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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

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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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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

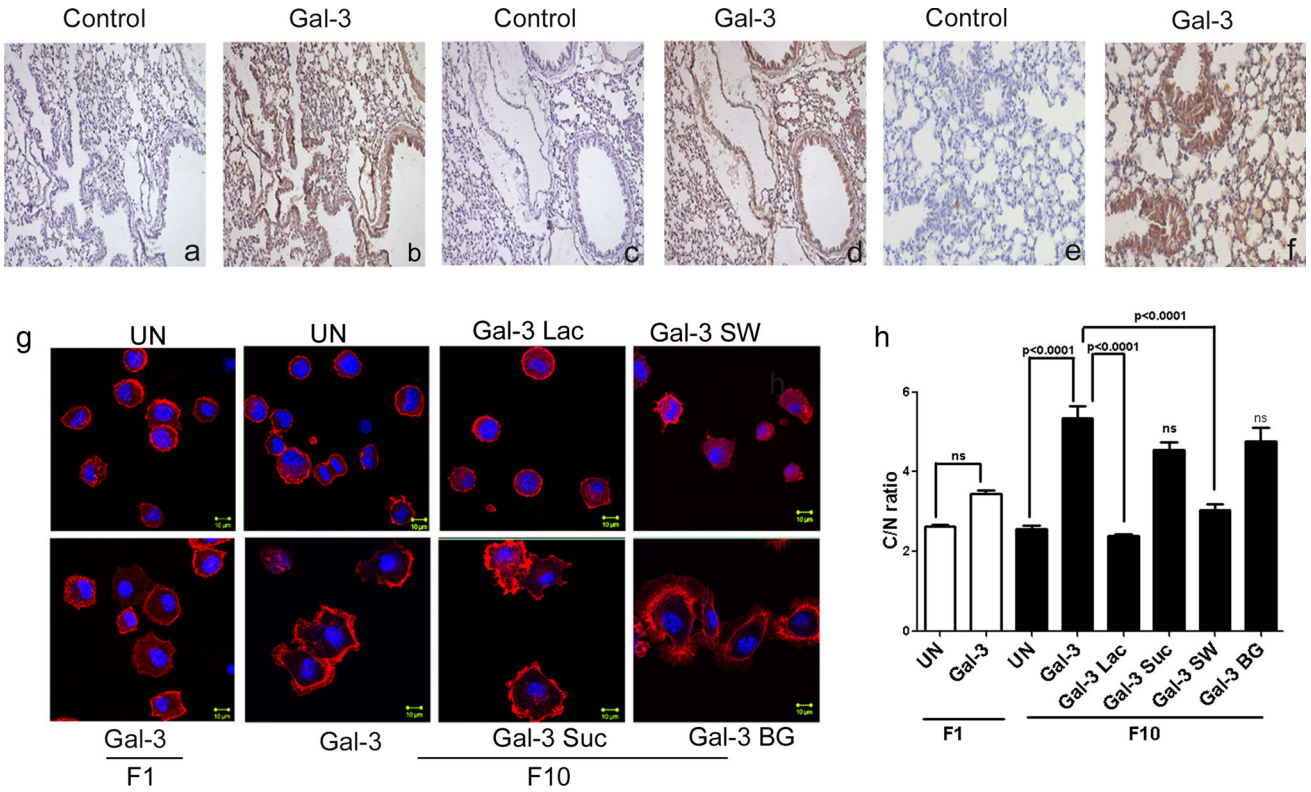

Fig. 1 Galectin-3 is expressed in all the major compartments of lungs and it facilitates spreading of melanoma cells. **a**–**f** Immuno-histochemical staining of mouse lung sections treated with rat anti-mouse galectin-3 antibody at ×10, ×20 and ×40 magnifications, respectively. The control sections 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

