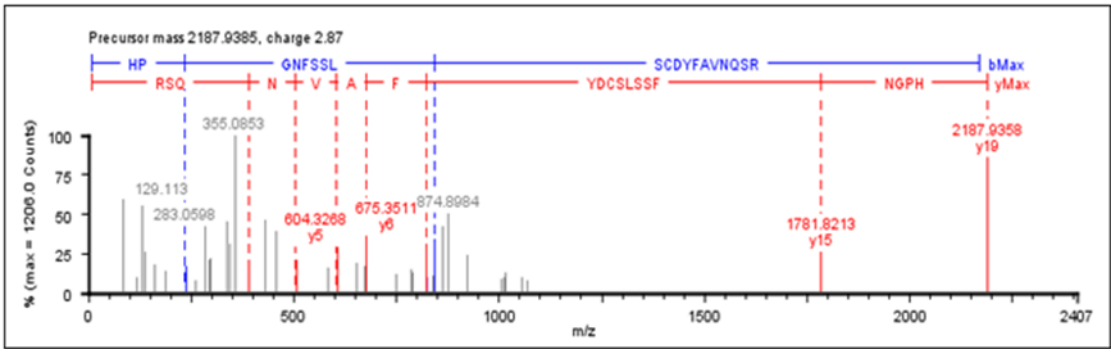
Embigin

216 221

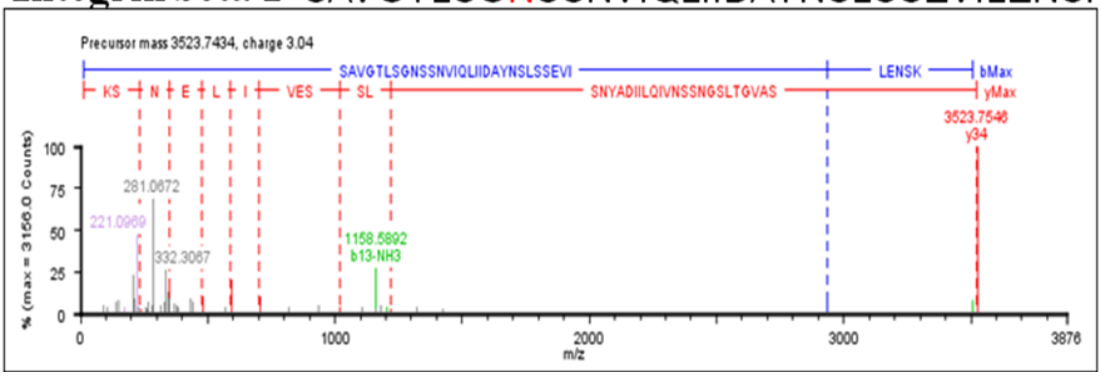
YIINGSHANETR



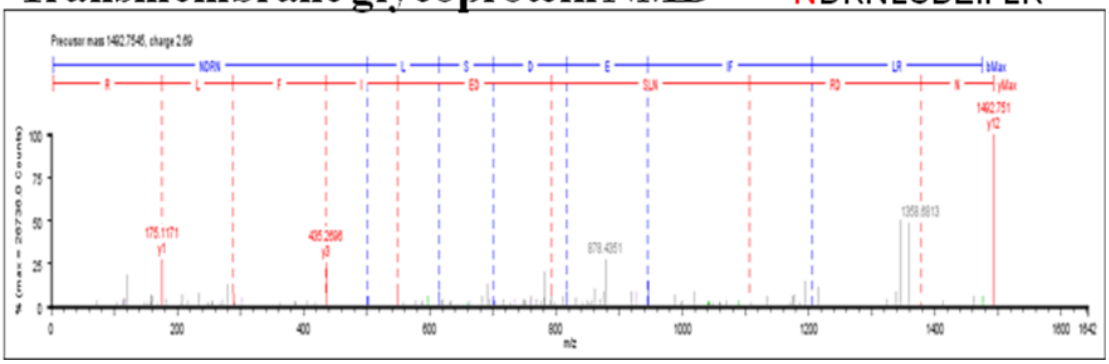
Integrin alpha 5 715 HPGNFSSLSCDYFAVNQSR 727



Integrin beta 1 363 SAVGTLSGNSSNVIQLIIDAYNSLSSEVILENSK



Transmembrane glycoprotein NMB 246 NDRNLSDEIFLR



Sulfated glycoprotein-1 333 FSELIVNNATEELLVK

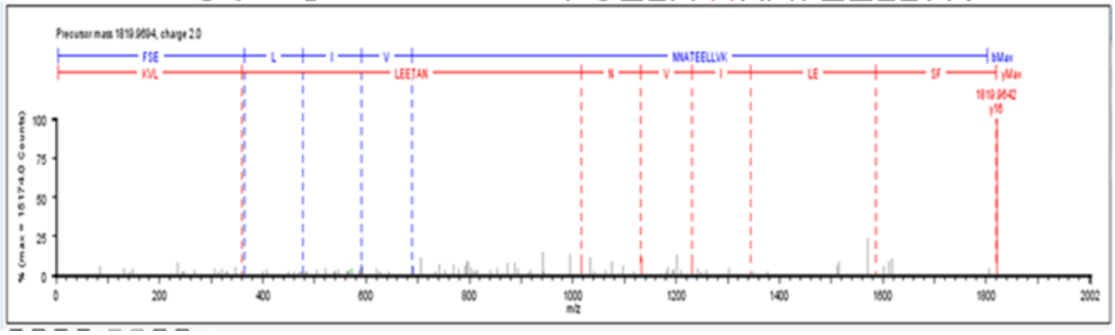
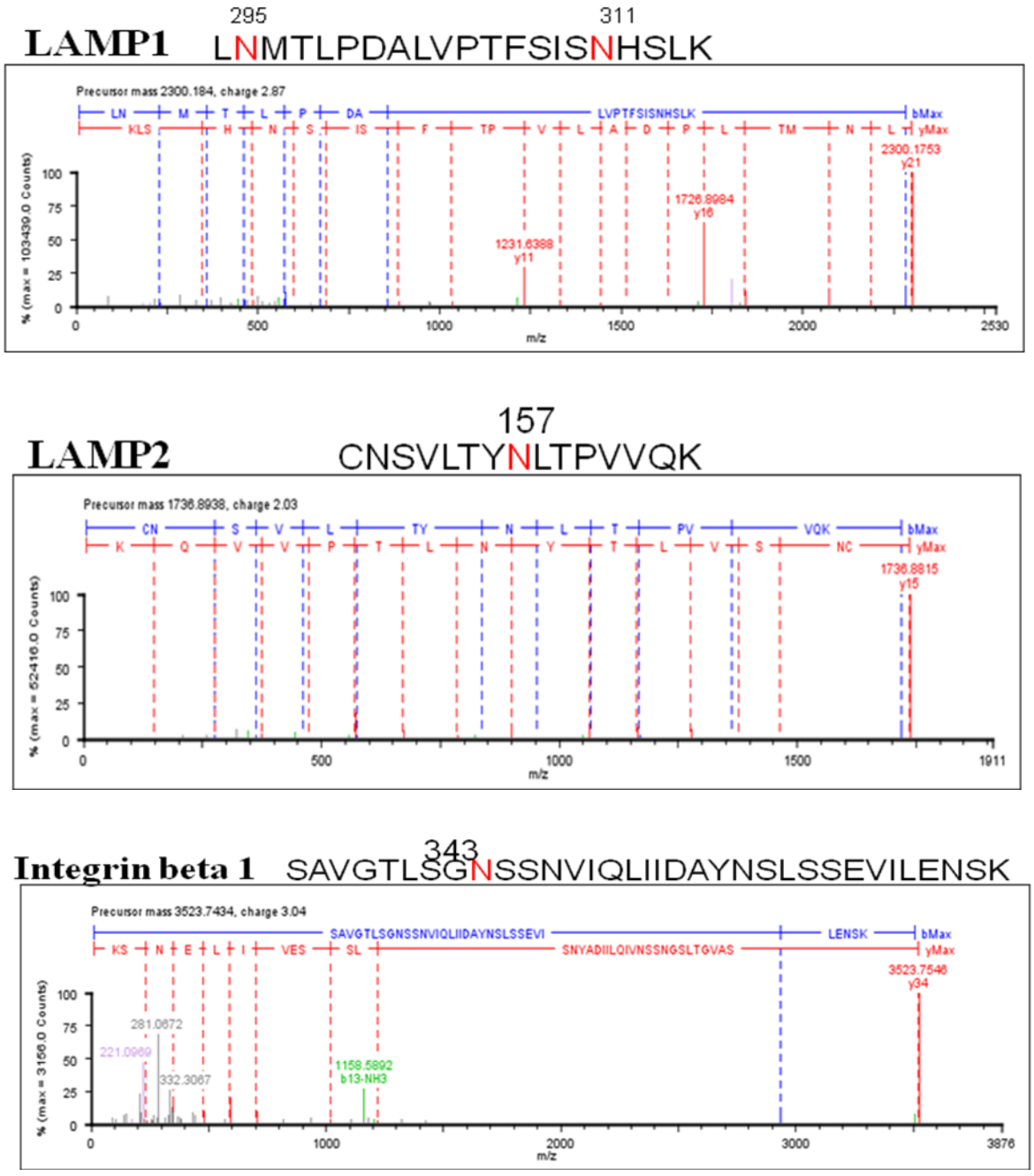


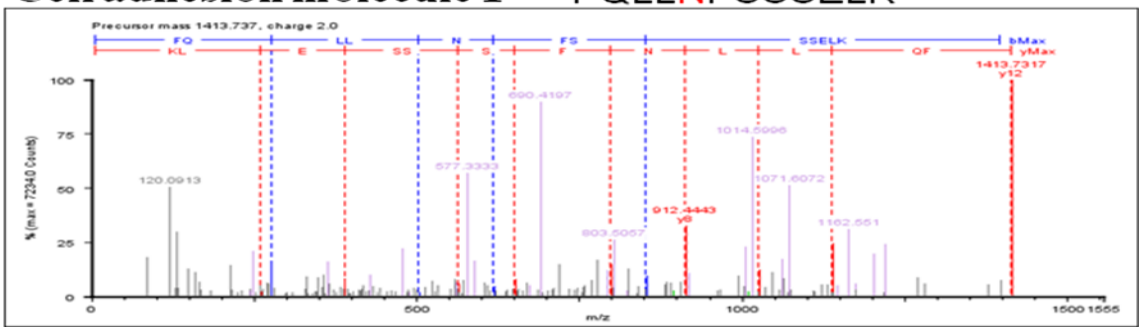
Table 6: Summary of identified L-PHA binding proteins

Protein name	Accession number	Molecular weight	Sequence Coverage (%)	Number. of glycosylated peptides	Amino acids modified
Integrin alpha 5	P43406	114971	18.13	2	715,727
Integrin beta 1	P09055	88173	33.7	1	363
LAMP1	P11438	43837	48	22	70,78,97,101,159, 177, 252,296, 311
LAMP3	P41731	25749	29.83	3	130, 143, 172
LAMP2	P17047	45652	42.7	7	115, 161, 265, 322, 156, 361
Cell surface glycoprotein MUC18	Q8R2Y2	71500	42.3	3	58
Nicastrin	P57716	78442	40.4	5	529, 530, 611, 434
Transmembrane glycoprotein NMB	Q99P91	63635	37.8	5	200, 246, 249, 275
Cell adhesion molecule 1	Q8R5M8	49757	17.1	2	104,116
Basigin	P18572	42418	36.8	3	160,275,297
Embigin	P21995	37040	34.5	8	55, 118, 216, 221, 270
5, 6 dihydroxyindole 2 carboxylic acid	P07147	60722	62.2	6	175, 181, 242, 256, 304, 350,
L dopachrome tautomerase	P29812	58473	50.0967	6	342, 230, 300, 246
Tyrosine protein phosphatase non receptor type substrate 1	P97797	56389	15.6	3	246, 312, 320
4F2 cell surface antigen	P10852	51578	37	1	259
Sulfated glycoprotein -1	Q61207	61381	26.3914	3	214

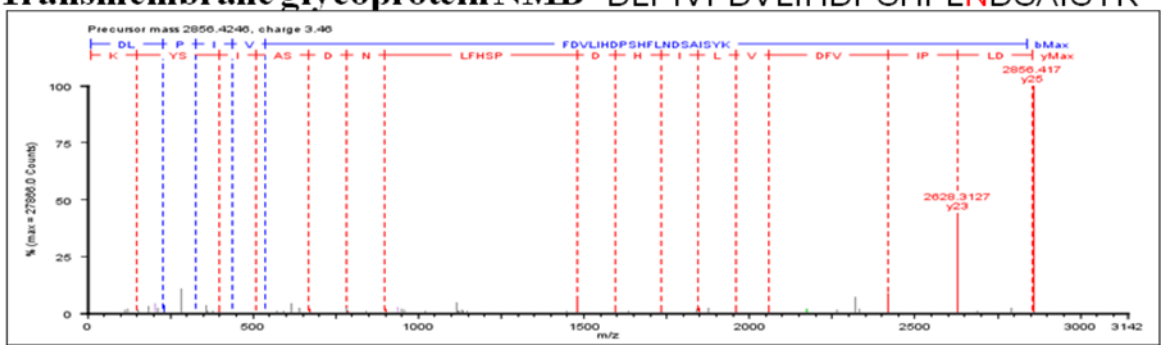
Figure 11: Representation of glycopeptide fragmentation pattern of identified L-PHA binding proteins



Cell adhesion molecule 1 114 FQLLNFSSSELK



Transmembrane glycoprotein NMB 275 DLPIVFDVLIHDPSHFLNDSAISYK



Cell surface glycoprotein MUC18 58 ASGNFSQVDWFLIHK

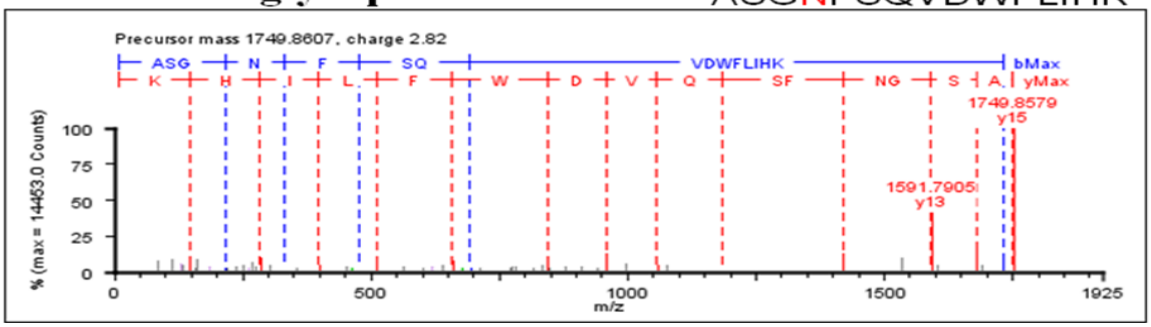


Table 7: Comparison of modified N-glycosylation sites of identified proteins

Glycoproteins carrying β1,6 branched N-glycans and polyLacNAc	**N-glycosylation sites modified only with polyLacNAc (blue) or β1,6 branched N-glycans (green) or both (red)**
Integrin β1	**212, 571, 669, 363**
Integrin α5	**472, 581, 185, 678, 715, 727**
Basigin	**160, 270, 275, 297,309**
Embigin	**55, 118, 216, 221, 253,270**
Cell adhesion molecule 1	**104,116**
LAMP1	**70, 78, 97, 101, 159, 177, 252, 296, 311**
LAMP2	**115, 156, 161,175, 265, 312, 322, 361**
LAMP3	**130, 143, 172**
Transmembrane protein NMB	**94,134, 197, 200, 246, 249, 275**
L dopachrome tautomerase	**230, 237, 242, 246, 300, 342**
5, 6 dihydroxyindole 2 carboxylic acid	**132, 175, 181, 256, 280, 304, 350**
MUC 18	**58, 167, 304**
Nicastrin	**44, 434, 529, 530, 461, 611**

3.7 Validation of identified proteins by immunoblotting and their functional involvement in one of the galectin-3 mediated processes

Identities of some of the proteins including LAMP1, LAMP3, α5 and β1 integrin, Basigin and Embigin were confirmed by immunoblotting. Galecctin-3 affinity enriched fraction indeed showed presence of these proteins. Proteins from F10 lysate probed with respective antibodies served as positive control (Figure 13).

