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Galectin-3 interacts with the cell surface glycoprotein CD146 (MCAM, MUC18) and induces secretion of metastasis-promoting cytokines from vascular endothelial cells

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Running title: galectin-3-CD146 interaction induces endothelial cytokine secretion

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Abstract

The galactoside-binding protein galectin-3 is increasingly recognized as an important player in cancer development, progression, and metastasis via its interactions with various galactoside-terminated glycans. We have previously shown that circulating galectin-3, that is increased by up to 30-fold in cancer patients, promotes blood-borne metastasis in an animal cancer model. This effect is partly attributable to galectin-3's interaction with unknown receptor(s) on vascular endothelial cells and causes endothelial secretion of several metastasis-promoting cytokines. Here, we sought to identify the galectin-3-binding molecule(s) on the endothelial cell surface responsible for the galectin-3-mediated cytokine secretion. Using two different galectin-3 affinity purification processes we extracted four cell-membrane glycoproteins, CD146/MCAM/MUC18, CD31/PECAM-1, CD144/VE-cadherin, and CD106/Endoglin, from vascular endothelial cells. CD146 was the major galectin-3-binding ligand and strongly co-localized with galectin-3 on endothelial cell surfaces treated with exogenous galectin-3. Moreover, galectin-3 bound to N-linked glycans on CD146 and induced CD146 dimerization and subsequent activation of AKT signalling. siRNA-mediated suppression of CD146 expression completely abolished the galectin-3-induced secretion of IL-6 and GCSF cytokines from the endothelial cells. Thus, CD146/MCAM is the functional galectin-3-binding ligand on endothelial cell surfaces responsible for galectin-3-induced secretion of metastasis-promoting cytokines. We conclude that CD146/MCAM interactions with circulating galectin-3 may have an important influence on cancer progression and metastasis.

Introduction

Galectin-3 is a galactoside-binding protein that is expressed by many types of human cells. Overexpression of galectin-3 commonly occurs in most types of cancer (1,2) and is increasingly recognized to influence cancer cell-cell and cancer-microenvironment communications and contributes to cancer development, progression and metastasis as a result of galectin-3 interaction with various galactose-terminated glycans carried on the cell surface and in the extracellular matrix(2).

Earlier studies by ourselves as well as by other groups have revealed that the concentration of circulating galectin-3 is markedly increased up to 30-fold in the bloodstream of cancer patients including those with colorectal, breast, lung, bladder, pancreatic, head and neck cancers and melanoma(2-8). Patients with metastatic disease tend to have higher concentrations of circulating galectin-3 than those with only localized tumours (3). Our recent study has revealed that increased circulating galectin-3 is an important promoter of blood-borne metastasis in a mouse model (9). This effect of galectin-3 is shown to be partly attributed to galectin-3 interaction with the oncofetal

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Thomsen-Friedenreich (Galβ1,3GalNAcα-, TF) [1] disaccharide expressed on the cancer-associated transmembrane mucin protein MUC1(10,11), which is expressed by the majority of epithelial cancer cells (12). The galectin-3-MUC1 interaction induces MUC1 cell surface polarization leading to exposure of the underlying adhesion molecules. This results in increased tumour cell heterotypic adhesion to the vascular endothelium as well as increased tumour cell homotypic aggregation to form tumour emboli (13). Circulating galectin-3 is also shown to promote metastasis by interaction with as yet unknown molecules on the endothelial cell surface and induces endothelial secretion of cytokines Interleukin-6 (IL-6), Granulocyte Colony-Stimulating Factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Intercellular Adhesion Molecule-1(sICAM-1) (14).

Pro-inflammatory cytokines such as IL-6 and G-CSF are important metastasis-promoters and are known to enhance various steps in the metastatic cascade including cancer-endothelial adhesion, cell proliferation, migration and angiogenesis(15,16). We showed that the increased secretion of these cytokines from the blood vascular endothelium generated by exogenous galectin-3 increases the expression of various adhesion molecules on the endothelial cell surface, including integrins and E-selectin either in an endocrine and/or paracrine way, resulting in increased cancer cell-endothelial adhesion, endothelial cell migration and angiogenesis (14). The galectin-3 binding ligand(s) on the endothelial cell surface responsible for galectin-3 mediated-endothelial secretion of these metastasis-promoting cytokines however has remained unknown.

We report in this study that CD146/MCAM (melanoma cell adhesion molecule, also known as MUC18) is the galectin-3 binding ligand on endothelial cell surface responsible for circulating galectin-3-mediated endothelial secretion of cytokines.