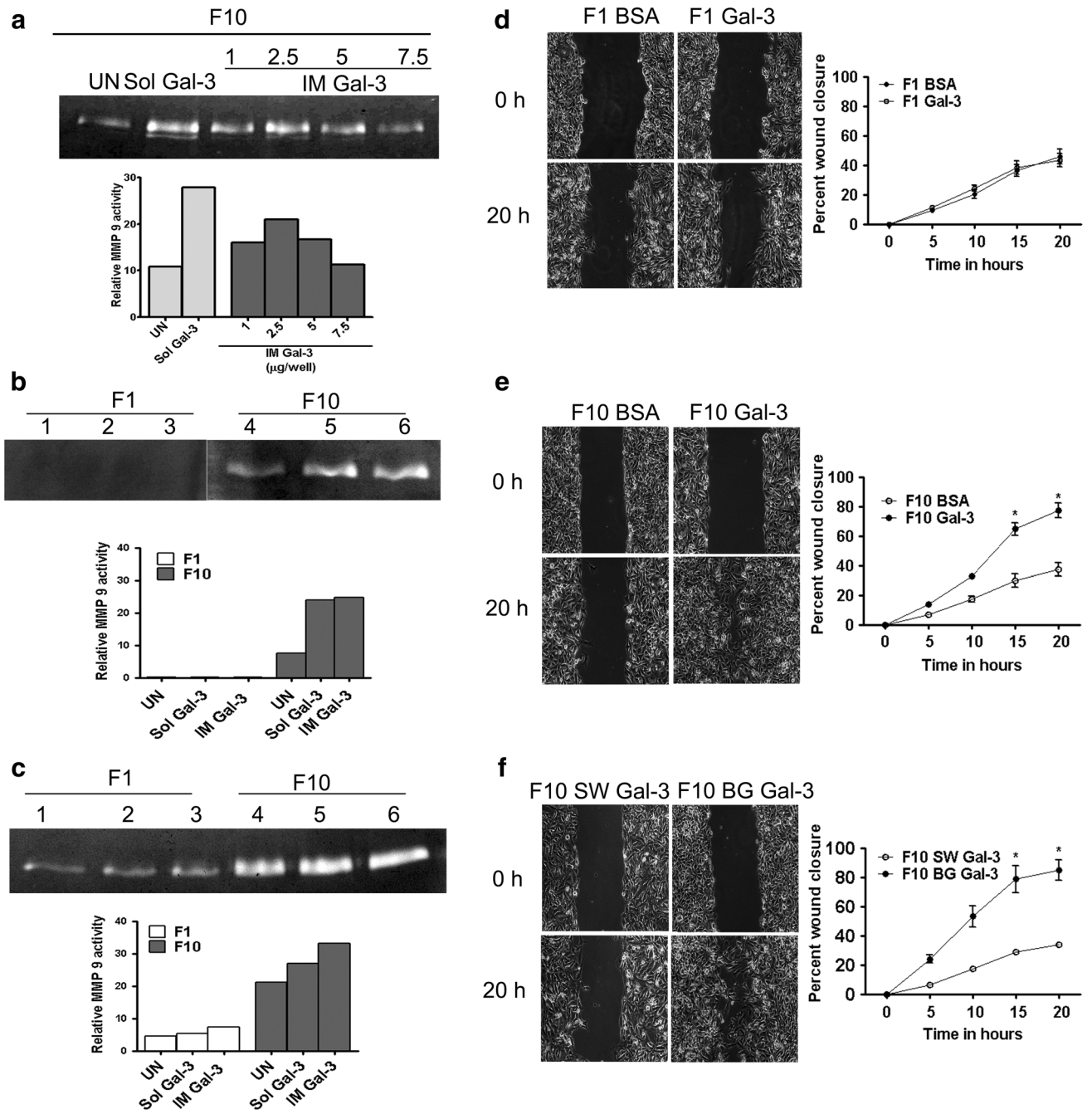

Fig. 2 Galectin-3 in both soluble (Sol Gal-3) as well as immobilized (IM Gal-3) form induces secretion of MMP-9 and IM Gal-3 promotes motility of melanoma cells. **a** Conditioned media of F10 cells grown on uncoated wells was compared with those grown in presence of 0.5 μg/ml sol gal-3 or on different 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