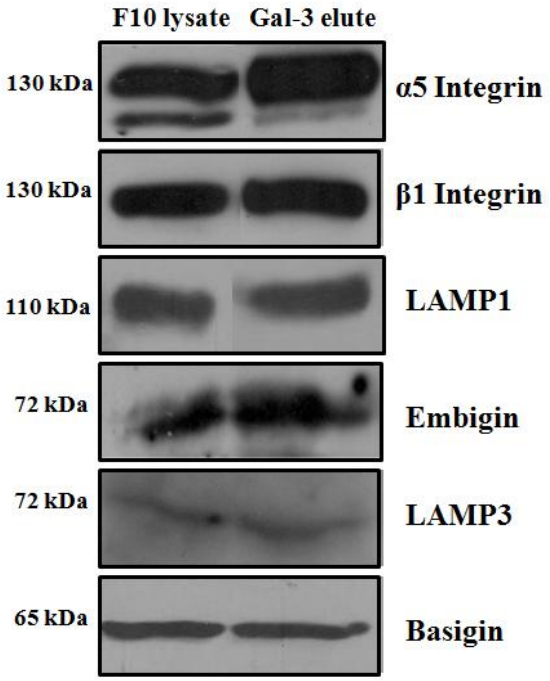


Figure 12: Validation of identified galectin-3 binding proteins using immuno blotting

Detection of integrins (α5 and β1), LAMPs (LAMP1 and 3), Basigin and Embigin in F10 cell lysate (100 µg) and galectin-3 enriched fraction (100 µg equivalent protein) using western blotting.

Earlier, LAMP1 and β1 integrin were identified as galectin-3 binding proteins and it was also shown that blocking these proteins using specific antibodies, affected galectin-3 mediated adhesion of melanoma cells. Here, using a similar strategy, we have looked at the effect of blocking galectin-3 binding proteins on cell spreading which is another event associated with metastasis. Apart from using antibodies to block LAMP1 and β1 integrin,

we have used antibody against another protein, basigin, which has been reported to be associated with galectin-3 and integrins at the cell surface. Blocking LAMP1 and β1 integrin affected formation of typical membrane protrusions which were seen when cells were incubated with coverslips coated with galectin-3. However, quantitatively the maximum inhibition was seen in cells treated with basigin antibody (Figure 13A and B). Cells treated with non specific Rat IgG antibody served as a control.

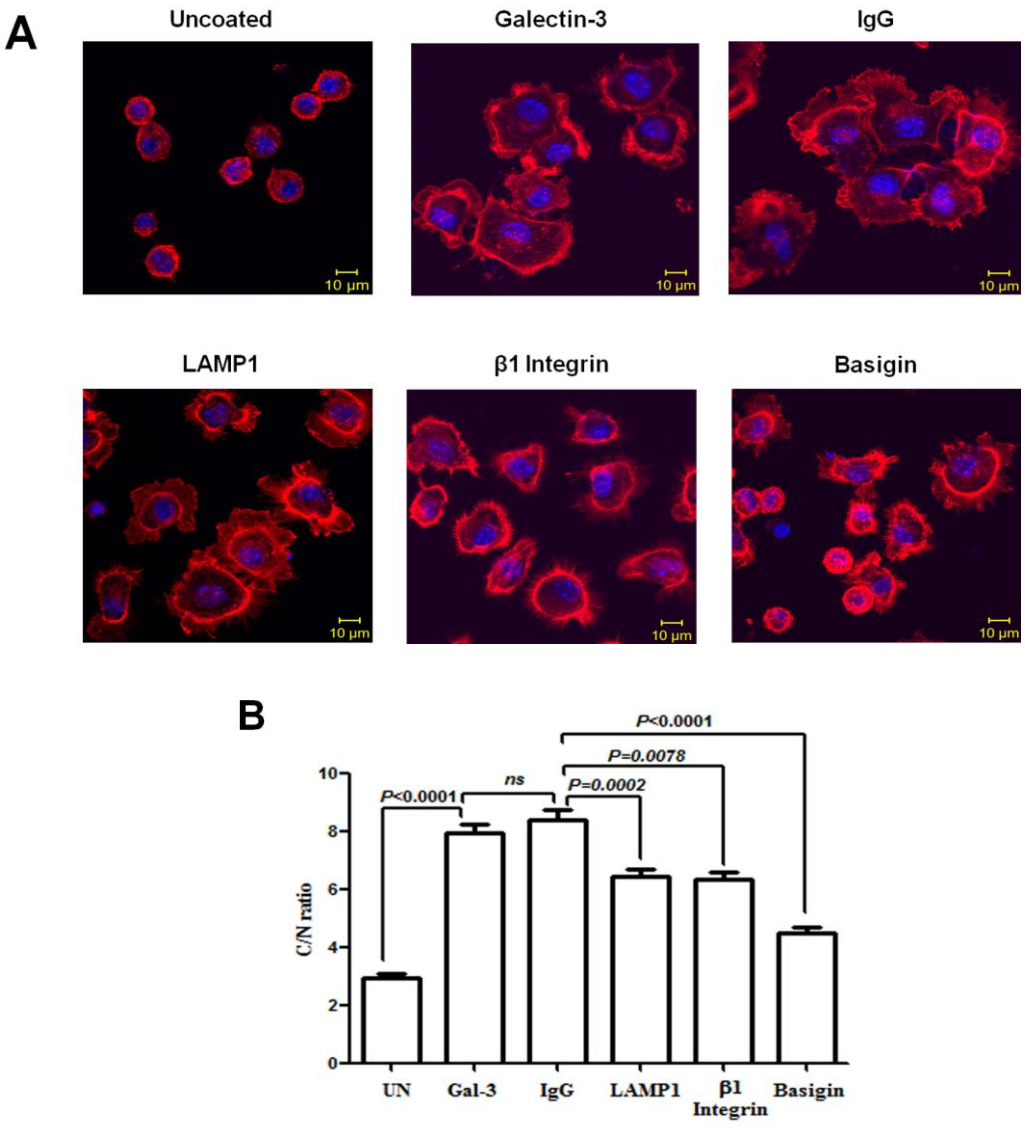


Figure 13: Role of galectin-3 binding proteins in cell spreading of melanoma cells on galectin-3 (A) Cell spreading assay of F10 cells incubated on galectin-3 coated coverslips in presence of antibodies to LAMP1, β1 integrin and Basigin. Cells incubated with Rat

IgG served as control. All antibodies were used at 10 μg/ml concentration. (B) Quantitation of cell spreading results by calculating the ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments. Unpaired students t-test was employed for statistical analysis and p value < 0.05 was considered significant

In summary, we have successfully identified and validated galectin-3 binding proteins which carry polyLacNAc on β1,6 branched N-glycans. Inhibition of some of the identified proteins including LAMP1, β1 Integrin and Basigin affected galectin-3 mediated cell spreading, which is an important event in extravasation of tumor cells. The role of these proteins in regulating galectin-3 mediated processes should be studied at cellular signalling level which would be the final confirmation for function of these proteins in metastatic events.

Objective III: To elucidate the downstream signalling events of galectin-3 induced MMP9 secretion and involvement of identified proteins in this process.

Galectin-3 is a nucleo-cytoplasmic protein that also gets secreted in a non-classical manner. The secreted galectin-3 can get associated with the cell surface or can become a part of the ECM and BM. Earlier; we have shown that both forms of extracellular galectin-3 i.e. soluble and immobilized galectin-3 induce MMP9 secretion. Induction of MMP9 expression was a dose dependent effect, maximum induction observed when F10 melanoma cells were grown in presence of 50 µg/ml of immobilized galectin-3. Cells grown on plastic alone served as negative control while cells grown on immobilized fibronectin served as positive control (Figure 14).

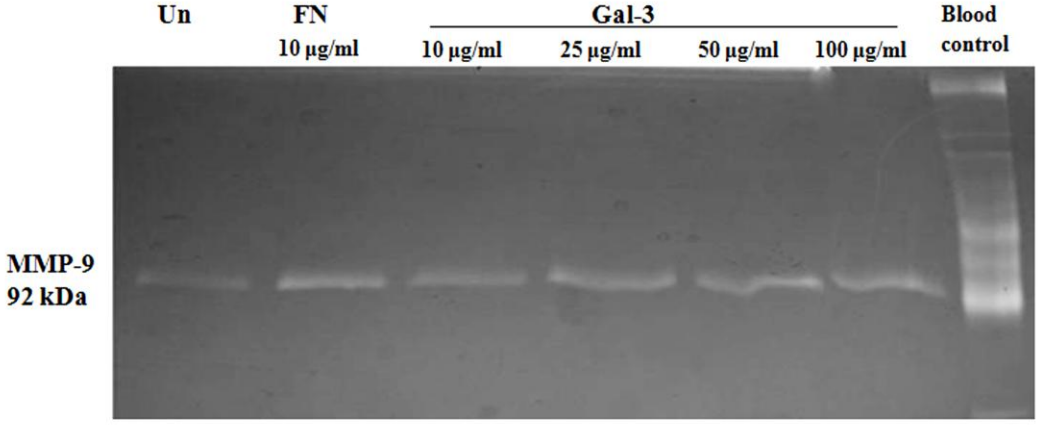


Figure 14: **Zymography gel showing levels of MMP9 in culture supernatants of F10 cells grown in presence of different concentrations of immobilized galetin-3 (10-50 µg/ml)**. Mice blood mixed with 1X non reduced sample buffer served as control for gelatin gel.

Though it is known that intracellular galectin-3 can regulate the expression of MMP9, role of extracellular galectin-3 in regulating MMP9 expression has not been explored. Thus

exact mechanism of galectin-3 induced MMP9 secretion including the role of galetin-3 binding proteins and downstream signalling pathways was explored.

Matrix metalloproteinases (MMPs), especially gelatinases, MMP2 and MMP9 are the key molecules involved in cancer cell invasion and tissue remodelling. MMPs are tightly regulated at multiple levels as it may impact tissue integrity. Multiple mechanisms have evolved to regulate the action of MMPs. 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 (192). 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 (193).

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

The levels of transcripts for MMP9 in cells grown on uncoated plastic plates were compared to those grown on fibronectin or galectin-3 coated plates by real time PCR. Cells grown on immobilized galectin-3 expressed significantly increased levels of MMP9 transcripts as compared to cells grown on plastic alone. Fibronectin, a well studied ECM protein and a known inducer of MMP9 secretion (9,194), served as a positive control (Figure 15A). These results suggest that galectin-3 in extracellular form can indeed induce the transcription of MMP9.

ECM proteins regulate the cellular signalling pathways involved in matrix remodelling by interacting with their receptors on the cell surface to initiate outside in signalling and *vice versa* (195). 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 either ERK, PI3K or NF-κB pathways. To understand which downstream pathways are activated by extracellular galectin-3, inhibitors for PI3K and MAPK (ERK, p38 MAPK and JNK) pathways were used.

Comparison of transcript levels of MMP9 in cells treated with different signalling inhibitors showed that inhibition p38 MAPK pathway had major effect on transcript levels of MMP9 (Figure 15B). Cells grown on galectin-3 coated plates treated with vehicle alone served as control. These results were further confirmed upon evaluation of the conditioned medium collected from cells grown on galectin-3 coated plates in absence or presence of different signalling inhibitors for the presence of MMP9. Both zymography and immunoblotting confirmed that in comparison to cells grown in presence of ERK, PI3K and JNK inhibitors, cells treated with p38 MAPK inhibitor (SB) showed maximum reduction in MMP9 levels (Figure 16).

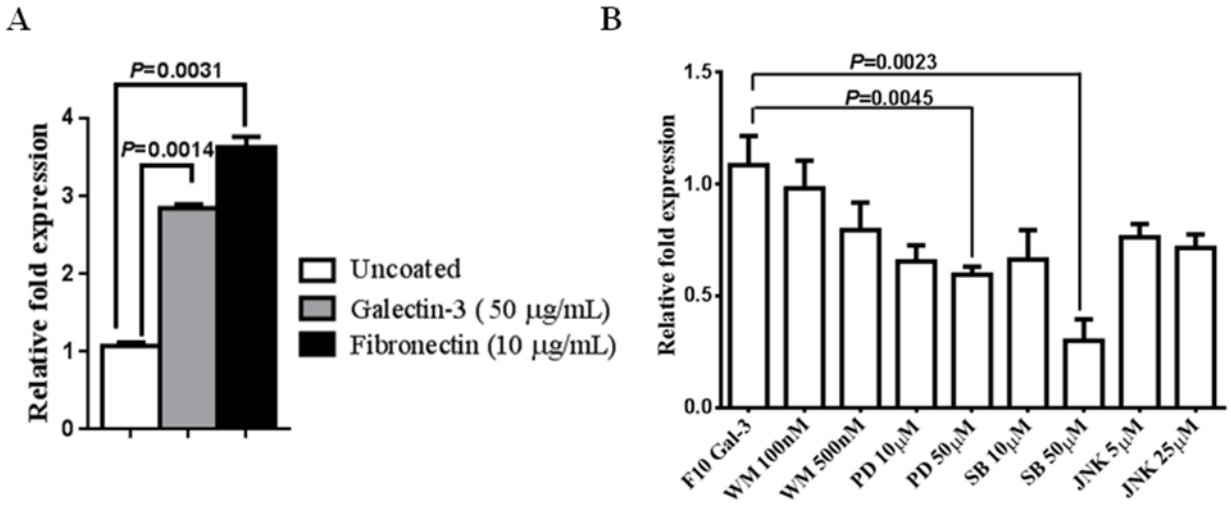


Figure 15: Immobilized galectin-3 induces expression of MMP9 at transcript level and inhibition of p38 MAPK affects induction of MMP9 expression.

(A) Bar graph represents comparison of levels of MMP9 transcripts by real time PCR in F10 cells grown on immobilized galectin-3 or on plastic alone. Error bars indicate standard errors of mean from three independent experiments performed in duplicates. (B)

Detection of MMP9 transcript levels by real time PCR in F10 cells grown on immobilized galectin-3 in presence of signalling inhibitors, PI3K (Wortmannin-WM, 100 nM and 500 nM), ERK (PD, 10 µM and 50 µM), p38 MAPK (SB, 10 µM and 50 µM) and JNK (5 µM and 25 µM). Cells grown on immobilized galectin-3 in presence of vehicle served as control. For statistical analysis, unpaired student's t-test was employed and p value < 0.05 was considered significant.

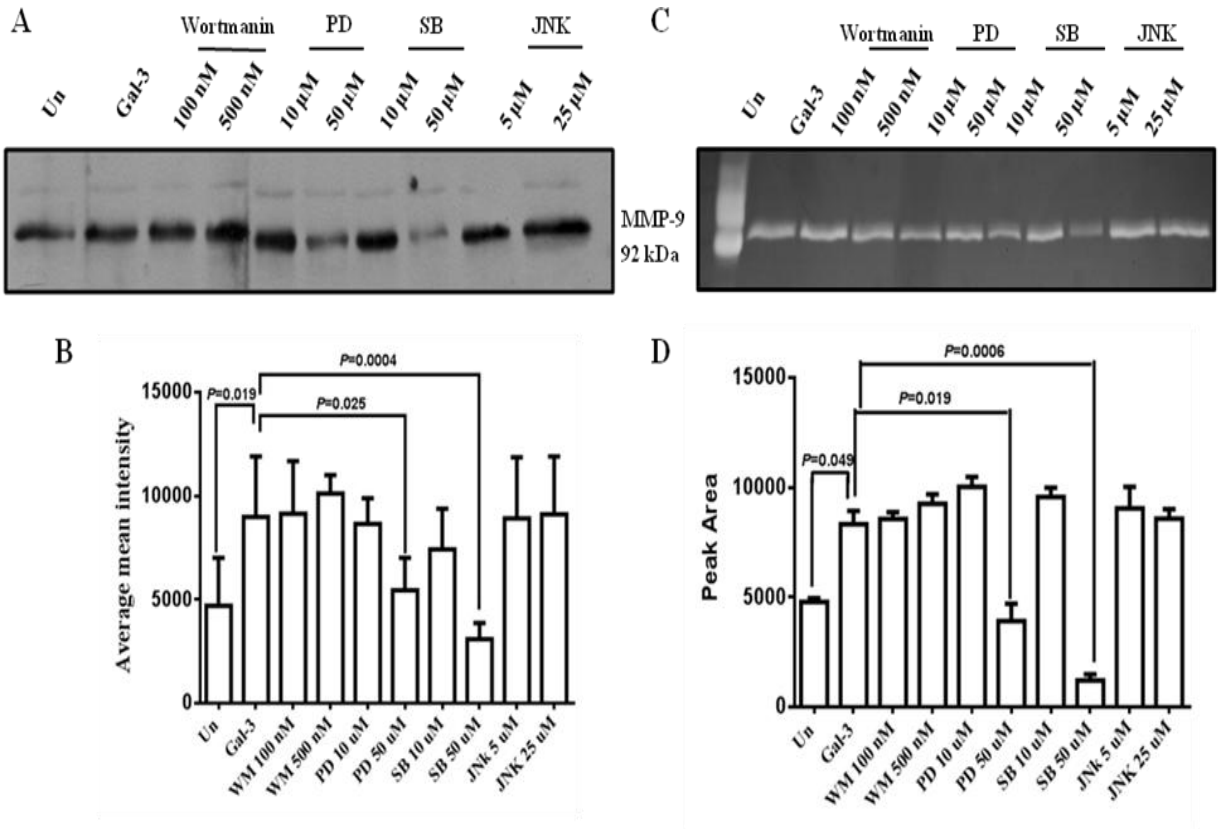


Figure 16: Inhibition of p38 MAPK affects galectin-3 mediated MMP9 secretion at protein levels.