[1] **Abbreviations:** Gal3, galectin-3; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HUVECs, human umbilical vein endothelial cells; HMVEC-Ls, human microvascular lung endothelial cells; sICAM-1, soluble intercellular adhesion molecule-1; IL-6, Interleukin-6; MCAM, melanoma cell adhesion molecule, CD146, MUC18; PECAM, CD31, platelet endothelial cell adhesion molecule.

Results

Galectin-3 induces cytokine secretion by micro- and macro-vascular endothelial cells. Our earlier study has reported that circulating galectin-3 induces secretion of IL-6, G-CSF, GM-CSF and ICAM-1 from human micro-vascular endothelial cells (14). Before proceeding to identify the endothelial cell surface galectin-3 binding molecules for galectin-3-induced cytokine secretion, we first compared the effect of galectin-3 on secretion of these cytokines from human micro- and macro-vascular endothelial cells. It was found that the presence of galectin-3 at concentrations similar to those seen in cancer patients (3) increased secretion of IL-6, G-CSF, GM-CSF and ICAM-1 from human micro-vascular lung endothelial cells (HMVEC-Ls) and also from macro-vascular umbilical vein endothelial cells (HUVECs) (Fig 1). This suggests that the effect of galectin-3 on endothelial secretion of these cytokines can occur in both micro- and macro-vasculature.

Four endothelial cell membrane glycoproteins were extracted by galectin-3 affinity purification

Galectin-3 blotting/overlay demonstrated similar binding of galectin-3 to several proteins in HUVECs and HMVEC-Ls (Fig 2A). Two galectin-3 affinity purification approaches, His-tagged recombinant galectin-3 conjugated to Nickel (Ni) and to agarose, were used to extract galectin-3 binding proteins from endothelial cells. After removal of unbound proteins, bound proteins were eluted from the columns with lactose. Various proteins ranging from ~50 to ~250kDa were seen to be extracted by both the affinity purification processes shown by silver staining following SDS-PAGE (Fig 2B). Mass spectrometry analysis revealed a number of cell membrane and cytosolic proteins. The top scored cell membrane proteins in both the galectin-3-Ni and galectin-3-agarose affinity extracts were CD146/MCAM (melanoma cell adhesion molecule, MUC18), CD31/PECAM-

1(Platelet endothelial cell adhesion molecule), CD144/VE-cadherin, and CD106/Endoglin (11~28 peptides of each protein).

CD146/MCAM is the major galectin-3 binding protein on the endothelial cell surface. To confirm interaction of galectin-3 with these cell membrane proteins, we treated the cells with galectin-3 followed by immunoprecipitation with antibodies against each of the four identified proteins. Galectin-3 was predominately found in the immunoprecipitates of CD146/MCAM and CD106/Endoglin (Fig 3A), suggesting possible binding ligands among these proteins. In addition to galectin-3 and the antibody/immunoglobulin heavy and light chains, two other protein bands were also seen in the blots of the immunoprecipitates (Fig 3). As these two protein bands occurred in all the immunoprecipitates including the control IgG immunoprecipitates, they reflect non-specific antibody/immunogloblin binding.

To further determinate interaction of galectin-3 with these cell membrane proteins, we cross-linked the cell surface proteins with a cleavable crosslinker DTSSP after treatment of the cells with recombinant galectin-3. Subsequent immunoprecipitation with antibodies against each of these proteins revealed presence of galectin-3 only in the CD146/MCAM immunoprecipitates (Fig 3B). This indicates that CD146/MCAM represents the major galectin-3 binding ligand on endothelial cell surface. Analysis by confocal microscopy showed weak expression of endogenous galectin-3 by the cells (Fig 4). Strong cell surface co-localization of CD146/MCAM with galectin-3 occurred when exogenous galectin-3 was introduction (Fig 4).

Galectin-3 binds to N-linked glycans expressed on CD146/MCAM.
To gain insight into the interaction of galectin-3 with CD146/MCAM, endothelial cells were first treated with N-glycanase (PNGaseF) or *O*-glycanase (from *Streptococcus pneumoniae*) before treatment with recombinant galectin-3. The cells were lysed and immunoprecipitated with anti-CD146/MCAM antibody. Treatment of the cells with N-glycanase reduced the size of CD146/MCAM molecular weight in SDS-PAGE as a result of reduction of N-linked sugar chains (Fig 5, top panel). Reduction of N-glycans on CD146/MCAM substantially reduced (35%) the presence of galectin-3 in the immunoprecipitates (Fig 5, bottom panel) whilst treatment of the cells with O-glycanase showed no effect on galectin-3 presence in CD146/MCAM immunoprecipitates. This suggests that galectin-3 binds predominantly to N-linked glycans on CD146/MCAM.