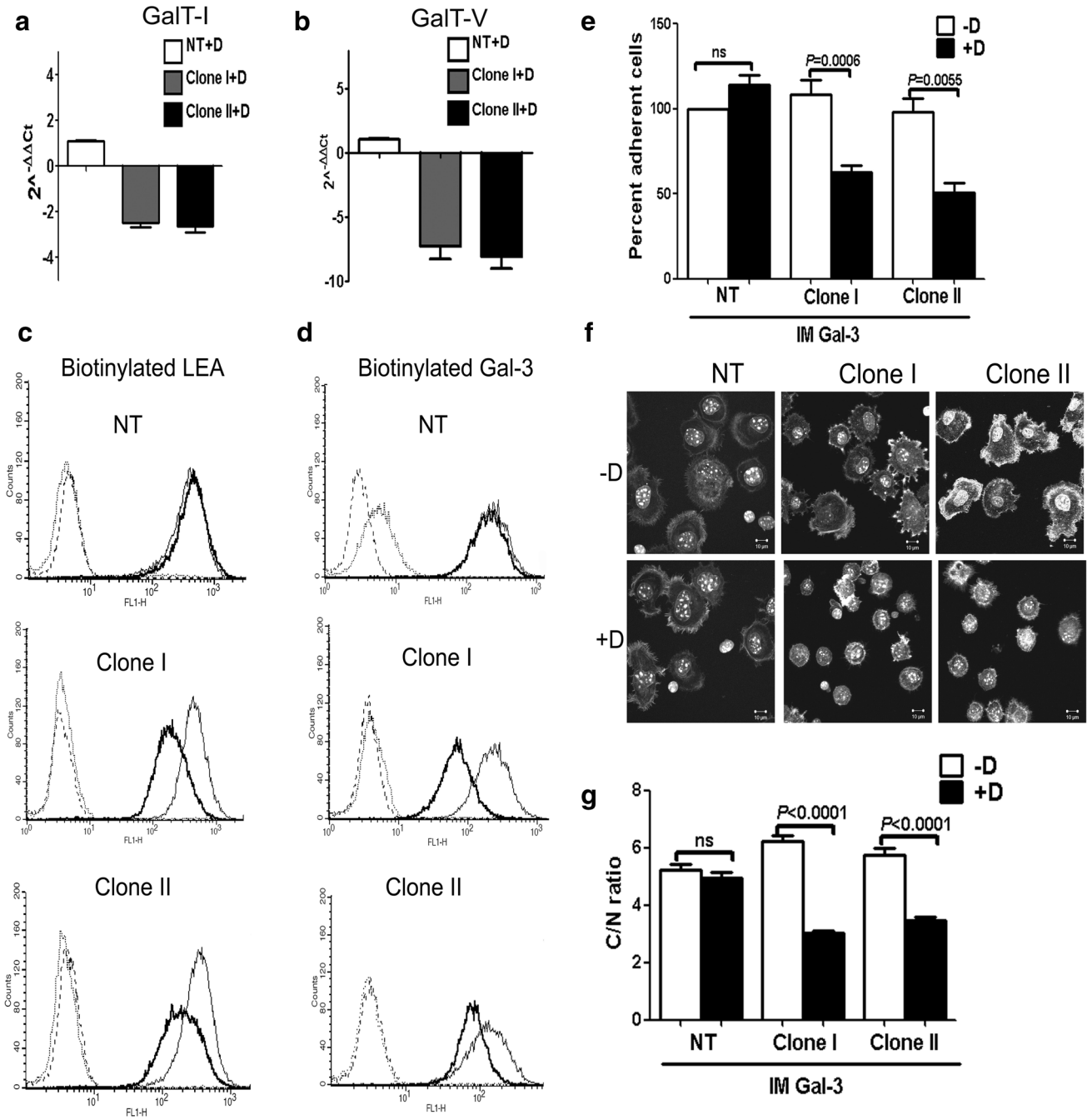

Fig. 3 Validation of down regulation of GalT-I and -V genes in clones and its effect on polyLacNAc expression, cell adhesion and spreading. **a**, **b** Represents transcript levels of beta 1,4 GalT-I and GalT-V in clones I and II respectively, after doxycycline induction by real time PCR. *NT* non targeting 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