(A) Western blotting of culture supernatants collected from F10 cells grown in presence of signalling inhibitors probed with anti MMP9 antibody. (B) Densitometric analysis of bands in (A) by Image J analysis. Each bar represents intensity of bands in respective lanes and error bars represent standard errors of mean from three different experiments.

(C) Zymography of culture supernatants collected from F10 cells grown in presence of signalling inhibitors. (D) Densitometric analysis of bands in (C) by Image J software using Each bar represents intensity of bands in respective lanes and error bars represent standard errors of mean from three different experiments. Unpaired student's t-test was employed for statistical analysis and p value < 0.05 was considered significant.

3.9 Downregulation of polyLacNAc affects galectin-3 induced MMP9 secretion through p38 MAPK pathway

Down regulating the enzymes involved in polyLacNAc synthesis not only affected binding of galectin-3 (Figure 2C) but also affected galectin-3 induced MMP9 induction as assessed by gelatin zymography and immunoblotting (Figure 5). Since galectin-3 induces MMP9 at transcript level (Figure 16A) therefore it was important to understand if high affinity galectin-3 ligand, polyLacNAc is involved in regulating MMP9 expression at transcript level.

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 (Figure 2). Induction of GalT shRNA lead to reduction in levels of MMP9 transcripts which clearly suggests that polyLacNAc is indeed involved in mediating induction of MMP9 at mRNA level (Figure 17A).

If inhibition of p38 MAPK pathway affected MMP9 induction (Figure 17), then it would be interesting to investigate if the activation of the same pathway is hampered in polyLacNAc downregulated clones. Cell lysates of these clones (untreated or treated with doxycycline for inducing shRNA expression) were probed with phospho-specific antibody for p38 MAPK. Lysates probed with antibody for detecting total levels of p38 MAPK served as loading control. Both the clones upon doxycycline induction showed

significantly reduced levels of phospho-p38 MAPK (Figure 17B and C), suggesting that polyLacNAc also signals galectin-3 mediated processes via this pathway.

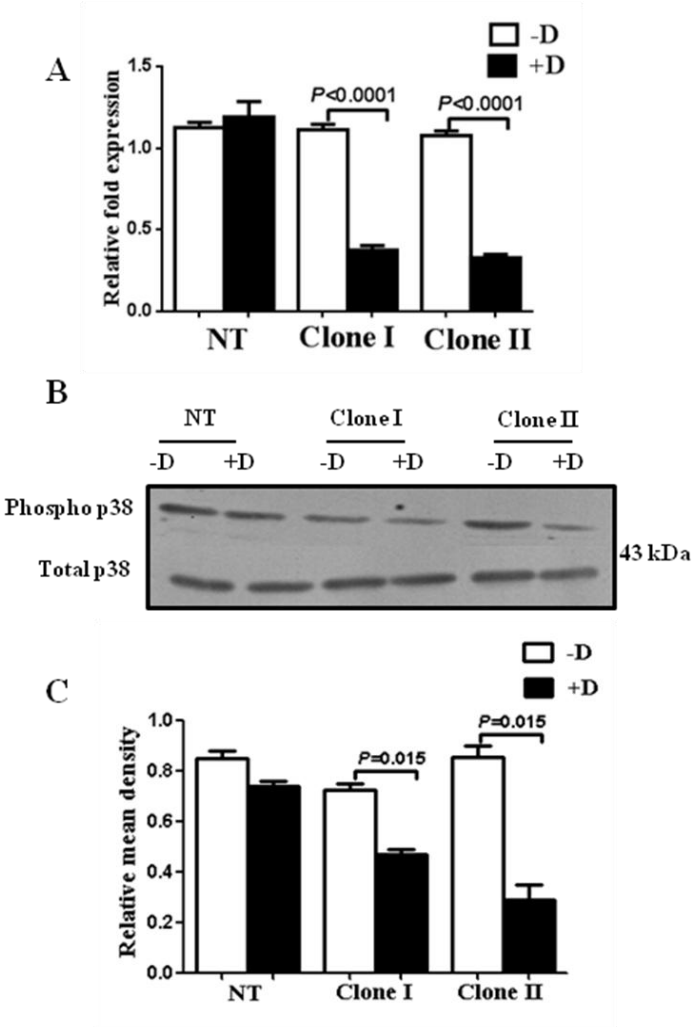


Figure 17: Downregulation of polyLacNAc affects MMP9 induction via activation of p38 MAPK pathway (A) Bar graph represents analysis of MMP9 transcript levels in polyLacNAc downregulated clones I and II grown on immobilized galectin-3 in doxycycline treated and untreated conditions (-D and +D respectively). Non targeting clones in presence or absence of doxycycline (NT-D and NT+D) served as controls served as vector controls. Error bars indicate standard errors of mean from three independent experiments performed in duplicates. (B) Western blotting for detection of phospho-specific forms of p38 in NT, clone I and clone II (-D and +D) grown on immobilized galectin-3. Total levels of p38 served as loading control. (C) Densitometric analysis of

bands in (B) by Image J software. Each bar represents ratio of intensity of phospho-p38 to total p38 levels in respective lanes and error bars represent standard errors of mean from three different experiments. Unpaired student's t-test was employed for statistical analysis and p value <0.05 is considered to be statistically significant.

3.10 LAMP1 identified as a major carrier of polyLacNAc can regulate galectin-3 induced MMP9 secretion

LAMP1 and β1 integrin are among the major proteins identified to carry polyLacNAc substituted β1,6 branched N-oligosaccharides on these melanoma cells. LAMP1 is a highly glycosylated protein that lines the lysosomes (196). More than 60% of its weight is contributed by carbohydrates and each molecule carries about 17-20 N-glycans that are highly substituted (196,197). In metastastic cells, LAMP1 is known to get translocated to the cell surface. 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. In addition, glycosylation in these cells has been shown to modulate the surface expression of LAMP1(198). LAMP1 is reportedly a known ligand for galectin-3 (199,200). 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 (198,201). It is thus possible that LAMP1 can be involved in galectin-3 mediated signalling that induces MMP9 transcription and secretion.

To investigate the role of LAMP1 in MMP9 expression, melanoma cells expressing shRNAs for LAMP1 were used. Real time PCR results show that downregulation of LAMP1 in F10 cells (Sh1 and Sh2) severely affects galectin-3 induced transcription of MMP9 (Figure 18A). This is further reflected in significantly reduced secretion of MMP9 by LAMP1 downregulated clones grown on galectin-3, as analyzed by both

immunoblotting and zymography (Figure 18B-D) suggesting that LAMP1 can be an upstream regulator in MMP9 induction. This was corroborated by decreased levels of activated (phospho) p38 MAPK in the lysates of LAMP1 downregulated clones (Figure 19E and F).

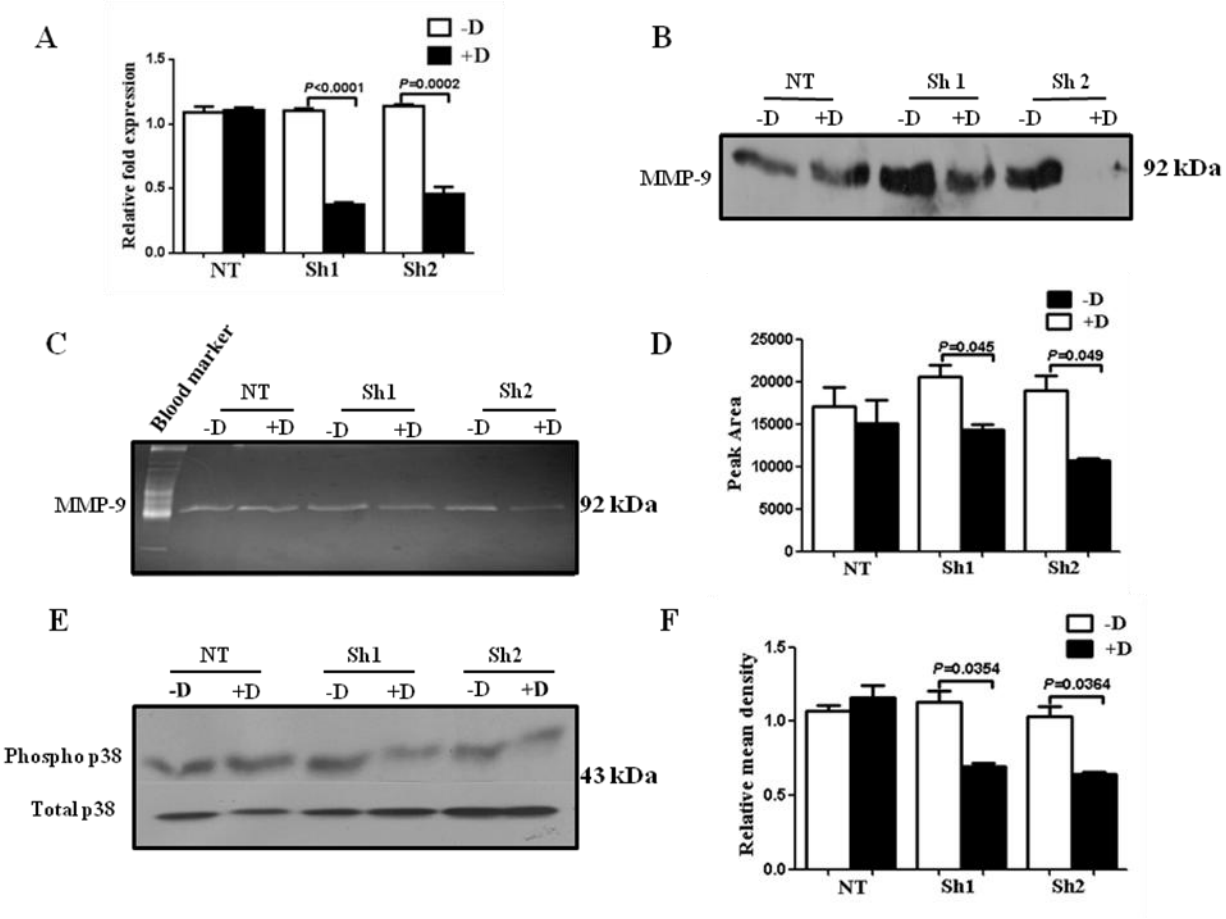


Figure 18: Downregulation of LAMP1 affects galectin-3 induced MMP9 expression via p38 MAPK pathway. (A) Bar graph represents analysis of levels of MMP9 transcripts by real time PCR for NT and LAMP1 downregulated clones Sh1 and Sh2 (-D and +D). Error bars indicate standard errors of mean from three independent experiments performed in duplicates. (B) Western blotted culture supernatants of NT, Sh1 and Sh2 clones probed with anti-MMP9 antibody. (C) Levels of MMP9 in culture supernatants of NT, Sh1 and Sh2 cells grown in presence and absence of doxycycline on immobilized

galectin-3 as detected by zymography. (D) Densitometric analysis of bands in (C) by Image J software. Each bar represents intensity of bands in respective lanes and error bars represent standard errors of mean from three different experiments. (E) Detection of phospho-specific forms of p38 in NT, Sh1 and Sh2 clones (-D and +D) grown on immobilized galectin-3. (F) Densitometric analysis of bands in (E) by Image J software. Each bar represents ratio of intensity of phospho-p38 to total p38 levels in respective lanes and error bars represent standard errors of mean from three different experiments. Unpaired student's t-test was employed for statistical analysis and p value <0.05 is considered to be statistically significant.

CHAPTER 4

DISCUSSION

4. Discussion

Metastasis is the major cause of cancer related deaths. Despite recent technological advances in science, the molecular mechanism behind metastasis is still not completely understood. Metastasis cascade involves multiple events which tumor cells should effectively bypass for establishing a metastastic colony. To metastasize, tumor cells must break free from the primary site, create space for their movement, get into and survive in circulation. Once in circulation, they are able to reach almost all organ sites (1). However, some metastasize in the anatomic vicinity, while others bypass several organs and colonize very specific organ sites (34,59). The patterns of circulation and mechanical factors appear to dictate the regional spread. However, organ specific metastasis is believed to be facilitated by specific adhesive interactions between the molecules on the tumor cells and the target organ, growth environment and chemotactic factors released from the target organ (202,203). Tumor cells show several metastasis associated modifications. Aberrant 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 invasive and metastatic potential (4,112,115). These evidences establish a strong link between association of β1,6 branched N-oligosaccharides with metastasis. Further, it is also reported that cell lines carrying these oligosaccharides home specifically to either the liver or to the lungs (4,112,114). At mechanistic level, these glycans may mediate organ specific metastasis in two ways. Firstly, β1,6 branch serves as the most favoured site for further substitutions like polyLacNAc, Lewis antigens, sialic acids and fucose which may serve as ligands for several endogenous lectins such as selectins, galectins, siglecs and yet unidentified endogenous lectins (3,138,204). For example, tumor cells expressing sialyl Lewis antigens, the E-selectin ligands, on these branches facilitate liver homing possibly

because of sinusoidal type of capillaries in liver which permits greater interaction of tumor cells with endothelium resulting in induction of expression of selectins on them. (78,139,205). Similarly, expression of polyLacNAc substituted β1,6 branched N-oligosaccharides, the galectin-3 ligands, has been proposed to facilitate lung metastasis (5,162). 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. Recently it has been shown that these glycans determine the cell surface residency of carrier proteins and which in turn can possibly affect their involvement in cellular signalling pathways. Proteins known to carry β1,6 branched structures include cell adhesion molecules such as integrins cadherins, CD44, growth factor receptors such as EGFR, ECM components like laminin and others like Lysosome-Associated Membrane Proteins (LAMPs) (3,128-130,142,206).

Using B16 melanoma model, we have previously shown that expression of polyLacNAc substituted β1,6 branched N-oligosaccharides correlates with metastastic potential of low metastastic, F1 and high metastastic, F10 cells. Galectin-3 expressed in highest amounts in lungs and constitutively on its vascular endothelium appears to serve as the high affinity receptor for polyLacNAc expressed on melanoma cells and thus facilitates tumor cell arrest in lungs (5). Further, galectin-3 as a component of all the other major compartments of lungs including vascular BM and organ parenchyma, apparently participated in all the major events associated with extravasation of tumor cells including spreading, degradation of matrix by secreting MMPs and cell migration into the organ parenchyma (6). The role of galectin-3 in metastasis was confirmed by using truncated gal-3 (tgal-3) which lacks the multimerization N-terminal domain and therefore lacks functional activity. Preincubation of melanoma cells with the tgal-3 consisting of only Carbohydrate recognition domain

(CRD) domain inhibited the lung metastasis of melanoma cells, by blocking the availability of polylacNAc on melanoma cells for binding to endogenous galectin-3 on the lungs. Alternatively, blocking of endogenous galectin-3 by feeding mice with modified citrus protein (MCP, polysaccharide rich in β-galactosides) also lead to reduction in metastasis potential of melanoma cells (6). On the basis of these findings it was established that galectin-3 plays and indispensible role in metastasis of melanoma cells.

Though β1,6 branched N-oligosaccharides is the preferred site for addition of polyLacNAc, even O-glycans carry them. Therefore it was important to understand if polyLacNAc on O-glycans contribute to the metastasis of melanoma cells. Treatment of melanoma cells with BG, an inhibitor for synthesis of O-glycans did not alter the metastastic potential of melanoma cells which conclusively ruled out any participation of these structures in metastasis of melanoma cells (162). However, treatment of melanoma cells with SW, an inhibitor for synthesis of complex type of N-glycans including β1,6 branched N-glycans lead to reduction in polyLacNAc expression, affected galectin-3 mediated adhesion, spreading, matrix degradation and significantly affected the metastasis of melanoma cells (6). Although, these results highlighted the importance of expression of β1,6 branched N-glycans on melanoma cells in metastasis, they did not conclusively establish the specific involvement of polyLacNAc in metastasis. This can be achieved only by targeting the genes which code for enzymes involved in synthesis of polyLacNAc. This strategy provides a tool to precisely study the role of specific terminal sugars (polyLacNAc in this case) in metastasis as opposed to broad action inhibitors which target the overall glycosylation pathway at early steps.