Suppression of CD146/MCAM expression abolishes galectin-3-induced cytokine secretion.
To determine whether CD146/MCAM is the functional ligand responsible for galectin-3-induced cytokine secretion of endothelium, we suppressed CD146/MCAM expression by siRNA. SiRNA treatment caused 80% reduction of cellular CD146/MCAM expression in comparison to the cells treated with control siRNA (Fig 6A). Suppression of CD146/MCAM expression completely abolished the effect of galectin-3 on endothelial secretion of IL-6 and G-CSF (Fig 6B and C). This indicates that CD146/MCAM is the galectin-3 binding receptor responsible for galectin-3-induced endothelial secretion of the cytokines.

Galectin-3 induces CD146/MCAM dimerization and activation of down-stream AKT signalling.
CD146/MCAM activity is known to be associated with CD146/MCAM dimerization (17,18) and downstream activation of AKT signalling (19-21). To determine whether galectin-3-CD146/MCAM interaction affects CD146/MCAM activity, we assessed CD146/MCAM dimerization and AKT activation in cell response to galectin-3. Introduction of galectin-3 caused a time-dependent increase of CD146/MCAM dimerization which was detected in non-denatured (Fig 7A) but not denatured SDS-PAGE condition (Fig 7B). Galectin-3 treatment also caused similarly time-dependent increase of AKT phosphorylation (Fig7C) as CD146/MCAM dimerization.

Galectin-3 activates several downstream signalling pathways.
To gain further insight into the global effect of galectin-3 on endothelial cytokine secretion, we compared the activity of 46 tyrosine kinases in response to galectin-3 using a human Phospho-Kinase array. Phosphorylation of several

tyrosine kinases was substantially increased in response to 24 hr treatment of endothelial cells with galectin-3 (Fig 8). These include HSP60 (39 fold), WINK1 (13 fold), p27 (6.9 fold) β-catenin (4.4 fold), c-jun (4 fold), p70 S6 Kinase (3.6 fold), Creb (2.2 fold), p53 (S392 and S15, 2.8 fold and 1.8 fold respectively) and PRAS40 (1.5 fold). This suggests that endothelial secretion of cytokines in response to galectin-3, which was shown previously to take several hours (14) and is shown in this study to be associated with binding of galectin-3 to cell surface CD146/MCAM (Fig6, 7), may involve multiple signalling pathways. The increased phosphorylation of PRAS40, a substrate of AKT, after 24 hr treatment of the cells with galectin-3 is in keeping with the increase in CD146/MCAM dimerization and AKT activation.

Discussion

This study shows that galectin-3 at concentrations similar to those found in the blood of cancer patients induces secretion of cytokines IL-6, G-CSF, GM-CSF and ICAM-1 from both micro- and macro-vascular endothelial cells. CD146/MCAM is identified as the galectin-3 binding ligand on the endothelial surface responsible for galectin-3-mediated secretion of cytokines. Galectin-3 binds to N-glycans on CD146/MCAM and induces CD146/MCAM dimerization and downstream AKT activation.

CD146/MCAM, also known as MUC18(22), is a type I transmembrane glycoprotein that contains 8 putative N-glycosylation sites on its extracellular domain(17). Its glycosylation accounts for about 35% of the molecular weight (23). CD146/MCAM was previously considered as an endothelial cell marker and used for identification of circulating endothelial cells(24). It is now known to be expressed also by melanoma cells(17) and associated with melanoma metastasis (25-27). There are two CD146/MCAM isoforms, CD146-long and CD146-short. The CD146-long form contains a 63-aa intracellular domain including 2 putative endocytic domains and 5 potential protein kinase recognition sites, and the CD146-short form contains a truncated cytoplasmic tail lacking both of the endocytosis motifs and one of the protein kinase sites. Endothelial cells express both the long and short forms whereas melanoma cells express primarily the long isoform.

CD146 is a member of the immunoglobulin superfamily of cell adhesion molecules and a key cell adhesion protein on the vascular endothelial cell surface. It is involved in various cell-cell and cell-matrix interactions in vascular endothelial activities such as cell migration and angiogenesis(17) and also engages in outside-in signalling. Although the natural CD146 ligand still remains elusive, its engagement is known to activate several signalling pathways including PI3K/AKT, p38 and PKC (17). CD146/MCAM interaction with VEGFR-2 on the endothelial cell surface has been shown to activate AKT and p38 signalling and increase cell migration (21). Interaction of CD146 with Laminin-411 facilitates Th17 cell entry into the central nervous system (28). Binding of Netrin-1 to CD146/MCAM was reported to activate an array of downstream signalling and increase endothelial cell proliferation, migration and angiogenesis (29). Recently, CD146 was reported to interact with exogenously introduced galectin-1 in endothelial cells and protects against galectin-1-induced cell apoptosis (30). Endogenous galectin-3 expression in HUVECs was found to be low (Fig 4). Strong interaction and co-localization of CD146/MCAM with galectin-3 occurred when exogenous galectin-3 was introduced (Fig 3 and 4).

