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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Cell lines and reagents

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

2.2. Cell culture

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

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

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

LAMP1 shRNA1 forward primer:

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

LAMP1 shRNA1 reverse primer:

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

LAMP1 shRNA2 forward primer:

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

LAMP1 shRNA2 reverse primer:

5′GTTGAATTCCGAGGCAGTAGGCA**GCGTTCAACATCAGCCCAAAT**TACATCTGTGGCTTC3′.

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

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

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

2.5. Flow cytometric analysis

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

2.6. Purification of recombinant human galectin-3

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

2.7. Cell spreading assays

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

2.8. Wound healing assays

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

2.9. Experimental metastasis assay

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

2.10. Statistical analysis

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

3. Results and discussion

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

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

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

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

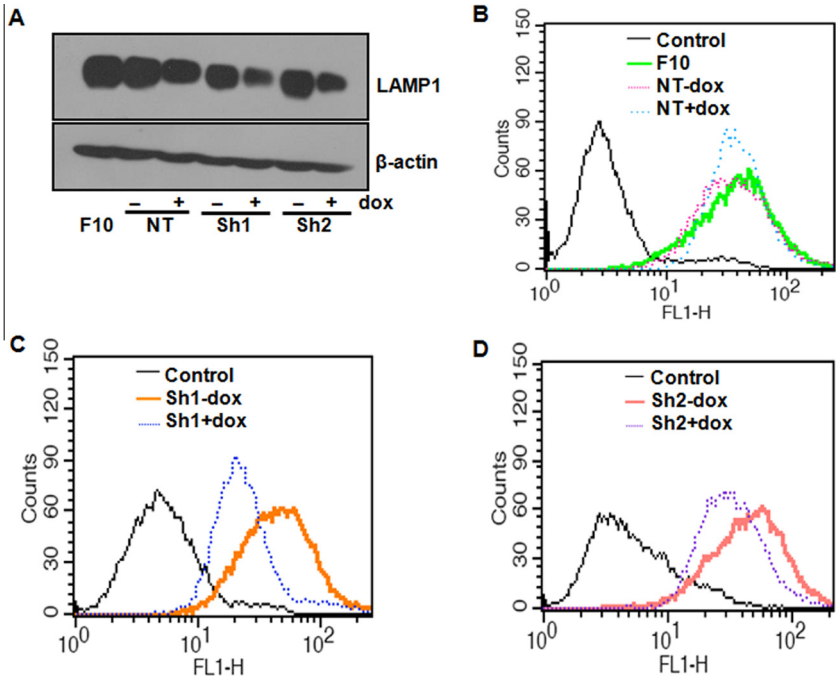

Fig. 1. Analysis of total and surface expression of LAMP1 in B16F10 cells transduced with LAMP1 shRNAs. Comparison of LAMP1 expression in B16F10 cells and its clones transduced with either non-targeting shRNA (NT), shRNA1 (Sh1) and shRNA2 (Sh2) induced with doxycycline (+dox) or under uninduced condition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

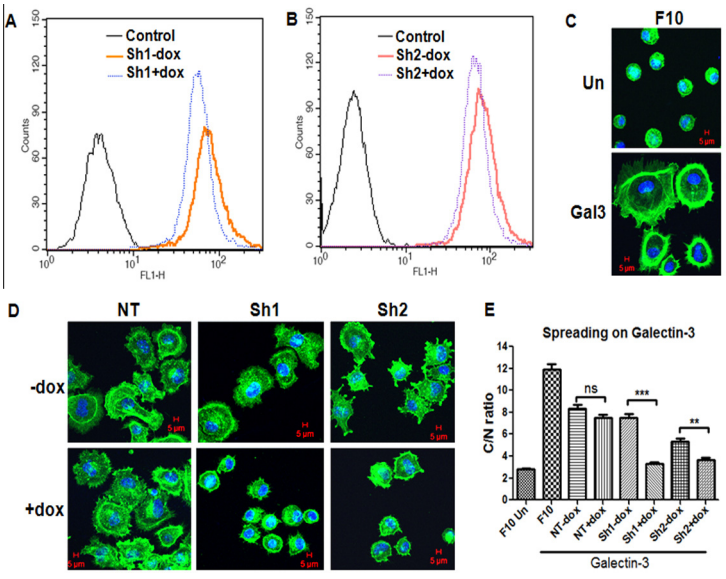

Fig. 2. Effect of decreased surface expression of LAMP1 on galectin-3 binding and spreading of melanoma cells on galectin-3. Comparison of galectin-3 binding by flow cytometry using galectin-3-FITC on surface of B16F10 cells transduced with LAMP1 shRNA clones, Sh1 (A) and Sh2 (B), either uninduced (−dox)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