PolyLacNAc is synthesized by the sequential addition of N-acetylglucosamine and galactose by the enzymes β1,3 N-acetyl glucosaminyl transferases or GnTs and β1,4 galactosyltransferases- GalTs (105). The length of polyLacNAc extensions varies

according to action of these two enzymes (207). Targeting either GnTs or GalTs can affect synthesis of polyLacNAc. Since GalTs have been shown to be involved in synthesis of polyLacNAc on β1,6 branched N-glycans and their expression correlates with metastastic potential of tumor cell lines, they were chosen for shRNA mediated downregulation. Out of the seven GalTs known to be involved in synthesis of polyLacNAc, GalT-VII is involved in synthesis of polyLacNAc on proteoglycans (208) therefore its levels were not analysed in melanoma cells. Out of six GalTs, GalT-I and –V are expressed in melanoma cells in a metastatic potential dependent manner (6). The nucleotide sequences of GalT-I and –V were analysed and a common 22 nucleotide sequence was chosen for shRNA mediated downregulation. Earlier studies from lab indicated that melanoma cells transfected with plasmid vector constitutively expressing GalTshRNA had severe alterations on growth and morphology. Therefore, to avoid any non specific effect of downregulation of GalTs on cellular properties of melanoma cells, shRNA was cloned in an inducible lentiviral vector (pTRIPZ) (Figure 1). The first major advantage of using inducible system is that it allows tight temporal control of expression for shRNA and second, the shRNA mediated effects can be rescued in absence of inducer which also acts as an internal control. GalT downregulated clones (I and II) generated after transduction of F10 melanoma were validated for expression of GalT-I and –V at transcript level. The common shRNA was able to target both the GalTs, but as compared to GalT-I, decrease in the levels of GalT-V transcripts was three folds higher, possibly due to difference in the expression levels of these two genes (Figure 2A).

Reduction in expression of GalT-I and –V affected the polyLacNAc synthesis in GalT downregulated clones (Figure 2B and C). Reduction in levels of polyLacNAc also affected the galectin-3 binding of GalT downregulated clones (Figure 2C) which indicated that galectin-3 mediated metastasis associated processes including tumor cell adhesion,

spreading, degradation of matrix and movement into the organ parenchyma could also be affected.

Adhesion to organ endothelium is one of the most critical step involved in extravasation of tumor cells. Galectin-3 in immobilized form has been shown to mediate adhesion of tumor cells (5). Total percentage of adhesion of melanoma cells on immobilized galectin-3 is more than twofold higher as compared to that on uncoated wells (Figure 3A). Downregulation of GalT-I and –V lead to reduction in the galectin-3 mediated adhesion of melanoma cells to the same levels as seen in uncoated wells (Figure 3B). Thus expression of polyLacNAc on melanoma cells has a crucial role in adhesion of melanoma cells.

The next crucial step during organ colonization involves cell spreading. Tumour cells arrested in the target organ would need to spread immediately, to counteract the high haemodynamic shear forces in circulation, which may otherwise dislodge them. Galectin-3 in immobilized form induces formation of membrane protrusions such as lamellopodia (Figure 4A and B). These structures not only strengthen the adhesive interactions but also facilitate the movement of tumor cells. Targeting GalT-I and –V affected the galectin-3 mediated spreading of melanoma cells (Figure 4C and D) which clearly suggest that reduction in polyLacNAc levels affects the galectin-3 induced spreading.

However, just adhesion and spreading of cells on the target organ is not enough for establishment of a metastatic colony. In order to gain access into the organ parenchyma, tumour cells would need to displace the endothelial cells and degrade the vascular basement membrane to create space for movement. Degradation of matrix is one of the most crucial events involved in extravasation of tumor cells. MMPs play a major role in degradation of underlying BM and facilitate tumor cell entry into organ parenchyma (209). There are multiple reports which have studied the various facets of galectin-3 and MMP9 association. Galectin-3 in different cellular compartments has been shown to regulate

MMP9 expression. Overexpressation of galectin-3 in the cytoplasmic/nuclear compartments of cells has been shown to regulate expression of MMPs, especially MMP-1, MMP2 and MMP9 and promote invasion. Nuclear galectin-3 in gastric cancer cells has been shown to interact with AP-1 transcription factor and regulate the expression of MMP-1 (210). Further, silencing the expression of galectin-3 in human tongue carcinoma and pancreatic cell lines affected β-catenin levels which in turn correlated with reduced levels of MMP2 and MMP9 (211,212). MMPs recognise the collagenase like domain in galectin-3 which can act as a cleavage site for MMPs and cleaved form appears to serve as a marker for cancer progression (213). However, the possibility that galectin-3 as an ECM protein has any role in regulating MMP9 expression has not been previously investigated. Results show that extracellular galectin-3 induces secretion of MMPs in melanoma cells and downregulation of polyLacNAc affects induction of MMP9 secretion (Figure 5A and B). From these results the role of polyLacNAc becomes evident in galectin-3 induced MMP9 secretion. MMP9 mediates degradation of ECM but it has to be coupled with movement for effective extravasation of tumor cells.

Extracellular galectin-3 has been reported to induce movement of variety of cells. Using wound healing assays, it was demonstrated that immobilized galectin-3 facilitates movement of melanoma cells (Figure 6A). Reduction in polyLacNAc expression affected galectin-3 induced migration of melanoma cells (Figure 6B-D). It was thus concluded that expression of polyLacNAc on melanoma cells is a critical factor involved in tumor cell extravasation. Since tumor cell extravasation ultimately determines the fate of metastastis, experimental metastasis assay was performed to determine the metastastic potential of GalT downregulated clones. The overall reduction in number of metastastic colonies in lungs of mice injected with GalT downregulated clones (Figure 7A and B) emphasise that

polyLacNAc is indeed a major player along with galectin-3 in metastasis of melanoma cells.

On this background it can be suggested that polyLacNAc carrying glycoproteins which can serve as ligands for galectin-3 can play an important in regulating the steps of metastasis. Previous studies have clearly demonstrated that polyLacNAc only on N-glycans and not on O-glycans play a crucial role in metastasis of melanoma cells (5) therefore the role of proteins modified with polyLacNAc substituted N-glycans in metastasis was explored.

Currently very few subsets of proteins adhesion molecules including integrins (β1, α5, α3), CAMs, growth factor receptors (EGFR, VEGFR, TGF β receptor), lysosomal proteins (LAMP1, 2 and 3) are known to carry polyLacNAc substitutions. However to understand the mechanism through which metastasis associated processes are regulated, it was important to identify the complete spectrum of proteins modified with polyLacNAc substitutions.

Enrichment of glycoproteins is essential prior to identification as these proteins constitute minute fraction of total cellular proteins. Lectin affinity chromatography is the most suitable technique for enrichment of glycoproteins because of their ability to differentiate patterns of glycan structures, easy availability and high binding capacity for their ligands (169). To enrich proteins carrying polyLacNAc on β1,6 branched N-glycans both galectin-3 and L-PHA affinity approaches were used as these lectins have broad specificity in recognition of their ligands (Illustration 7). Post enrichment glycoproteins were resolved by one dimensional SDS PAGE and subjected for sample preparation of mass spectrometry (Figure 8A and 9).

To avoid interference of glycans in identification of proteins, enzymatic deglycosylation strategy was employed (Figure 8B). Deglycosylation of tryptic peptides by PNGase F

treatment not only increased the sequence coverage of identified proteins but also helped in annotating the modified N-glycosylation sites (Table 5 and 6). After comparative analysis, proteins which have affinity for both galectin-3 as well L-PHA were short listed (Table 7). The identified proteins represented in Table 7 can be broadly classified into four major categories. Adhesion molecules including integrins (α5, α3 and β1), immunoglobin superfamily proteins (Basigin, Embigin, Cell adhesion molecule 1), lysosomal proteins (LAMPs, Sulphated glycoprotein-1, cation dependent mannose 6 phosphate receptor and lysosomal acid phosphatase), and proteins involved in melanin metabolism (5,6-dihydroxyindole-2-carboxylic acid or Tyrp1, L dopachrome tautomerase or Tyrp2 and transmembrane protein NMB). Mass spectrometry results were validated by probing galectin-3 enriched fraction with antibodies against proteins including LAMP1, LAMP3, Basigin, Embigin, Integrin β1 and α5 (Figure 12).

Integrins are well reported carriers of β1,6 branched N-glycans. Recently, it has been shown that expression of β1,6 branched N-glycans on β1 integrin facilitates its interaction with MT1-MMP to aid invasion of melanoma cells (124). Interaction of galectin-3 with αVβ3 integrin regulates migration of endothelial cells (214). Similarly, galectin-3 mediated cross-linking of α3β1 and α5β1 integrin induces formation of membrane protrusions including lamellopodia and motility of tumor cells (171,215). On the basis of these reports, the involvement of β1 integrin in galectin-3 mediated processes such as cell spreading of melanoma cells was investigated. Blocking β1 integrin using anti β1 integrin antibody affected galectin-3 mediated spreading of melanoma cells (Figure 13A and B) which suggests that as a galectin-3 binding protein β1 integrin indeed can participate in metastasis associated events.

It is evident from the mass spectrometry results that variety of lysosomal proteins could be modified with polyLacNAc substituted β1,6 branched N-glycans. For e.g. All LAMPs

were found to be modified these structures. LAMP1 was found to be major carrier amongst all the identified proteins as in total nine glycosylation sites (N-70, 78, 97, 101, 159, 177, 252, 296, 311) were found to be modified with these structures (Table 7). LAMP1 has earlier been shown to get translocated to the surface of B16 melanoma cells in a metastatic potential dependent manner (5). Inhibiting LAMP1 by using anti LAMP1 antibody affected the spreading of melanoma cells (Figure 13A and B). LAMP2 is another lysosomal membrane protein which is known to interact with galectin-3. However, LAMP2 does not get translocated to the cell surface in melanoma cells, and hence its role in metastasis was ruled out (5). LAMP3 also called as CD63 is the first tetraspanin to be characterized (216). The cell surface expression of CD63 is regulated by it glycosylation status (217). In addition, expression of CD63 has been found to be associated with metastastic potential of tumor cells (218). As a galectin-3 binding protein its role in metastasis should be explored.

Immunoglobin superfamily (IgSF) member proteins including Basigin and Embigin have been identified as galectin-3 binding proteins carrying polyLacNAc on β1,6 branched N-glycans. Basigin also called as CD147 has been recently shown to carry β1,6 branched N-glycans and interact with galectin-3 (219). CD 147 is often found to be overexpressed in tumor cells (220,221). There are in total five glycosylation sites on CD 147 reported to carry N-glycans including Asn 160, 270, 275, 306 and 309 (190,191). Asn 160 is potentially modified with polyLacNAc substituted β1,6 branched N-glycans (Table 7) and therefore this particular glycosylation site might play a important role in interaction of basigin with galectin-3. CD 147 is also called as EMPPRIN for its extracellular matrix metalloproteinase inducer activity. Recently, it has been reported that association of CD147 with galectin-3 is required for MMP-9 induction (222). Galectin-3 induced cell spreading of melanoma cells was inhibited at maximum level in presence of anti CD147

antibody in comparison to blocking LAMP1 and β1 integrin (Figure 13A and B). Embigin is a transmembrane glycoprotein and is another member of same subset of IgSF. It shares more than 50% sequence similarity with basigin. The Embigin protein is 34 kDa in mass, but due to presence of 12 potential N-glycosylation sites it appears as a 66-90 kDa molecule (190,191,223). Embigin was found to be modified with polyLacNAc on β1,6 branched N-glycans at Asn 55,216 and 221 positions (Table 7). The expression of Embigin has been found to be upregulated in embryonic carcinoma cells (224). However, role of importance of glycosylation on Embigin in tumor progression has not been studied in detail.

These galectin-3 binding proteins are possibly the major determinants which regulate the extravasation and organ colonization of melanoma cells. On galectin-3 binding, these proteins would initiate the activation of downstream signalling pathways which in turn regulate the cellular processes associated with tumor cell extravasation. For instance, it has been shown that galectin-3 in association with α5β1 regulates adhesion and migration of epithelial cancer cells by activating FAK and PI3K signalling pathways (171,225). Apart from adhesion and migration, degradation of matrix is another important event during metastasis. Extracellular galectin-3 induces MMP-9 secretion in melanoma cells in dosage dependent manner (Figure 14). However, whether extracellular galectin-3 has any direct role in regulation of MMP9 expression is still not clear.

Multiple modes of mechanisms have evolved to regulate the action of MMPs. 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. Galectin-3 induced MMP9 expression is regulated at transcript level which is the first point of regulation (Figure 15A). Regulation at transcription level indicated that galectin-3/polyLacNAc pair might regulate one or of the three major signalling pathways including

MAPK, PI3K and NF- κB which are involved in regulation MMP9 expression. Using chemical inhibitors it was shown that galectin-3 induced MMP9 expression at transcript level occurs through p38 MAPK pathway (Figure 15B). Both zymography and immunoblotting confirmed that in comparison to cells grown in presence of ERK, PI3K and JNK inhibitors, cells treated with p38 MAPK inhibitor (SB) showed maximum reduction in MMP9 levels (Figure 16A-D). Though it was understood that galectin-3 activates p38 MAPK pathway to induce MMP9 still it was clear if this indeed occurs through polyLacNAc. This was confirmed by using GalT downregulated clones. Reduction in expression of polyLacNAc in GalT downregulated clones was associated with lowered levels of MMP-9 transcripts and activated p38 MAPK which indeed confirmed that polyLacNAc expression at cell surface is essential for activating the galectin-3 mediated downstream events (Figure 17A-C). Recently, the mechanism by which extracellular galectin-3 regulates cellular processes has been hypothesized. On binding to polyLacNAc on β1,6 branched N-glycans, galectin-3 oligomerises to form lattices/microdomains through which the cellular signalling pathways is activated (135). The glycoproteins which carry polyLacNAc substituted β1,6 branched N-glycans act as determining factors in formation of galectin-3 lattices. In melanoma cells, LAMP1 and β1 integrin are the major proteins which have been identified to carry polyLacNAc substituted β1,6 branched N-glycans. Mass spectrometry data also suggests that LAMP1 carries nine potential glycosylation sites which could be modified by polyLacNAc substituted β1,6 branched N-glycans (Table 7). Thus LAMP1 can act as major ligand for galectin-3 and thus participate in galectin-3 mediated events. LAMP1 expression at cell surface correlates with metastastic potential of melanoma cells and recently it has been shown that its cell surface residency is determined by its glycosylation status (5,198). Downregulation of LAMP1 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 (198,201). Thus on the basis of these findings we studied the involvement of LAMP1 in galectin-3 induced MMP9 expression using inducible lentiviral LAMP1 downregulated clones developed by Agarwal *et al* (201).

Induction of shRNA expression in LAMP1 downregulated clones lead to reduction in transcript levels of MMP9 (Figure 18A). Zymography and immunoblotting experiments also confirmed that downregulation of LAMP1 indeed affects galectin-3 induced MMP9 expression (Figure 18B-D). Further activation of p38 MAPK pathway was also affected in LAMP1 downregulated clones as it was shown to be affected in GalT downregulated clones (Figure 17B and C) which emphasizes that signalling probably occurs through similar mechanism in clones (Figure 18E and F). These findings suggest that LAMP1 as a polyLacNAc carrier has an important role in induction in MMP9 expression, though it may not be the only galectin-3 binding protein involved in inducing MMP9 expression.

There are two potential mechanisms through which LAMP1 can regulate MMP9 expression. The first mechanism involves direct involvement of LAMP1 through interaction with signalling proteins at cytoplasmic end. LAMP1 has a very short cytoplasmic tail (consisting of only eleven amino acids) (196) 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. Ezrin can function as a crosslinker between membrane proteins and cytoskeletal proteins to modulate cellular adhesion and motility (226). The loss of spreading and motility on galectin-3 in LAMP1 downregulated clones is possibly via some such mechanism (201). It is possible that induction of MMP9 by surface LAMP1 is also mediated by similar protein(s) that interact with LAMP1 and activate the pathway leading to induction of MMP9 transcription and secretion.

Alternatively, as a glycosylated cell surface protein, high levels of polyLacNAc on LAMP1 may initiate formation of galectin-3 mediated lattices/membrane microdomains (147). 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 (135,142,227-230). 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.