It remains to be determined exactly how CD146/MCAM activation in response to galectin-3 binding induces endothelial secretion of cytokines. The rapid activation of AKT indicates its involvement at the initial stage of signalling transduction. The activation of cellular WINK1, β-catenin, and HSP60 after 24 hr galectin-3 treatment suggests possible involvement of multiple downstream signalling pathways at later stages of the process. Toll-like receptor-2-associated IL-6 secretion from megakaryocytes has been shown to be associated with WNT β-catenin signalling(31) and the presence of humanized anti-HSP60 antibody suppresses IL-6 secretion from PBMCs (32). These studies suggest a role of WNT and HSP signalling in galectin-3-mediated cytokine secretion.

In addition to CD146/MCAM, CD31/PECAM-1 and CD144/VE-cadherin were also seen in

this study to be extracted by galectin-3 affinity purification from endothelial cells (Fig 2 and 3). CD146 has been reported to be able to interact with vascular endothelial growth factor receptor-2 (VEGFR-2) and induce activation of AKT(21). Interestingly, a complex formed by CD31/VE-cadherin and CD144/PECAM is reported to be associated with VEGFR-2 in endothelial cells (33) and their interaction shows to affect VEGFR-2-associated AKT signalling. It is therefore possible that CD146/MCAM, CD31/VE-cadherin and CD144/PECAM may form a super-complex on the endothelial cell surface through VEGFR-2. This may explain the extraction of these four proteins by galectin-3 affinity purification (Fig 2) even though galectin-3 may only bind to CD146/MCAM (Fig 3). The discovery that exogenous galectin-3 increases AKT activation (Fig 7), a downstream CD146/MCAM signalling that is associated also with VEGFR-2 activation, is in keeping with this.

CD146/MCAM is thus the functional galectin-3 binding ligand on the endothelial cell surface responsible for galectin-3-induced endothelial secretion of metastasis-promoting cytokines. Many previous studies have shown that higher serum levels of pro-inflammatory cytokines (e.g. IL-6 and TNFα) correlate with advanced metastatic stages and poor survival in various types of cancer (34). These cytokines enhance various cell activities including proliferation, invasion, angiogenesis and metastasis (15,35). The increased secretion of IL-6, G-CSF and other cytokines from the vascular endothelium induced by interaction of circulating galectin-3 with endothelial CD146/MCAM in cancer may therefore have an important influence on cancer progression and metastasis.

Experimental procedures

Materials

Human Interleukin-6 (IL-6) and Granulocyte Colony-Stimulating Factor (G-CSF) ELISA kits were purchased from Peprotech, London, UK. Antibodies against CD146/MCAM (mab 932), CD144/PECAM-1 (BBA7), CD31/VE-Cadherin (MAB9381), Galectin-3 (mab 1154), biotinylated-anti-Galectin-3 (BAF1154) and Proteome Profiler Human Phospho-Kinase Array Kits (Ary003b) were from R&D Systems, Abingdon, UK. Antibodies against Endoglin (SC-18838) and pan-actin 5 were from Santa Cruz Biotechnology (Heidelberg, Germany) and Neomarkers (Fremont, Ca), respectively. Antibodies against AKT (9272S) and Phospho-AKT (Phospho T308, 13038S) were purchased from Cell Signalling (Hitchin, UK). DTSSP was purchased from Thermo Fisher Scientific, Runcorn, UK.

Cells

Human microvascular lung endothelial cells (HMVEC) and human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Basel, Switzerland) and cultured in EGM and EGM-2-MV media respectively. Cells with less than 6 passages were used in all experiments.

Cytokine quantification

HUVECs or HMVEC-Ls were seeded in 12-well plates at $5x10^4$cells/well and cultured for 24 hr at 37^0C before introduction of recombinant galectin-3 for 24 hr. The culture medium was collected and centrifuged at 1000rpm to remove any cell debris. The supernatant was used for determination of IL-6 and G-CSF concentration using the IL-6 and G-CSF ELISA kits according to the manufacturer's instructions.

Production of recombinant galectin-3

Full-length recombinant human galectin-3 and His-tagged recombinant human galectin-3 were produced in *E.coli* as previously described(36).