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

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

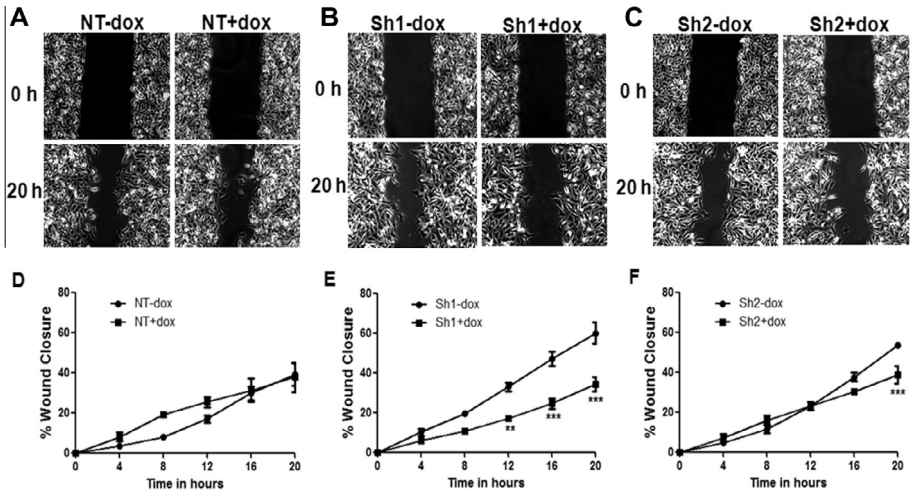

Fig. 3. Effect of decreased surface expression of LAMP1 on motility of melanoma cells on galectin-3. Motility on galectin-3 coated plates of B16F10 cells transduced with either (A) non-target shRNA (NT) or LAMP1 shRNAs, Sh1 (B) & Sh2 (C) without (−dox) or with (+dox) induction of shRNA by doxycycline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

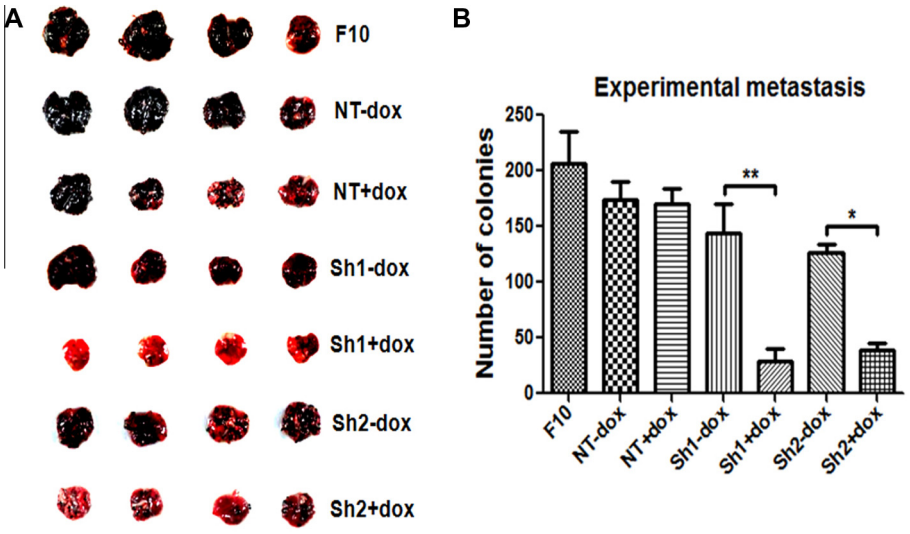

Fig. 4. Effect of decreased surface expression of LAMP1 on lung metastasis. (A) Melanoma colonies on lungs of C57BL/6 mice injected with F10, NT−dox, NT+dox, Sh1−dox, Sh1+dox, Sh2−dox and Sh2+dox cells. (B) Graphical representation of mean number of lung colonies. One-way analysis of variance followed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

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

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

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

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

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