Taken together the present study not only demonstrates the importance of glycosylation in extravasation of tumor cells but also investigates the involvement of cellular signalling mechanisms mediated by glycosylation carriers in regulation of metastasis associated events.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 Summary

- Downregulation of GalT-I and –V in melanoma cells leads to reduction in polyLacNAc levels and affects their binding to galectin-3.
- Decreased levels of polyLacNAc in GalT downregulated clones affects participation of melanoma cells in galectin-3 mediated extravasation events including cell adhesion, spreading, matrix degradation and migration.
- Decreased levels of polyLacNAc on melanoma cells significantly reduce their lung metastatic ability.
- Galectin-3 binding proteins carrying β1,6 branched N-glycans were enriched using galectin-3 and L-PHA affinity chromatography.
- Enriched proteins were identified proteins using nano LC MS/MS and potential sites carrying polyLacNAc substituted N-glycans were annotated.
- Galectin-3 binding proteins including LAMP1, β1 integrin and Basigin were shown to participate in galectin-3 mediated cell spreading of melanoma cells.
- Extracellular galectin-3 induces secretion of MMP-9 in dosage dependent manner.
- Induction of MMP-9 expression occurs at transcript level and inhibition of p38 MAPK pathway affects MMP9 expression
- Downregulation of polyLacNAc inhibits p38 MAPK regulated MMP9 expression.
- LAMP1, a carrier of polyLacNAc, serves as galectin-3 receptor for MMP9 induction.

5.2 Conclusions

The present study demonstrates the importance of expression of polyLacNAc on N-glycans in mediating organ colonization of melanoma cells. PolylacNAc in association with galectin-3 act as major determinants of organ specific metastasis. Lectin affinity chromatography in conjunction with mass spectrometry helped in identifying the galectin-3 binding proteins and deglycosylation strategies facilitated locating the possible polyLacNAc carrying N-glycosylation sites on identified proteins. Such multistep approach is prerequisite in the field of glycoproteomics which has major implications in tumor biology. Extracellular galectin-3 in association with polyLacNAc regulates the expression of MMP9 through p38 MAPK pathway. LAMP1 as a galectin-3 binding protein acts as one of the major players involved in galectin-3 induced MMP9 expression. In summary it can be concluded that glycosylation has a major role in mediating organ specific metastasis of melanoma cells.

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RESEARCH PAPER

Galectin-3 expressed on different lung compartments promotes organ specific metastasis by facilitating arrest, extravasation and organ colonization via high affinity ligands on melanoma cells

Manohar C. Dange · Nithya Srinivasan · Shyam K. More · Sanjay M. Bane · Archana Upadhya · Arvind D. Ingle · Rajiv P. Gude · Rabindranath Mukhopadhyaya · Rajiv D. Kalraiya

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Abstract Interactions between molecules on the surface of tumor cells and those on the target organ endothelium play an important role in their arrest in an organ. Galectin-3 on the lung endothelium and high affinity ligands poly-*N*-acetyllactosamine (polyLacNAc) on N-oligosaccharides on melanoma cells facilitate such interactions. However, to extravasate and colonize an organ the cells must stabilize these interactions by spreading to retract endothelium, degrade exposed basement membrane (BM) and move into parenchyma and proliferate. Here, we show that galectin-3 is expressed on all the major compartments of the lungs and participates in not just promoting adhesion but also in spreading. We for the first time demonstrate that both soluble and immobilized galectin-3 induce secretion of MMP-9 required to breach vascular BM. Further, we show that immobilized galectin-3 is used as traction for the movement of cells. Downregulation of galactosyltransferases-I and -V resulted in significant loss in expression of polyLacNAc and thus reduced binding of galectin-3. This was accompanied with a loss in adhesion, spreading, MMP-9 secretion and motility of the cells on galectin-3 and thus their metastasis to lungs. Metastasis could also be inhibited by blocking surface polyLacNAc by pre-incubating cells with truncated galectin-3 (which lacked oligomerization domain) or by feeding mice with modified citrus pectin in drinking water. Overall, these results unequivocally show that polyLacNAc on melanoma cells and galectin-3 on the lungs play a critical role in arrest and extravasation of cells in the lungs and strategies that target these interactions inhibit lung metastasis.

Keywords Organ specific metastasis · Lungs · Galectin-3 · Extravasation · Poly-*N*-acetyllactosamine · β1,6 branched N-oligosaccharides

Introduction

In spite of being the major cause of mortality in cancer patients; the underlying molecular mechanisms of metastasis are still poorly understood possibly due to the complexity of this multistep process [1]. To metastasize, tumor cells must break free from the primary site, create space for their movement, get into and survive in circulation [2]. Once in circulation, they are able to reach almost all organ sites. However, some metastasize in the anatomic vicinity, while others bypass several organs and colonize very specific organ sites [3]. The patterns of circulation and mechanical factors appear to dictate the regional spread [4]. However, organ specific metastasis is believed to be facilitated by specific interactions between the molecules on the tumor cells and the target organ, growth environment and chemotactic factors released from the target organ [3, 5–7].

Manohar C. Dange, Nithya Srinivasan have contributed equally to this work

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M. C. Dange · N. Srinivasan · S. K. More · S. M. Bane · A. D. Ingle · R. P. Gude · R. Mukhopadhyaya · R. D. Kalraiya (✉)
Kalraiya Lab, KS 131, Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Tata Memorial Centre, Sector 22, Kharghar, Navi Mumbai 410210, India
e-mail: rkalraiya@actrec.gov.in

A. Upadhya
Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, SVKM's NMIMS, Mumbai, India

Vascular endothelium is the first barrier that a tumor cell must overcome to colonize an organ. Organ endothelium also provides specific receptors/ligands for organ specific homing of cancer cells [8, 9]. Extravasation of leucocytes at the inflamed site has provided useful clues to the overall process of extravasation which involves rolling, adhesion and extravasation [10]. Selectins and their ligands promote rolling and retard the movement of leucocytes. Firm endothelial adhesion is facilitated by activated integrins and their counter receptors. This is followed by diapedesis which involves endothelial retraction, degradation of vascular basement membrane (BM) and movement into organ parenchyma [10, 11].

Tumor cells are also believed to utilize similar mechanisms for extravasation and each of these steps could be rate limiting [12, 13]. The vascular endothelium has been shown to express specific set of surface molecules on different organs [14]. Tumors reportedly adhere preferentially to the endothelial cells or the 'outside out' endothelial cell membrane vesicles, derived from their metastatic site [15]. VE-cadherin, integrins, Ig class of cell adhesion molecules, selectins, carbohydrates and their lectin receptors are among the major class of molecules on the endothelial cells and on the cancer cells, which are believed to aid adhesion of cancer cells to the target organ [7, 16–19]. Constitutive expression of E-selectin on vessels of the bones or on inflamed organ endothelium has been shown to facilitate organ homing of cells expressing specific E-selectin ligands [10, 20].

However, the participation of E-selectins and its ligand in promoting metastasis in the lungs appears remote. Extravasation occurs predominantly in the micro-vascular capillaries in pulmonary circulation, which are too small to allow rolling [21, 22]. Lung colonizing cancer cells have been shown to get redirected to liver upon forced expression of ligands for E-selectin on hepatic cells [23]. Other receptors/ligands implicated in lung specific interactions include dipeptidyl peptidase IV (DPP-IV), Lu-ECAM1, VCAM-1, CLCA2 on the lung endothelium and their counter receptors like fibronectin; CXCR4, β4 integrin on tumor cells [24–26]. Galectin-3 on the organ endothelium has also been implicated in promoting organ homing [27, 28]. It is a nucleo-cytoplasmic β-galactoside specific lectin that gets secreted out in a non classical manner and gets incorporated onto the cell surface and as part of the matrix and BM [29]. In mice, lungs were shown to express highest amounts of galectin-3 and express it constitutively on its vascular endothelium [27]. Several reports implicate T/Tn antigens on tumor cells in mediating both homophilic interactions and heterophilic interactions with endothelial cells via galectin-3 [19, 30, 31]. Apart from these interactions, galectin-3 in the host may also facilitate melanoma metastasis by modulating immune response, in particular innate antitumor immunity [32, 33].

Using low and high metastatic variants of B16 melanoma cells, previous work by our group has shown that polyLacNAc substituted β1,6 branched N-oligosaccharides on cancer cells may serve as very high affinity, easily accessible form of ligands for galectin-3 [27, 28]. Galectin-3 shows >200-fold higher affinity towards polyLacNAc as compared to T/Tn antigens [34]. Galectin-3 on the lung microvascular endothelium appeared to promote lung metastasis by serving as an anchor to arrest circulating tumor cells carrying polyLacNAc substituted β1,6 branched N-oligosaccharides [27, 28]. Under flow conditions galectin-3 has been shown to bind to the glycoproteins carrying its ligands with high affinity as compared to the selectins to their ligands (Kd of 1 vs. 100–300 μM for selectins and is comparable to the interactions mediated by integrins) [35, 36]. However, just adhesion to vascular endothelium is not enough to establish metastatic foci.

The tumor cells need to displace endothelium, interact with and degrade the exposed vascular BM, move into organ parenchyma and proliferate within for effective metastasis [5, 7]. This was elegantly demonstrated by monitoring adhesive interactions with organ microvasculature and invasion by intra-vital microscopy of colon cancer cell lines differing in their metastatic potential. Although, adhesion occurred in micro-vasculatures of metastatic target organ only, their migration into organ parenchyma correlated with metastatic potential [12].

In the present communication, we demonstrate that galectin-3 present on all the major compartment of the lungs participates not just in promoting adhesion to vascular endothelium but also in all the subsequent events of extravasation. Further, we show that polyLacNAc substituted N- and not O-oligosaccharides participate in all these processes. Inhibition of expression of polyLacNAc or competitive inhibition of their interaction with the host galectin-3 both inhibited all these processes and thus metastasis.

Materials and methods

Reagents

TRIzol and Superscript TM amplification system for RT-PCR and Calcein AM were from Invitrogen, USA. Anti-mouse galectin-3 rat antibody was from R&D Biosystems, USA, and anti-Rat HRPO, anti-Goat HRPO from Santa Cruz Biotechnology, USA. *E. coli* BL 21 with pET3C plasmid containing a full-length human galectin-3 was a kind gift from Prof. Hakon Leffler, Lund University, Sweden. Biotinylated lectin *Lycopersicon esculentum* lectin (LEA), avidin–peroxidase, and streptavidin–FITC, were either from Sigma Chemical Company, USA or Vector

Labs, USA. Power SYBR Green PCR Master Mix was from Applied Biosystems. Anti-MMP-9 antibody, Primers for RT-PCR, Primers for real time PCR and for shRNA amplification, Phalloidin TRITC, Phalloidin FITC, DAPI, Pectin from citrus peel, Polybrene were purchased from Sigma Chemical Company. Dulbecco modified essential medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco, Invitrogen. All other chemicals were purchased locally and were of analytical grade. For experimental metastasis assay, inbred strain of C57BL/6 mice was used.

Cell lines

B16F1(F1) and B16F10 (F10) murine melanoma cell lines [37] were obtained from National Centre for Cell Sciences, Pune, India. The cell lines were expanded and frozen aliquots were stored in liquid nitrogen. Each aliquot was used only up to five passages in vitro. The metastatic potential of F10 cells is maintained by culturing melanoma colonies on the lungs obtained by performing experimental metastasis assay in C57BL/6 mice. Cell lines were routinely characterized for (C57BL/6) mouse specific origin and mycoplasma free status as described in supplementary methods.

Immunohistochemical detection of galectin-3 in mouse lungs

Immunohistochemical staining for galectin-3 was performed on 3-μm paraffin embedded sections as described in [27]. Sections were stained with rat anti-mouse galectin-3 monoclonal antibody followed by anti-rat horse radish peroxidase (HRPO) conjugate and developed with diaminobenzidine containing H_2O_2 as the substrate. Instead of the primary antibody, the control lung sections were treated with rat IgG in the concentration similar to the primary antibody. The slides were later counter stained with hematoxylin.

Purification of recombinant human galectin-3

Galectin-3 was purified as described in [38].

Adhesion assays

For Adhesion assays, either calcein AM labeled or tritiated thymidine labeled melanoma cells were used and were performed in 96 well plates coated overnight with galectin-3 (50 μg/ml) as described previously [39]. For labeling with calcein, melanoma cells were incubated with DMEM medium containing 3 μg/ml calcein. Fluorescence was measured in 96 well plate reader from Berthold Mithras LB-940 machine (Excitation filter-485 nm and Emission filter-535 nm). The percentage adhesion was calculated by considering F10 cells bound to galectin-3 as 100 %.

Cell spreading assay

Melanoma cells were harvested, washed free of serum and 0.5 million cells were seeded in serum free DMEM on the coverslips coated overnight with 50 μg/ml galectin-3 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 TRITC or Phalloidin FITC staining solution made in PBS for 15 min at 37 °C. Nuclei were stained with 5 μg/ml of 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for one minute. The stained cells were mounted and images were acquired using LSM510 software on a Carl Zeiss Laser confocal Microscope at 63× magnification. The ratio of cytoplasmic/nuclear (C/N) area of approximately 100 cells was measured using Image J software to quantitate cell spreading.

Detection of MMPs by zymography and Western blotting

Melanoma cells seeded at a density of 15,000 cells in 100 μl of complete DMEM were grown in 96 well plates for 24 h at 37 °C. Cells were subjected to serum starvation for additional 24 h in absence or presence of different concentrations (0.25 μg–0.75 μg/ml) of soluble galectin-3. To see the effect of immobilized galectin-3, wells were coated overnight with 100 μl of different concentrations of galectin-3 (10–75 μg/ml) at 4 °C in serum free DMEM. The cells were grown on immobilized galectin-3 in complete medium at 37 °C for 24 h in a CO_2 incubator followed by serum starvation for 24 h. Cells seeded on uncoated wells served as control. The serum free conditioned medium was collected from each well and analyzed by gelatin zymography on 10 % SDS-PAGE containing 0.1 % gelatin as per [40]. MMP-9 levels in culture supernatant were also detected by Western blotting with anti MMP-9 antibody.

Wound healing assay

35 mm culture dishes were coated overnight with galectin-3 (50 μg/ml) in serum free DMEM at 4 °C, followed by blocking of non-specific sites with 2 % BSA for 1 h. 0.75 million melanoma cells were seeded in coated plates and incubated at 37 °C for 24 h in a CO_2 incubator. The cells were serum starved for 24 h for cell synchronization. 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 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. Uncoated culture dishes, blocked only with BSA served as control.

Cloning of shRNA for targeting β1,4 galactosyltransferases-I and -V genes (GalTs)

Downregulation of polyLacNAc in F10 cells was performed by using short hairpin RNA (shRNA) against GalT-I and -V the genes involved in polyLacNAc synthesis, as per the guidelines outlined [41]. A 21 nucleotide sequence (5′-TGGGGCGGAGAAGATGACGAC-3′) from the open reading frame of GalT genes was chosen which is common and unique only to these two genes. The strategy for cloning shRNA into pSuperneo H1 vector is described in supplementary data.

For cloning shRNA into pTRIPz lentiviral vector, primers were designed according to pTRIPz manual (Open biosystems). Forward primer contained *Xho*I site followed by mir sequence (represented in italics), sense sequence (represented in italics bold) and loop sequence.

Forward GalT shRNA primer
5′GAACTCGAG*AAGGTATATTGCTGTTGACAGTGAG CG**TGGGGCGGAGAAGATGACGAC***TAGTGAAGCC ACAGA3′
Reverse primer contained *Eco*RI site followed by mir sequence (represented in italics), sense sequence (represented in italics bold) and loop sequence.
Reverse GalT shRNA primer
5′GTTGAATTC*CGAGGCAGTAGGCA**TGGGGCGGA GAAGATGACGAC***TACATCTGTGGCTTC3′

Using these primers shRNA sequence was amplified. The shRNA was cloned in pTRIPz lentiviral vector digested with *Eco*RI and *Xho*I sites. The ligated plasmid was 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 F10 cells. The clones were selected using puromycin (1 μg/ml) and subsequently maintained as separate stocks. For inducing shRNA expression cells were cultured in complete DMEM containing doxycycline (4 μg/ml) for 96 h.

Real time PCR

For detecting transcript levels of GalT-I and GalT-V genes specific primers were designed. RPL4 was used as housekeeping gene for relative quantification of transcript levels [42].

The real time PCR reaction was carried out in 7900HT system (ABI Prism) and for detecting amplicons Power SYBR green was used. The data represents mean of three different experiments carried out in duplicates with different batches of cDNA. The Ct values obtained were normalized to RPL4 values. Analysis was performed using $2^{-\Delta\Delta C_t}$ method [43].

Flow cytometric analysis

For flow cytometry, cells were either fixed overnight in 1.5 % glutaraldehyde or 1 % paraformaldehyde in PBS (pH 7.4) and were analyzed for surface expression of polyLacNAc using biotinylated galectin-3 (0.75 μg/ml) and biotinylated LEA (2 μg/ml) as described previously [28].