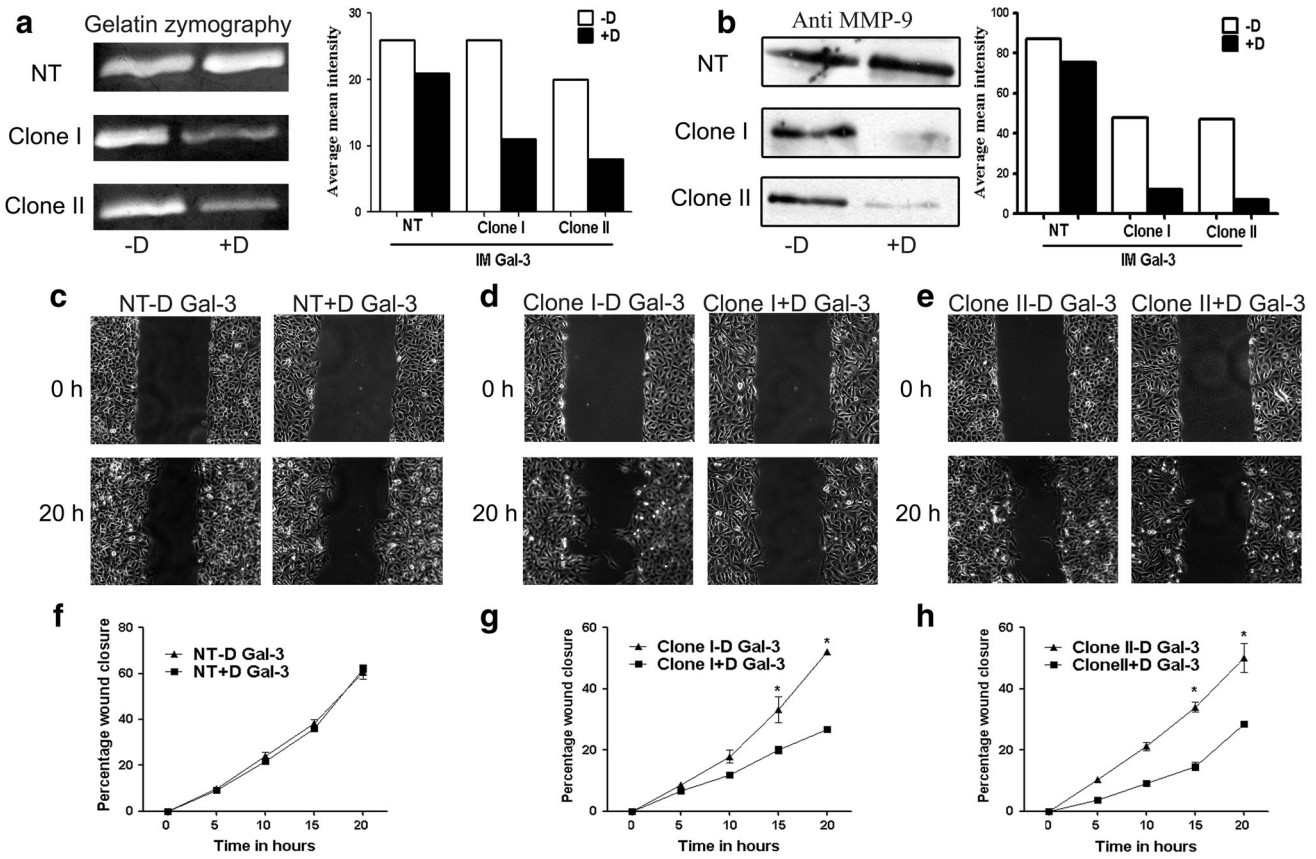

Fig. 4 Effect of polyLacNAc downregulation on secretion of MMP-9 and migration. **a** Levels of MMP-9 in culture supernatants of NT, clone I and II grown in presence and absence of doxycycline on immobilized galectin-3 as detected by zymography and **b** Western blotting. The adjacent right panel of **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