Galectin-3 affinity Purification

Confluent HUVECs were washed once with 100mM lactose/PBS and twice with PBS before being lysed in lysis buffer [PBS, 0.5% Triton X-100, 0.5% NP-40 (v/v), protease inhibitors]. The lysate was collected and sonicated 3x20 seconds on ice. The lysate was cleared by centrifugation at 16000 x g for 10 min at 4°C and before application to galectin-3 affinity columns. Galectin-3-Ni (Nickel) column was prepared by injection of 12 mg His-tagged recombinant galectin-3 to a His-Trap HP column (GE healthcare). Galectin-3-agarose affinity beads were prepared by conjugating 30 mg recombinant galectin-3 to 12.5 ml NHS-agarose slurry beads (Pierce) according to manufacturer instructions.

After removal of the unbound galectin-3 by three washes with PBS, the cell lysate was applied to the column three times. After 3

washes with PBS, the bound proteins were eluted with 0.2M lactose/PBS. The eluate was dialysed at 4°C for 24 hr against distilled water. The samples were freeze-dried and analysed by SDS-PAGE followed by silver staining or by mass spectrometry.

Mass spectrometry and protein identification

Sample preparation: A proportion of the freeze-dried eluate from both the galectin-3-agarose and galectin-3-Ni columns was reconstituted in 500µL of 25mM ammonium bicarbonate (NH_4HC0_3). 10µL of Strataclean resin (Agilent UK) was added to the sample followed by gentle vortexing for 1min. The samples were centrifuged at 2,000 x g for 2min and the protein-depleted sample aspirated and the resin washed three times with 1mL of 25mM NH_4HC0_3. The resin was re-suspended in 80µL of the same buffer and 5µL of 1%(w/v) Rapigest (Waters, UK) was added and the samples placed in a heating block set at 80^0C for 10min. Samples were reduced by the addition of 5µL of 60mM dithiothreitol in 25mM NH_4HC0^3 and incubated at 60^0C for 10min. The samples were alkylated by the addition of 180mM iodoacetaminde in 25mM NH4HC03 followed by incubation at room temperature in the dark for 30min. Trypsin (Promega Gold) 5µL was added from a 0.2mg/mL stock solution in 50mM acetic acid (~1:50 enzyme to protein ratio) and the samples incubated at 37°C overnight with continuous mixing.

Mass spectrometry analysis: Data-dependent LC-MSMS analyses were conducted on a QExactive quadrupole-Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano-liquid chromatograph (Hemel Hempstead, UK). Sample digest from the galectin-3 affinity chromatography was analysed using a 50min LC-MS method. Digest (1µL) was loaded onto a trapping column (Acclaim PepMap 100 C18, 75 µm x 2 cm, 3 µm packing material, 100 Å) using a loading buffer of 0.1 % (v/v) TFA, 2 % (v/v) acetonitrile in water for 7 min at a flow rate of 9 µL min-1. The trapping column was then set in-line with an analytical column (EASY-Spray PepMap RSLC C18, 75 µm x 50 cm, 2 µm packing material, 100 Å) and the peptides eluted using a linear gradient of 96.2 % A (0.1 % [v/v] formic acid):3.8 % B (0.1 % [v/v[formic acid in water:acetonitrile [80:20] [v/v]) to 50 % A:50 % B over 30 min at a flow rate of 300 nL min-1, followed by washing at 1% A:99 % B for 5 min and re-equilibration of the column to starting conditions. A shorter 30min LC-MS method was used for the digest from His-galectin-3 affinity chromatography due to the lower sample complexity. The column was maintained at 40^0C, and the effluent introduced directly into the integrated nano-electrospray ionisation source operating in positive ion mode. The mass spectrometer was operated in DDA mode with survey scans between m/z 300-2000 acquired at a mass resolution of 70,000 (FWHM) at m/z 200. The maximum injection time was 250 ms, and the automatic gain control was set to 1e6. The 10 most intense precursor ions with charges states of between 2+ and 5+ were selected for MS/MS with an isolation window of 2 m/z units. The maximum injection time was 100 ms, and the automatic gain control was set to 1e5. Fragmentation of the peptides was by higher-energy collisional dissociation using normalised collision energy of 28%. Dynamic exclusion of m/z values to prevent repeated fragmentation of the same peptide was used with an exclusion time of 20 sec.

Raw data files were searched in Proteome Discoverer (v1.4) against the UniProt human reviewed database (20,187 sequences) using the Mascot search engine. Trypsin was specified as the protease with one missed cleavage allowed and with fixed carbamidomethyl modification for cysteine and variable oxidation modification for methionine. A precursor mass tolerance of 10 ppm and a fragment ion mass tolerance of 0.01 Da were applied. A peptide false discovery rate of 1-5% was applied.