Cloning, expression and purification of mouse truncated galectin-3 (mtGal-3)

The coding DNA sequence of C-terminal carbohydrate binding domain of galectin-3 was cloned into the pET3a bacterial expression vector using forward 5′ATAGTCATCA TATCATCATAGTCGATCATATGGTGCCC3′ and reverse 5′GGTGGATCCTTAGATCATGGCGTGGTTAGC3′ primers. The total cDNA obtained from F10 cells served as a template. The *Nde*I and *Bam*HI restriction sites were incorporated in forward and reverse primers respectively to clone the amplified product into pET3a plasmid vector. The sequence of the positive clones was confirmed. This construct was transformed into *E. coli BL21* (*DE3*) strain and expression of mtGal-3 was induced by IPTG (1 mM). The expressed protein was of 15.4 kDa (amino acid sequence 130–264 of full length galectin-3). The mtGal-3 protein was purified using lactose sepharose column as described in [38].

Preparation of modified citrus pectin (MCP)

MCP was prepared from citrus pectin exactly as described in [44].

Experimental metastasis assay

Melanoma cells were routinely cultured in DMEM as described in [28], with or without glycosylation inhibitors Swainsonine (SW, 2 μg/ml) and benzyl-α-*N*-acetylgalactosamine (BG, 2 mM). For injecting GalT clones, mice were fed with doxycycline (1 mg/ml) in 5 % sucrose solution 24 h prior to injection and continued until sacrificed.

For injecting F10 cells treated with murine truncated galectin-3 (mtGal-3, carrying only CRD), 0.1 million F10 cells were pre-incubated with 0.1 ml of 500 μg/ml of mtGal-3 for 1 h on ice. The mice that received pre-treated

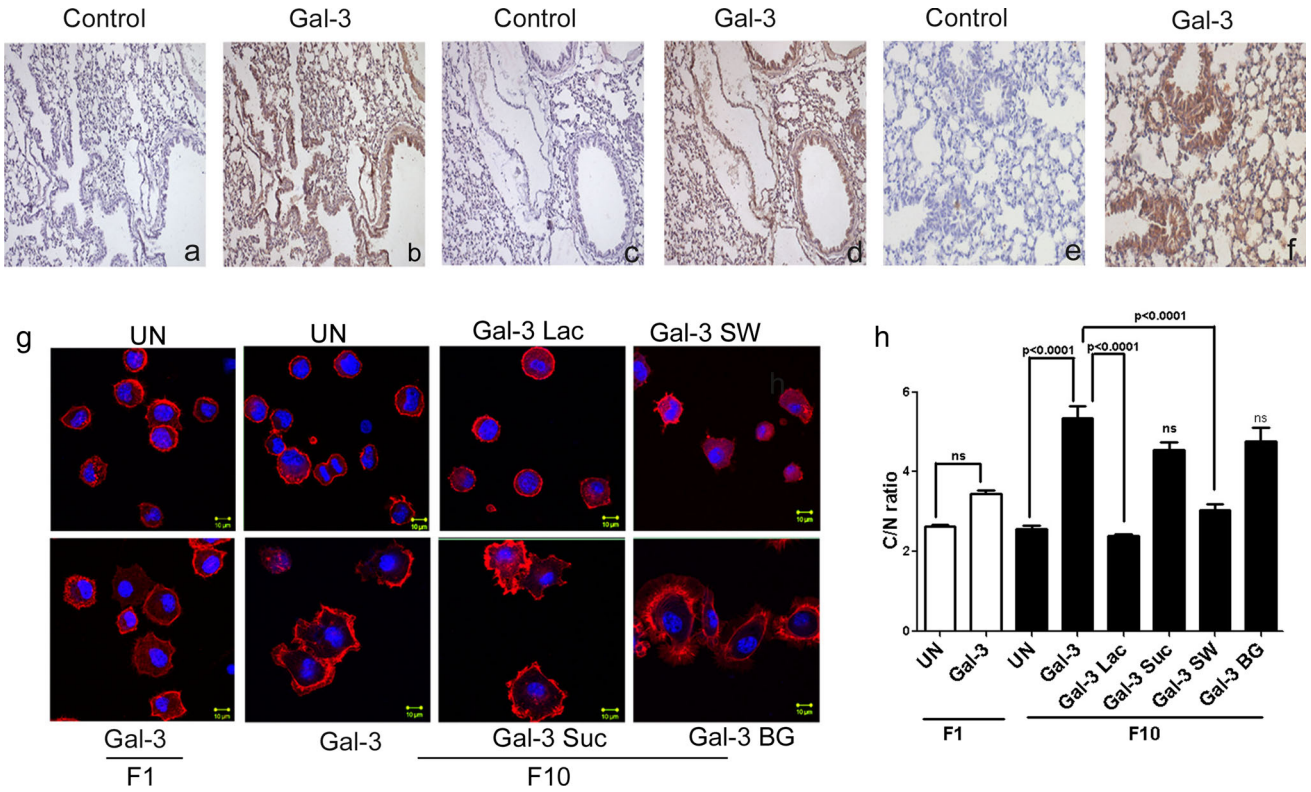

Fig. 1 Galectin-3 is expressed in all the major compartments of lungs and it facilitates spreading of melanoma cells. **a**–**f** Immuno-histochemical staining of mouse lung sections treated with rat anti-mouse galectin-3 antibody at ×10, ×20 and ×40 magnifications, respectively. The control sections were treated with pre-immune rat IgG. **g** Cell spreading of F1 and F10 cells on uncoated (UN) and galectin-3 (Gal-3) coated coverslips as assessed by Phalloidin-TRITC staining. DAPI was used to stain the nuclei (*blue*). Spreading of F10 cells was also seen on galectin-3 coated coverslips in presence of lactose (Gal-3 Lac) and sucrose (Gal-3 Suc), and after treatment with either SW (Gal-3 SW) or BG (Gal-3 BG). *Scale bar* 10 μm. **h** *Each bar* represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments. (Color figure online)

cells also received injections of mtGal-3 (250 μg in 0.1 ml, via intra muscular injection) on day 1 (2 h before and after injection of cells) and once on day 2.

For injecting F10 cells treated with MCP, 0.1 million F10 cells were resuspended in medium with and without MCP (0.05 %) and mice which received cells with MCP were on drinking water containing 1.5 % MCP from 5 days prior to injection till the day of sacrifice.

Statistical analysis

All the data is represented as mean ± SE unless stated. For comparison of two groups in case of cell spreading, cell adhesion, experimental metastasis assay student's *t* test was employed and multiple groups were compared by one way ANOVA. For wound-healing assays, 2-way ANOVA with the Bonferroni posttest was conducted. All the statistical analysis was performed using GraphPad Prism 5. $P < 0.05$ was considered significant.

The details of cloning of shRNA in pSupeneo H1, sequences of primers used for real time and semi quantitative RT-PCR have been described in supplementary methods. Total cell lysate, Protein estimation, SDS-PAGE and Western blotting were performed as described in [27].

Results

Galectin-3 is localized in all the major compartments of mouse lungs

In mice, lungs have previously been shown to express highest levels of galectin-3 and express it constitutively on the surface of its vascular endothelium. Immunohistochemistry results showed that not just endothelium, galectin-3 is localized in all the major tissue compartments of the lungs, including epithelia of bronchioles, alveoli and on the surface of vascular endothelium (Fig. 1a–f) and possibly thus may participate in different processes of organ colonization.

Galectin-3 facilitates spreading of melanoma cells in a metastasis and N-glycosylation dependent manner

Ability of cells to spread on immobilized galectin-3 was analyzed to see if it stabilizes the interactions of the cells

adhered on the vascular endothelium. F1 cells spread poorly with diffused organization of actin, on both uncoated and galectin-3 coated cover slips (Fig. 1g). In contrast, F10 cells showed significant spreading on galectin-3 as compared to uncoated cover slips, as characterized by organization of F-actin in lamellipodial projections and significantly increased C/N ratio (Fig. 1g, h). Like adhesion [28], the extent of spreading was also dependent on the metastatic potential of the cells which was inhibited by specific disaccharide lactose and not sucrose, and by inhibitor of N- but not O-glycosylation (Fig. 1g, h, Gal-3 Lac/Suc and Gal-3 SW/BG).

Galectin-3 induces secretion of proteases and motility in melanoma cells, thereby aiding invasion

Degradation of Basement membrane/Extracellular matrix (BM/ECM) and movement are the next major event required during extravasation. Assays were performed to see if soluble and immobilized galectin-3 promotes any of these processes. Zymography of the conditioned media collected from cells grown in the absence or presence of either soluble or immobilized galectin-3, showed that galectin-3 induces secretion of matrix degrading enzyme, MMP-9 in a dose and metastatic potential dependent manner (Fig. 2a–c).

For F10 cells, the highest induction with soluble galectin-3 was seen at 0.5 μg/ml (data not shown), whereas with immobilized galectin-3, it was 2.5 μg/well of a 96 well plate (Fig. 2a). The concentrations higher than these appeared to inhibit the induction of MMP-9 secretion. Comparison of the melanoma variants clearly showed that induction is dependent on the metastatic potential (Fig. 2b, c). No gelatin clear bands (as a result of MMP-9 activity) could be visualized in F1 lanes on 24 h incubation of the gel in renaturation buffer (Fig. 2b) whereas prolonged incubation resulted in saturation in F10 lanes due to substrate limitation (Fig. 2c). Since expression of polyLacNAc on N-glycans is also dependent on the metastatic potential of melanoma cells, the galectin-3 mediated induction of MMP-9 could be via polyLacNAc.

Movement of extravasated cells towards organ parenchyma is also a key event for metastatic establishment. Wound healing assays showed that galectin-3 is indeed used as traction by these cells for their movement. This again, was dependent on the metastatic potential and the N-glycosylation status of cells (Fig. 2d–f). O-glycosylation inhibitor BG had no effect on any of these cellular properties or on metastasis [28]. This reaffirmed our earlier observation that galectin-3 ligands only on N-oligosaccharides participate in these processes.

Downregulation of GalT-I and -V enzymes in F10 cells leads to significant reduction in the expression of polyLacNAc

Although, using Swainsonine, a broad range N-glycosylation inhibitor, we confirmed that N-glycans play an important role in metastasis of F10 cells; we needed to confirm that it is via polyLacNAc on them. PolyLacNAc is synthesized by the concerted action of the enzymes that sequentially add *N*-acetylglucosamine (β1,3 *N*-acetylglucosaminyltransferases or β3GnTs) and galactose (β1,4 galactosyltransferases—GalTs) [45]. Among the seven members of the GalT family GalT-VII adds galactose only onto proteins with proteoglycan core [46]. Comparison of transcripts of the remaining six members by semi-quantitative PCR showed up regulation of GalT-I and -V in the higher metastatic variant (Supplementary Fig. S1A). Both β4GalT-I and β4GalT-V reportedly promote addition of polyLacNAc preferentially on N-oligosaccharides [47, 48].

Both these genes were down-regulated using shRNA targeting a sequence common to both of them, using plasmid (pSuperneo H1) as well as inducible lentiviral (pTRIPz) vectors for cloning shRNA in F10 cells. The functional effects of downregulation were confirmed by in vitro as well as in vivo assays. The two F10 cell clones, sh3 and sh6, generated in pSuperneo H1 vector which constitutively expresses shRNA showed downregulation of transcripts and polyLacNAc on the cell surface. (Supplementary Fig. S1b, c).

The inducible lentiviral vector system (pTRIPz) allowed tight temporal control of target gene knock down with minimal off-target and deleterious effects on cells. The two F10 clonal cell lines (clone I and II) expressing the inducible GalT-I and -V shRNA were established. Simultaneously, clones of F10 cells expressing the inducible non-targeting shRNA (NT) were also established. Upon doxycycline induction, clone I and II showed significant reduction in the transcript levels of GalT-I and -V as compared to clone expressing non targeting sequence of shRNA (Fig. 3a, b). In contrast to NT clone, both the clones expressing specific shRNA showed significant reduction in the surface levels of polyLacNAc as assessed by flow cytometry, using biotinylated LEA and galectin-3, after doxycycline induction (Fig. 3c, d).

Downregulation of polyLacNAc results in decreased adhesion, spreading, MMP-9 secretion and motility of F10 cells on galectin-3 together with reduced experimental metastasis

Reduced expression of polyLacNAc in the induced clone I and II was associated with significantly decreased

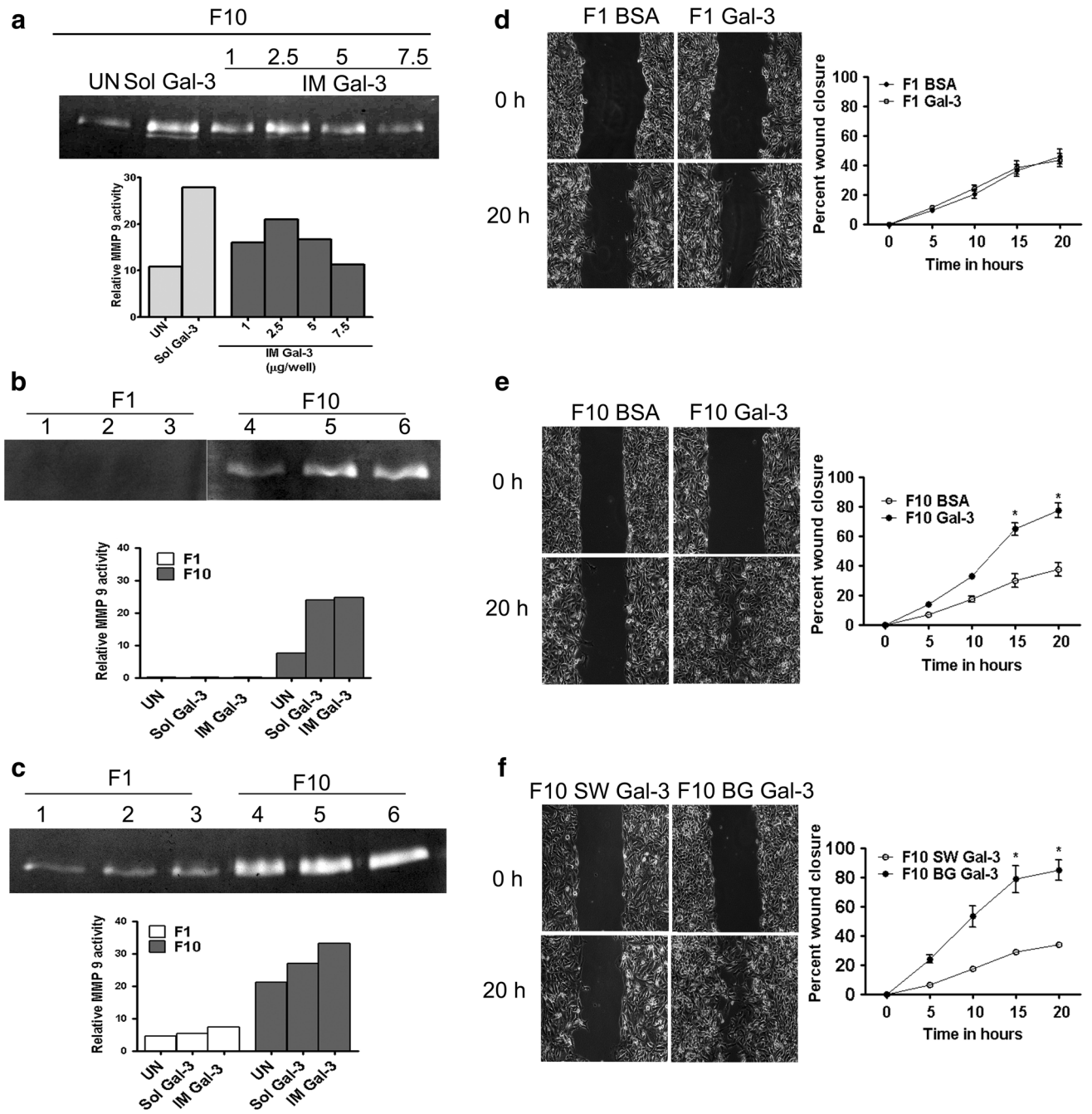

Fig. 2 Galectin-3 in both soluble (Sol Gal-3) as well as immobilized (IM Gal-3) form induces secretion of MMP-9 and IM Gal-3 promotes motility of melanoma cells. **a** Conditioned media of F10 cells grown on uncoated wells was compared with those grown in presence of 0.5 μg/ml sol gal-3 or on different amounts of IM gal-3 (0.1 ml/well of 10, 25, 50 and 75 μg/ml in 96 well plate). **b**, **c** Represent data comparing levels of MMPs in conditioned media of F1 and F10 cells grown on uncoated (UN, *lanes 1*, *4*) or in presence of soluble (Sol Gal-3, 0.5 μg/ml) (*lanes 2*, *5*) and immobilized (IM Gal-3, 0.1 ml/well of 50 μg/ml) (*lanes 3*, *6*) galectin-3. **b** The data from gels incubated for 24 h, and **c** from gels incubated for 48 h in renaturation buffer. For quantification, densitometry analysis was performed and is represented in *bar graphs* below **a**, **b**, and **c**, respectively. **d**–**f** Represent time lapse video microscopy images at 0 and 20 h of wound closure on 2 % BSA and on IM Gal-3. *Right panel* depicts graphical representation of percent wound closure of d–f at 5 h interval. Mean values of triplicate for each position of the wound width of each image frame from two different experiments, was analysed using Metamorph software. * indicates $P < 0.05$ which was considered significant

adhesion on galectin-3 (Fig. 3e). Downregulation of polyLacNAc also appeared to reduce their spreading on galectin-3 as compared to that of NT cells, as seen by microscopy images and C/N ratio (Fig. 3f, g). Similarly, induction of shRNA expression affected MMP-9 secretion by these clones on galectin-3 coated plates as compared