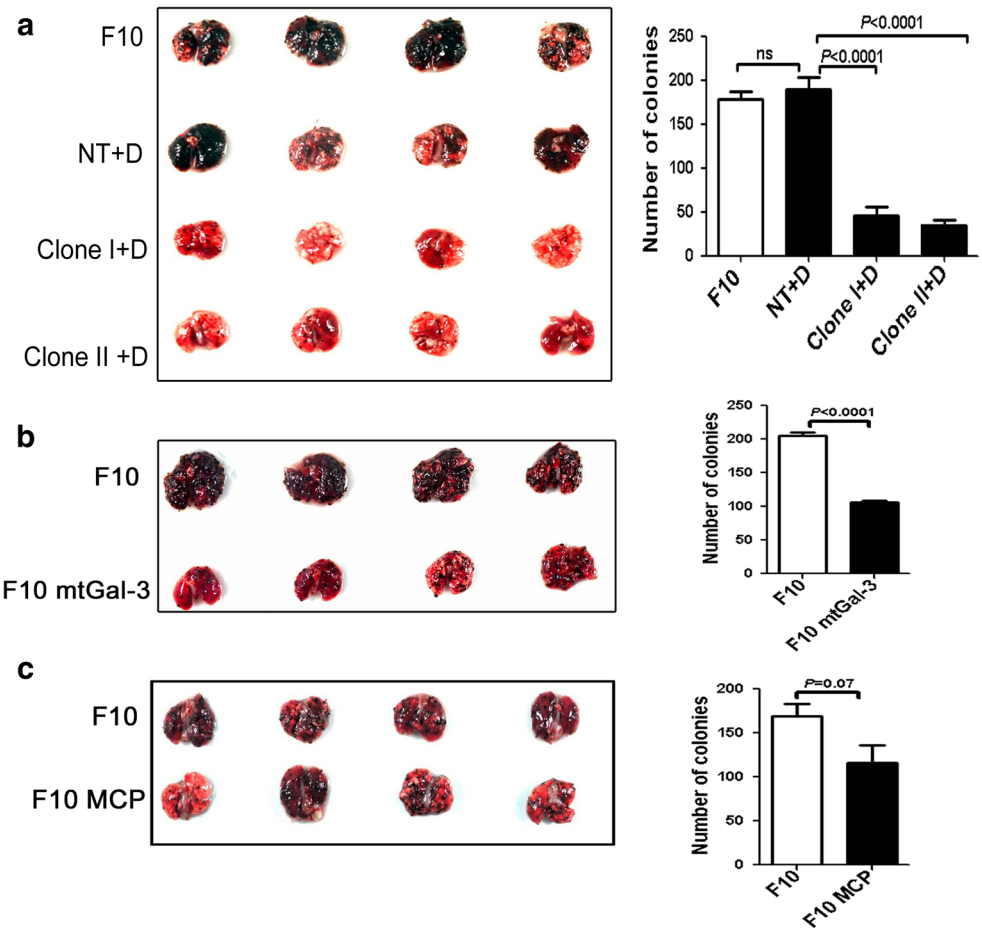

Fig. 5 Galectin-3/polyLacNAc pair plays an important role in lung metastasis. Experimental metastasis assay for **a** NT, clone I and II cells, **b**, **c** F10 cells treated with mtGal-3 and with MCP respectively (as described in "Materials and methods" section). Untreated F10 cells served as control. 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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