Immunofluorescence and confocal microscopy

Sub-confluent HUVECs or HMVEC-Ls were seeded into 12-well plates inserted with glass coverslips. After 24 hr culture, the cells were treated with either galectin-3 or BSA (4 µg/ml) for 1 hr. After washing with PBS and fixed with 4% paraformaldehyde, the cells were incubated with 1% BSA for 1 hr before application of biotinylated anti-galectin-3 (1:1000) or anti-MCAM (1:1000) in 2% BSA/PBS (w/v) for 1 hr at room temperature. After three washes with PBS, the cells were incubated with FITC-conjugated (1:2000) secondary antibody or

Texas Red-Avidin (1:2000) for 1 hr at room temperature. The cells were washed 5 times in PBS before being mounted using DAPI-containing fluorescent mounting media (Vector Laboratories, Burlingame, CA). The slides were analysed with 3i confocal microscope (Marianas SDC, 3i Imaging) and Slidebook 6 Reader version 6.0.4 (Intelligent-imaging).

Co-Immunoprecipitation

90% confluent HUVECs were first treated with galectin-3 (4µg/ml) for 1 hr at 37^0C before being washed with ice cold PBS and lysed with lysis butter (1% Triton X-100 in PBS) on ice for 10 minutes. In some of the experiments, after treatment with galectin-3 (4µg/ml), the cells were washed and crosslinked with 2mM DTSSP for 1 hr on ice. After quenching the reaction with 20mM Tris buffer for 15 minutes, the cells were lysed and centrifuged at 10,000 x g for 10 min at 4^0C. The supernatants were collected and pre-cleared with 30µl protein-A/G PLUS-Agarose beads (sc-2003, Santa Cruz) for 1 hr at 4^0C. The lysates were incubated with anti-MCAM antibody (2 µg/ml) or control isotype control IgG at 4^0C overnight. After addition of 30µl protein-A/G plus agarose beads for 4 hr, the beads were washed four times with ice cold PBS with 1% Triton X-100 (v/v). Proteins were eluted from the beads by boiling in SDS-sample buffer and analysed by SDS-PAGE and western blotting.

Western blotting

Cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked in 5% BSA(w/v)/0.05% Tween-20/TBS before incubation with primary antibodies against MCAM, PECAM-1, VE-Cadherin, Endoglin, galectin-3 or actin at a concentration of 1:1000 overnight at 4^0C. After 3 washes with 0.05% Tween-20/TBS, the blots were incubated with peroxidase-conjugated anti-mouse or anti rabbit antibodies (1:5000) for 1 hr. After 6 washes with 0.05% Tween-20/TBS, the blots were developed using chemiluminescence Super Signal kit and visualized with the Molecular Imager® Gel Doc™ XR System (Biorad).

Protein de-glycosylation

Following immunoprecipitation as described above, protein-A/G plus agarose beads were washed twice with ice cold 1% Triton X-100/PBS before incubation with 5U PNGase F (F8435, Sigma) or 10mU O-glycanase (GK80090, Prozyme) overnight at 37^0C. The beads were washed twice with PBS and proteins were eluted from the beads by boiling in SDS-sample buffer and analysed by SDS-PAGE and western blotting.

siRNA protein knockdown

Fifty percent confluent HMVEC-Ls were cultured in 12-well plates for 24 hr. MCAM siRNA (150pmol) (siGenome Smart pool human MCAM, Thermo Fisher) or control non-target siRNA (siGenome control siRNA #1, Thermo Fisher) were incubated with 100µl serum free medium for 5 min. DharmaFect Transfection Reagent-4 (Thermo Fisher) (2.5µl in 100µl serum free medium) was then introduced for 20 min at room temperature before 800 µl EGM2 medium was added. After 16 hr incubation, the transfection medium was replaced with fresh medium for 24 hr. The cells were then either lysed by SDS-sample buffer for subsequent analysis for MCAM expression by immunoblotting or introduced with galectin-3 for 24 hr at 37^0C before the IL-6 and G-CSF concentrations in the culture media were determined by ELISA.

CD146/MCAM dimerization

HMVEC-Ls were cultured in 12-wells plate at $5x10^4$cells/well for 1 day before treatment with galectin-3 (4µg/ml) for 0, 10, 30 and 60 min at 37^0C. Cells were washed in ice cold PBS and lysed in SDS-sample buffer with or without beta-mercaptoethanol before analysis by SDS-PAGE and western blotting.