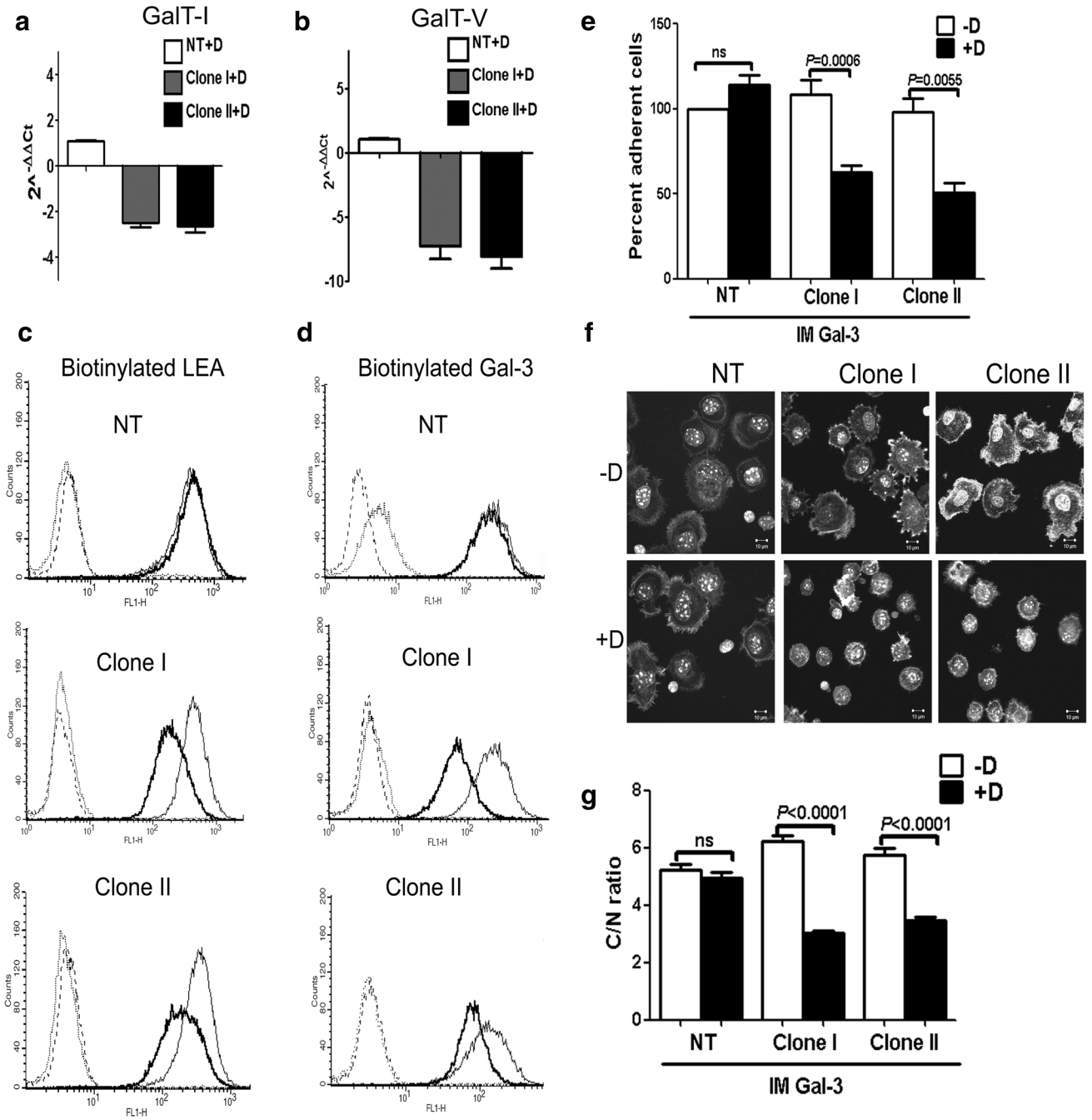

Fig. 3 Validation of down regulation of GalT-I and -V genes in clones and its effect on polyLacNAc expression, cell adhesion and spreading. **a**, **b** Represents transcript levels of beta 1,4 GalT-I and GalT-V in clones I and II respectively, after doxycycline induction by real time PCR. *NT* non targeting was used as the vector control. **c**, **d** Expression of polyLacNAc on the cell surface of clones I and II under doxycycline treated and untreated conditions by flow cytometry using biotinylated LEA and galectin-3 respectively. Cells treated with ExtraAvidin FITC only served as control, −doxycycline (*dotted line*), +doxycycline (*dashed line*). Test samples treated with doxycycline are represented as (*thick line*) and untreated samples as (*thin line*). **e** Adhesion of clones I, II and NT in presence or absence of doxycycline on galectin-3 coated plates. Values are mean ± SE of two independent experiments. **f** Spreading of NT, clone I and II on galectin-3 coated coverslips in presence or absence of doxycycline. *Scale bar* 10 μm. **g** Each *bar* represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments

to NT clone (Fig. 4a, b). The motility of clones I and II on galectin-3 was also significantly reduced on induction of shRNA expression (Fig. 4c–h).

These altered cellular properties as a result of loss of polyLacNAc had a major impact on the experimental metastasis of these clones as compared to non transduced

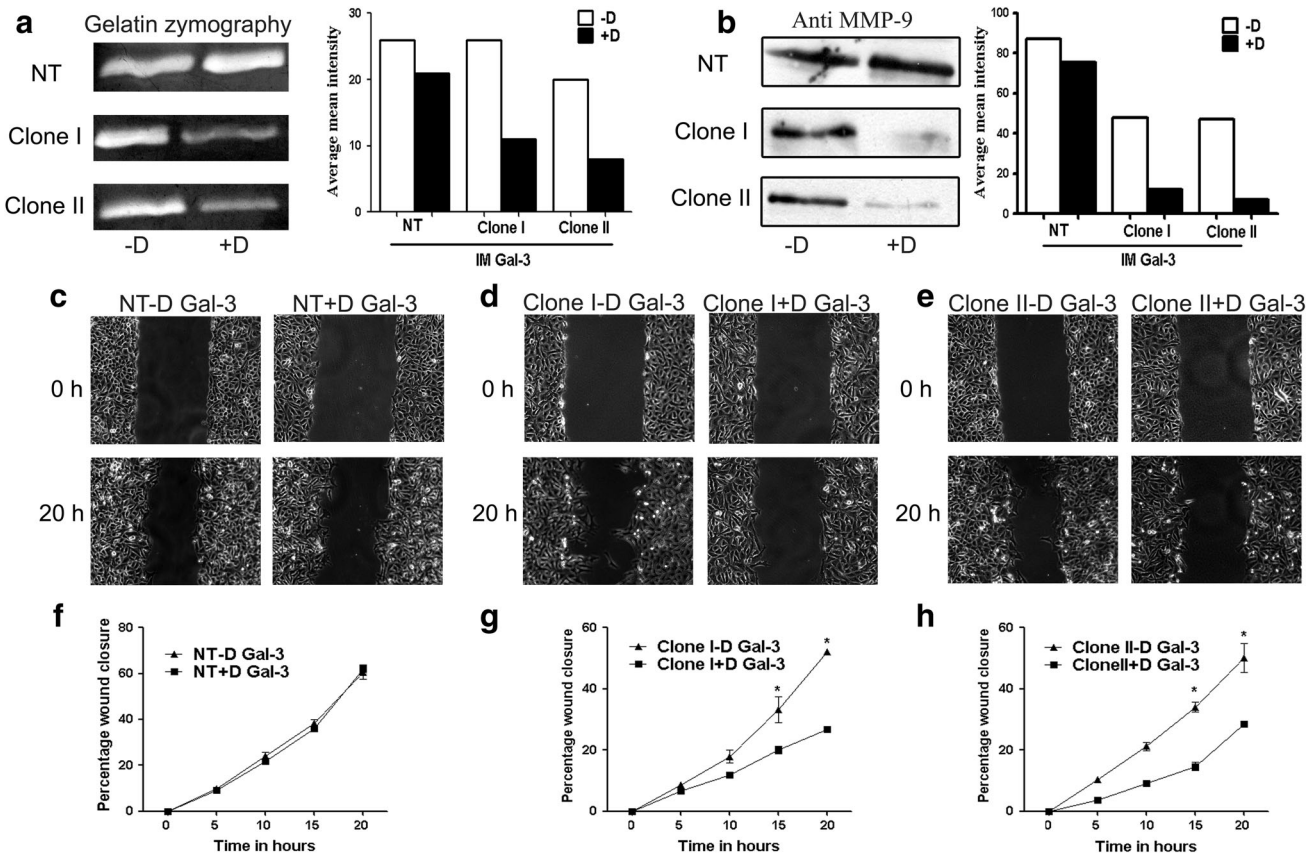

Fig. 4 Effect of polyLacNAc downregulation on secretion of MMP-9 and migration. **a** Levels of MMP-9 in culture supernatants of NT, clone I and II grown in presence and absence of doxycycline on immobilized galectin-3 as detected by zymography and **b** Western blotting. The adjacent right panel of **a**, **b** represent densitometry analysis. **c**–**e** Represent time lapse video microscopy images at 0 and 20 h of wound closure on immobilized galectin-3 of NT, clone I and II under doxycycline treated and untreated conditions, respectively. The data in **f**–**h** represents mean percent wound closure at 5 h interval. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. * indicates $P < 0.05$ which was considered significant

F10 cells or NT clones induced with doxycycline (Fig. 5a). This was also observed in the sh3 and sh6 clones generated by plasmid mediated constitutive shRNA expression. Reduced polyLacNAc levels affected galectin-3 mediated cell adhesion (Supplementary Fig. S1d), spreading and MMP-9 secretion (data not shown) resulting in significantly reduced metastatic potential of both sh3 and sh6 clones (Supplementary Fig. S1e, f).

These results collectively highlight that polyLacNAc on N-glycans on melanoma cells regulate key cellular processes that are critical for lung metastasis.

Effect of dominant negative inhibitor and the competitive sugar to galectin-3 on metastasis of F10 melanoma cells to the lungs

To confirm the role of galectin-3 as the major polyLacNAc binding lectin responsible for mediating lung specific colonization, two approaches were adopted. In the first case, all the available galectin-3 binding sites on melanoma cells were blocked using truncated galectin-3 which lacks oligomerization domain [49] and in second approach, we tried to block all the endogenous galectin-3 in mice by feeding them with MCP [44]. Both MCP and recombinant murine truncated galectin-3 (mtGal-3) were found to inhibit galectin-3 mediated spreading of F10 cells (Supplementary Fig. S2a, b). Blocking galectin-3 binding sites with excess mtGal-3 significantly reduced the metastatic potential of F10 cells (Fig. 5b). Similarly, injection of F10 cells into mice fed continuously with MCP resulted in profound decrease in their lung metastasis. MCP apparently competes with polyLacNAc on melanoma cells for binding to galectin-3 on the lungs thereby impacting metastasis (Fig. 5c).

Discussion

Interaction of specific molecules on organ endothelium and on the tumor cells is a major determinant of organ specific

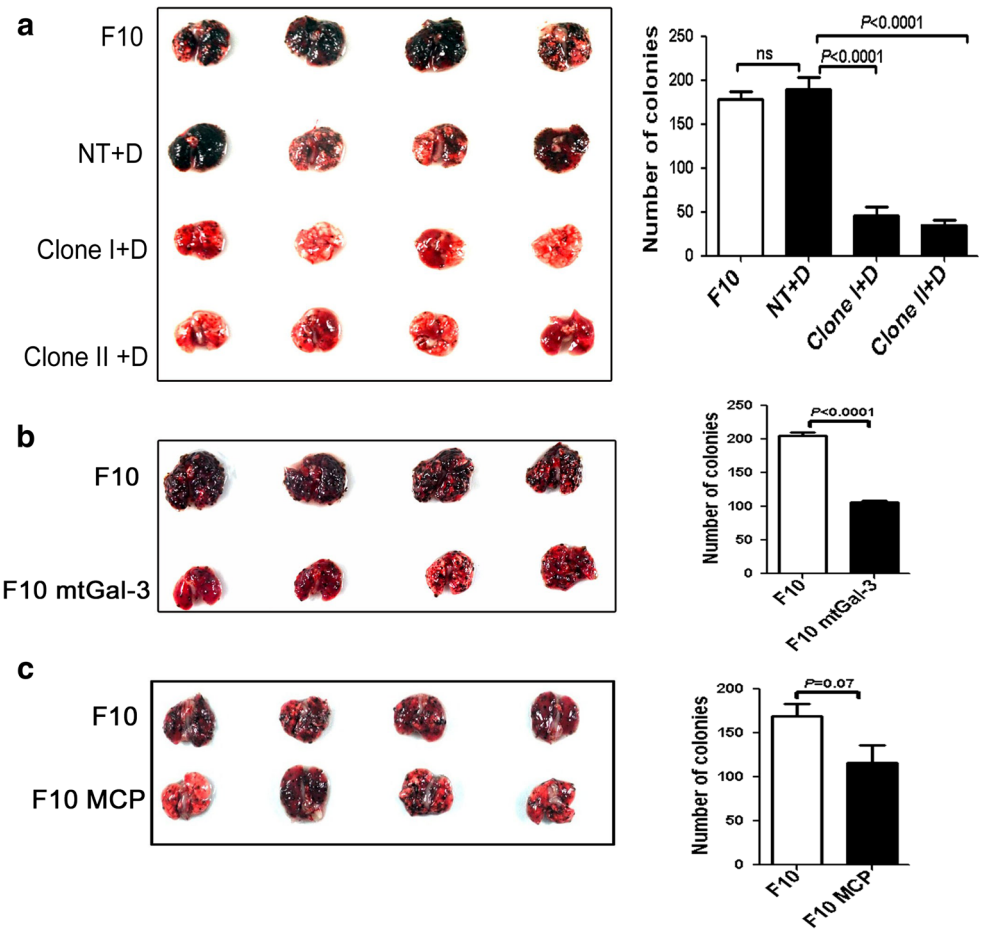

Fig. 5 Galectin-3/polyLacNAc pair plays an important role in lung metastasis. Experimental metastasis assay for **a** NT, clone I and II cells, **b**, **c** F10 cells treated with mtGal-3 and with MCP respectively (as described in "Materials and methods" section). Untreated F10 cells served as control. The *left panel* shows the lungs images while *right panel* is the graphical representation of the number of metastatic lung colonies

metastasis [3, 50]. The strength of these interactions, ability to invade the vascular BM, entry into organ parenchyma and survival in response to organ growth environment are the other key factors that determine the organ specificity of tumor cells [3, 7]. Constitutive expression of galectin-3 on the lung endothelium apparently aids arrest of tumor cells expressing high affinity easily accessible ligands in the form of polyLacNAc on *N*-oligosaccharides [27]. Lungs in mice have previously been shown to express highest levels of galectin-3 [27]. Here, we demonstrate that galectin-3 is expressed not just on the endothelial cells but on all the tissue compartments of the lungs including alveolar epithelium, bronchioles and on most pulmonary tissue spaces (Fig. 1a–f).

Galectin-3 is a multifunctional nucleo-cytoplasmic protein which is involved in different cellular functions. It can interact with transcription factors in the nucleus to regulate gene expression and also can perform anti or pro-apoptotic functions depending on its cytoplasmic or extracellular localization [29]. The secreted galectin-3 often gets incorporated on the cell surface, ECM or the BM by virtue of its ability to bind to the glycoprotein ligands and oligomerise/form lattices on cell surface [51, 52]. These studies investigated if galectin-3, present in abundance in all the major compartments of the lungs, has any role in establishing metastatic foci of cells expressing high levels of polyLacNAc on N-glycans on their surface. The interactions mediated by galectin-3 are much stronger than those via selectins and are comparable to those mediated by integrins [35, 36, 53]. However, the cells adhered to the organ endothelium via galectin-3 would need to stabilize these interactions to prevent them from being flown off under hemodynamic flow conditions and to initiate processes like vascular retraction, required to extravasate. This can be achieved by initiating the spreading of adhered cells [7].

Members like galectin-8 of the galectin family in their immobilized form have earlier been shown to regulate spreading of cells [54, 55]. We for the first time demonstrate that galectin-3 in the immobilized form induces the formation of membrane protrusions in melanoma cells which can be inhibited specifically via inhibitors of N- and not O-oligosaccharides (Fig. 1e, f).

The next barrier for effective organ colonization is the exposed vascular BM. MMPs play a major role in degradation of underlying BM and facilitate tumor cell entry into

organ parenchyma [56]. Overexpression of galectin-3 in the cytoplasmic/nuclear compartments of cells has been shown to regulate expression/secretion of MMPs, especially MMP-1, MMP-2 and MMP-9 and promote invasion. Nuclear galectin-3 in gastric cancer cells was shown to interact with AP-1 transcription factor and regulate the expression of MMP-1 [57]. Further, silencing the expression of galectin-3 in human tongue carcinoma and pancreatic cell lines affected β-catenin levels which in turn correlated with reduced levels of MMP-2 and MMP-9 [58, 59]. Galectin-3 has collagenase like repeats adjacent to its N-terminal domain which can act as a cleavage site for MMPs and cleaved form appears to serve as a marker for cancer progression [60]. Lungs express galectin-3 in highest amounts [27] which may be present in both soluble and immobilized form in different tissue compartments including vascular BM. We show that both immobilised as well as soluble forms of galectin-3 induce secretion of MMP-9 in a dose dependent manner (Fig. 2a–c). Secretion of MMP-9 by melanoma cells correlated with their metastatic potential (Fig. 2b, c). Ours is the first study which reports that extracellular galectin-3 induces the secretion of MMP-9 in melanoma cells, most likely via the polyLacNAc on N-glycans. MMP-9 mediates degradation of ECM but it has to be coupled with movement for effective extravasation of tumor cells.

Galectin-3 expressed in various compartments has been associated with motility of wide range of cell types. In the soluble form, it has been shown to induce reorganization of cytoskeleton which in turn facilitates motility of corneal epithelial cells [61]. Cell surface galectin-3 localises in lipid raft and its absence affects the formation of membrane ruffles and lamellipodia [62]. Overexpression of galectin-3 in cytoplasm of oral tongue squamous carcinoma cells (OTSCC) enhanced motility via wnt/beta-catenin signalling pathway [63]. Also, at low concentrations galectin-3 can act as chemoattractant, for monocytes and macrophages [64].

Using wound healing assays, we demonstrate that immobilised galectin-3 which often gets incorporated as part of ECM and BM can itself be used as traction for forward motility of melanoma cells. The dependence of cellular motility on the metastatic potential of the cells and their N- and not O-glycosylation status confirmed that it is indeed mediated by N-oligosaccharides on surface glycoproteins (Fig. 2d–f). Although, other members like galectin-8 have been shown to facilitate movement of cells in a similar manner [65], here we demonstrate that even immobilized galectin-3 can facilitate haptotactic motility. Galectin-3 mediated motility would be important for cells to move into lung parenchyma.

Beta 1,6 branched expressed on N-oligosaccharides of cell surface proteins is the preferred site for further substitution of polyLacNAc. Several proteins that carry β1,6 branched N-oligosaccharides may also carry polyLacNAc. Some of the possible carrier proteins include integrin subunits (α3, α5, αv and β1), growth factor receptors like EGFR and others like CD-44 (hyaluronate receptors) and lysosome associated membrane proteins (LAMPs) [66, 67]. The cancer cells most possibly use surface receptors expressing polyLacNAc on N-oligosaccharides for motility. Galectin-3 may also promote proliferation by sustained signalling via growth factor receptors by restricting them in the lattices and preventing their internalization [68, 69].

PolyLacNAc is synthesized by the sequential addition of *N*-acetylglucosamine and galactose by the enzymes β1,3 *N*-acetylglucosaminyltransferases or β3GnTs and β1,4 galactosyltransferases—GalTs [45]. Among the six possible enzymes that add galactose, the expression of GalT-I and -V correlated with metastatic potential of B16 melanoma cells (Supplementary Fig. 1a). Downregulation of these two genes by shRNA mediated plasmid and inducible lentiviral vectors, showed marked reduction in polyLacNAc expression (Fig. 3a, b, Supplementary Fig. 1b, c). This was accompanied with inhibition of all the galectin-3 mediated processes like adhesion, spreading, movement and induction of MMP-9 secretion (Fig. 3e–g, Supplementary Fig. 1d, 4a–h). Inhibition of experimental metastasis as a result of inhibition of all the galectin-3 mediated processes highlighted the importance of polyLacNAc and galectin-3 pair in facilitating lung colonization (Fig. 5a) (Supplementary Fig. 1e, f).

Galectin-3 is a monomeric lectin that forms oligomers on binding to its ligand via its N-terminal domain. Truncated galectin-3 devoid of the N-terminal domain has been shown to act as a dominant negative inhibitor of galectin-3. Truncated galectin-3 affected growth and lymph node metastasis of breast cancer cell line on sustained treatment [49]. Pre-incubation of B16F10 cells with truncated galectin-3 inhibited lung metastasis apparently by blocking polyLacNAc on melanoma cells making it unavailable for binding to galectin-3 on the lung vascular endothelial cells (Fig. 5b). MCP has been shown to affect several galectin-3 mediated processes including metastasis [70]. Inhibition of experimental metastasis in mice fed with MCP indicated that MCP in circulation possibly competes with polyLacNAc on melanoma cells for binding to endothelial cells (Fig. 5c). However, the contribution of galectin-3 as an immunomodulating agent also, cannot be ruled out in facilitating melanoma metastasis [32].

Our studies very clearly demonstrate the importance of galectin-3 and polyLacNAc in not just mediating adhesion to lung endothelium but also in several downstream processes critical for lung homing. It would be interesting to study the molecular pathways activated downstream of

galectin-3/polyLacNAc interactions which regulate the successive events involved in metastasis. We expect that confirmation of existence of similar mechanisms in lung metastasis of human tumors would open up several interesting avenues to explore and would also be crucial in developing effective strategies to prevent metastasis.

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Conflict of interest No potential conflicts of interest were disclosed.

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

Manohar C. Dange · Akhil Kumar Agarwal · Rajiv D. Kalraiya

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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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M. C. Dange · A. K. Agarwal · R. D. Kalraiya (✉)
Kalraiya Lab, KS 131, Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Tata Memorial Centre, Sector 22, Kharghar, Navi Mumbai 410210, India
e-mail: rajivkalraiya@gmail.com; rkalraiya@actrec.gov.in

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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).

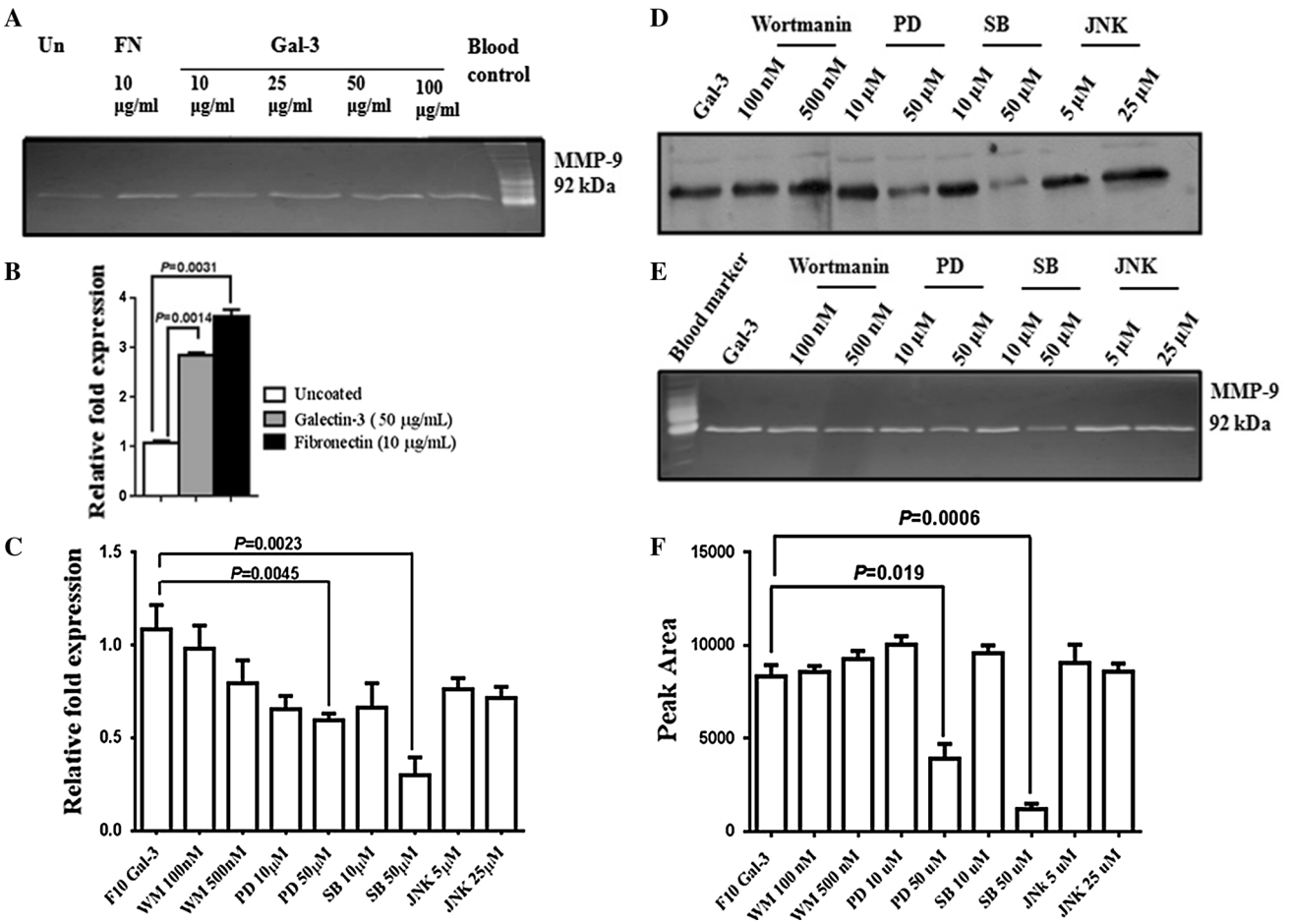

Fig. 1 Immobilized galectin-3 induces expression of MMP9 through activation of p38 MAPK pathway **a** MMP-9 levels in culture supernatant (conditioned media) of F10 cells grown under serum-free conditions for 24 h on uncoated wells were compared with those grown similarly on plates coated with fibronectin 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

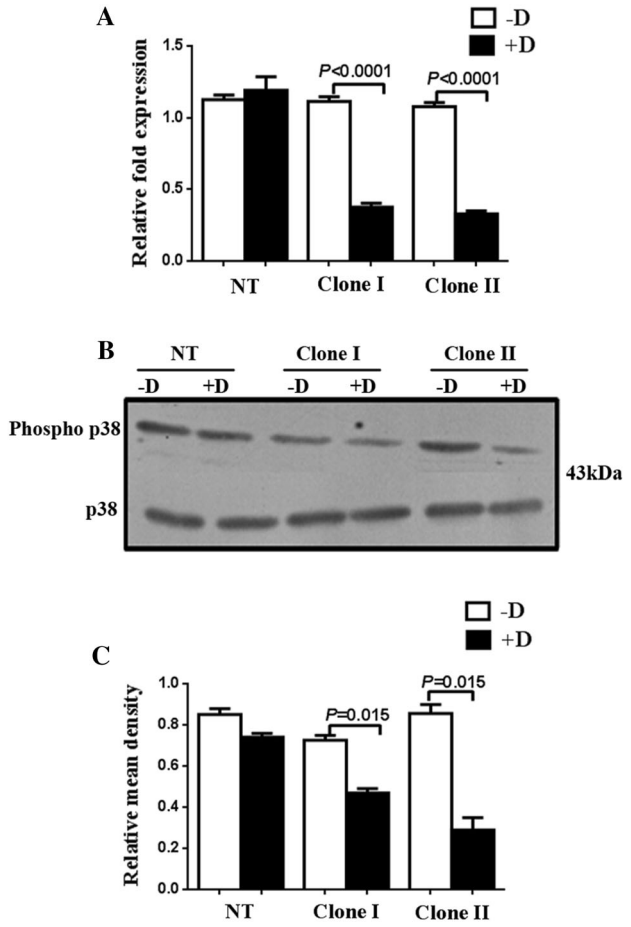

Fig. 2 Downregulation of polyLacNAc affects MMP9 induction via activation of p38 MAPK pathway. **a** *Bar graph* represents analysis of MMP9 transcript levels in polyLacNAc downregulated clones I and II grown on galectin-3-coated plates in doxycycline-treated and -untreated conditions (+D and −D, respectively). 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

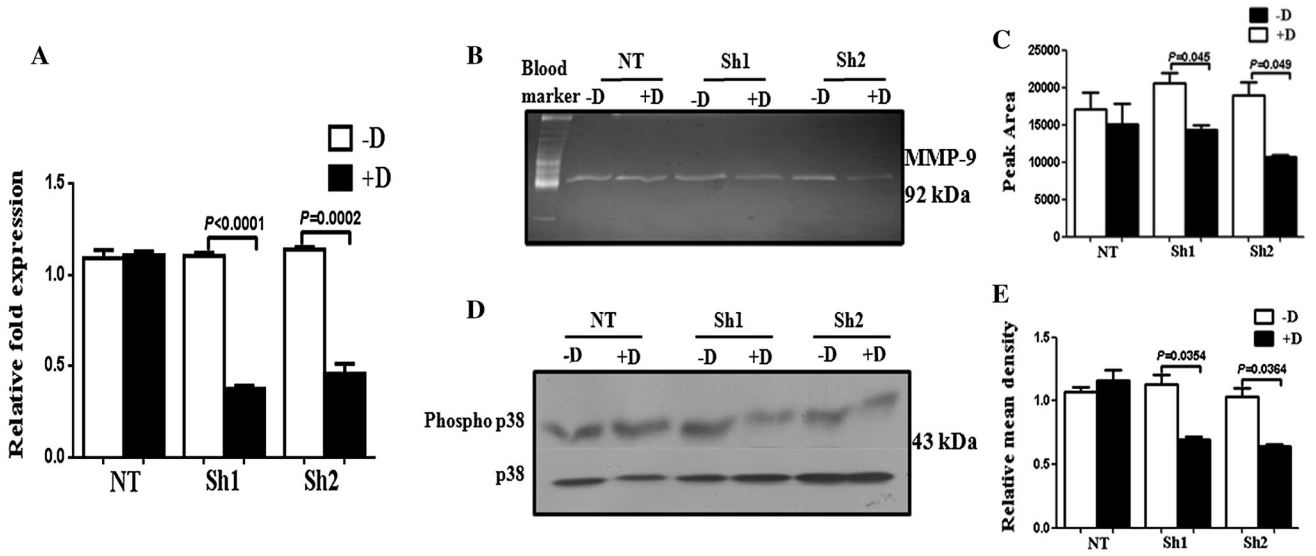

Fig. 3 Downregulation of LAMP1 affects galectin-3-induced MMP9 expression via p38 MAPK pathway. **a** *Bar graph* represents analysis of levels of MMP9 transcripts by real-time PCR for NT and LAMP1 downregulated clones Sh1 and Sh2, grown on galectin-3-coated plates in the absence (−D) and presence of (+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

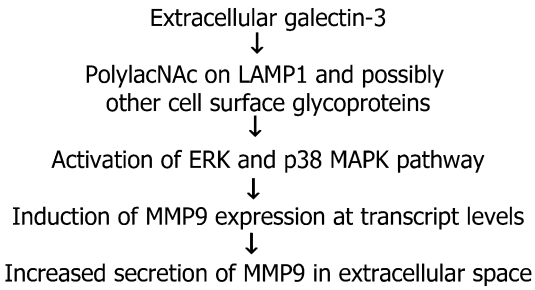

Fig. 4 Schematic representation summarizing the overall events involved in galectin-3 induced MMP9 expression in melanoma cells

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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