Protein phosphor-Kinase array

90% confluent HUVECs were treated with 4µg/ml galectin-3 or BSA for 24 hr. Cells were washed, lysed and analysed using Proteome Profiler Human Phospho-Kinase Array Kit (Ary003b) according to the manufacturer instructions. Membranes were visualized with the Molecular Imager® Gel Doc™ XR System and analysed using Image Lab software version 5.2.1 (Biorad).

Statistical analysis

Unpaired *t* test for single comparison or one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons (SPSS 24t) were used where appropriate.

Differences were considered significant when $p<0.05$.

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Abbreviations: Gal3, galectin-3; G-CSF, Granulocyte Colony-Stimulating Factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HUVECs, human umbilical vein endothelial cells; HMVEC-Ls, human microvascular lung endothelial cells; sICAM-1, Intercellular Adhesion Molecule 1; IL-6, Interleukin-6; MCAM, melanoma cell adhesion molecule, CD146; PECAM, CD31, platelet endothelial cell adhesion molecule.

Conflict of interest
The authors declare no conflict to interest

Author contributions
FC performed and analysed the experiments shown in Fig 2-9 and contributed to drafting the manuscript; WW performed and analysed the experiments shown in Fig 1 and part of Fig 2; MZ produced the recombinant galectin-3; DS performed the mass spectrometry analysis for protein identification; RB and JMR contributed to the design and interpretation of data; LGY designed the study, contributed to interpretation of data and drafted the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Figure legends

Fig 1. **Galectin-3 induces cytokine secretion from both human micro- and macro-vascular endothelial cells.** Human microvascular lung endothelial cells (HMVEC-Ls) (**A**) and macrovascular umbilical vein endothelial cells (HUVECs) (**B**) were treated with BSA or galectin-3 for 24 hr and the concentrations of G-CSF, GM-CSF, IL-6 and ICAM-1 in the culture medium were determined. Data are presented as mean ± SEM of at least 3 independent experiments each in triplicate. ***p<0.001, **p<0.01.

Fig 2. Identification of endothelial cell surface galectin-3 binding proteins. A: Galectin-3 blotting/overlay shows similar binding of galectin-3 to a number of proteins in HUVECs and HUVECs. B: Galectin-3 affinity purification and protein identification. HUVEC cell lysates were separated by SDS-PAGE and stained by silver staining (left panel), or applied to Ni- or agarose-conjugated galectin-3 columns. Bound proteins were released by lactose and identified by mass spectrometry, or separated by SDS-PAGE and stained by silver staining (right panel).

Fig 3. Identification of CD146/MCAM as the major galectin-3 binding protein. HUVECs were treated with galectin-3 (4µg) for 60 min. The cells were then treated without (A) or with (B) DTSSP crosslinker before immunoprecipitation with antibodies against MCAM, PECAM-1, PE-Cadherin, or endoglin and followed by immunoblotting with anti-galectin-3 antibody and peroxidase-conjugated secondary antibody. Co-immunoprecipitated galectin-3, the heavy (IgG-H) and light (IgG-L) chains of the antibody/immunoglobulin used in the immunoprecipitation are indicated by arrows. Additional arrows show non-specific (NS) antibody/immunoglobulin binding.

Fig 4. Co-localization of exogenous galectin-3 with CD146/MCAM in HUVECs. After treatment of HUVECs without (top panel) or with (bottom panel) galectin-3 (4 ug/ml) for 60 min, the cells were fixed and CD146/MCAM (green) and galectin-3 (red) immunohistochemistry was conducted and followed by confocal microscopy. Cell nuclei were stained by DAPI (blue).

Fig 5. Galectin-3 binds to N-linked glycans on CD146/MCAM. After immunoprecipitation of HUVECs with anti-CD146 antibody, the immunoprecipitates were treated with PNGaseF or O-glycanase before electrophoresis and immunoblotting with CD146/MCAM and galectin-3 antibody.

Fig 6. Suppression of CD146/MCAM expression abolishes galectin-3-induced cytokine secretion. HUVECs were treated with control or CD146/MCAM siRNA for 36 he. The cells were then either lysed and analysed for CD146/MCAM expression by CD146/MCAM immunoblotting (A), or further treated with galectin-3 (4ug/ml) for 24 hr before the levels of G-CSF (B) or IL-6 (C) in the culture medium were determined. *p<0.5 (unpaired *t* test).

Fig 7. Galectin-3 induces CD146/MCAM dimerization and down-stream AKT signalling. HUVECs were treated with galectin-3 (4ug/ml) for various times. The cells were lysed and applied to SDS-PAGE under non-denatured (A) or denatured (B) conditions and probed by anti-CD146/MCAM antibody. Cellular proteins were also probed with antibodies against p-AKT and AKT (C).

Fig 8. Activation of multiple downstream signalling pathways. HUVECs were treated with BSA (top panel) or galectin-3 (4ug/ml) (bottom panel) for 24 hr before the cells were lysed and analysed by Profiler human Phospho-Kinase Array.

Fig 1

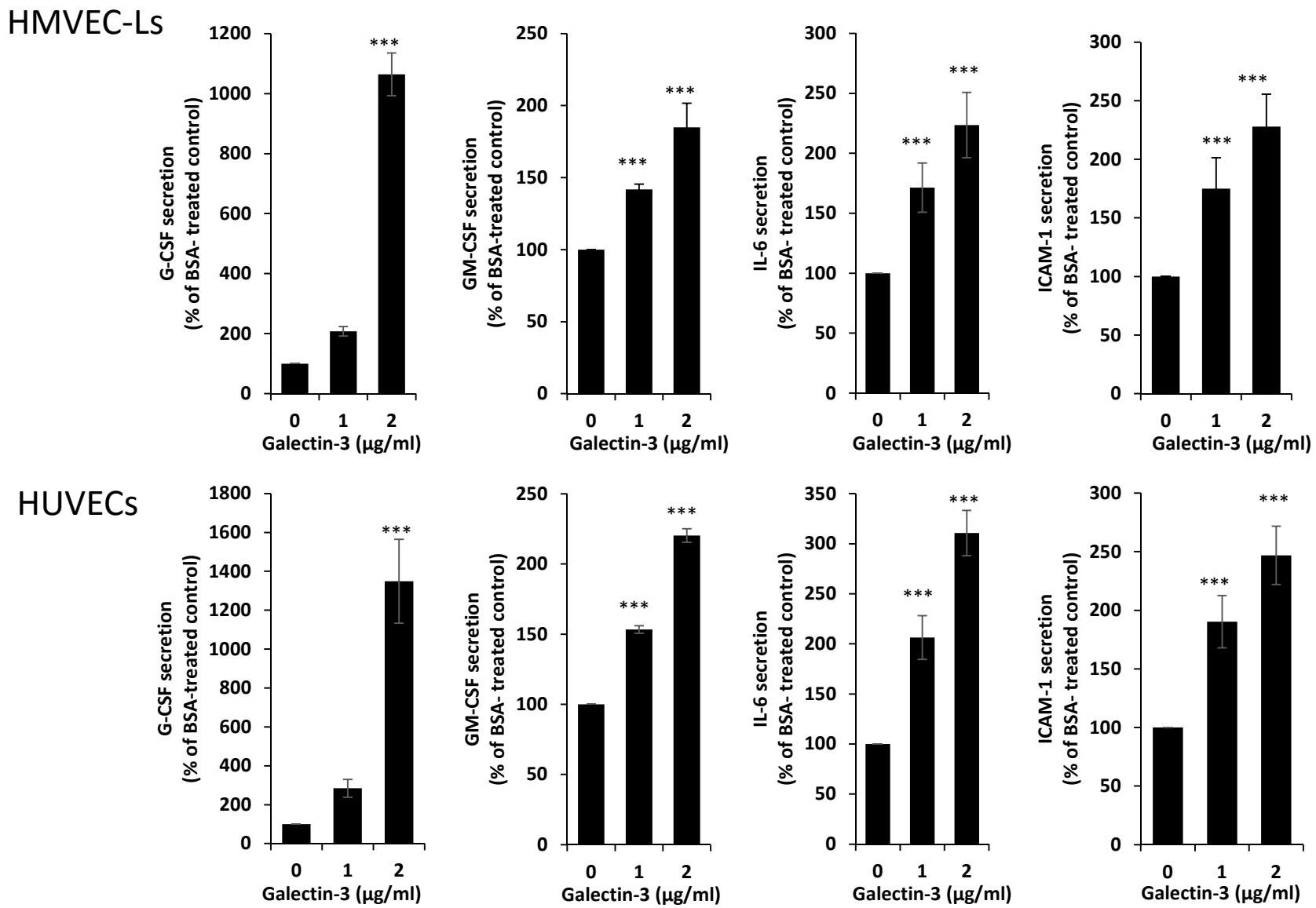

HMVEC-Ls
G-CSF secretion (% of BSA- treated control)
GM-CSF secretion (% of BSA-treated control)
IL-6 secretion (% of BSA- treated control)
ICAM-1 secretion (% of BSA- treated control)
Galectin-3 (µg/ml)
***
HUVECs
G-CSF secretion (% of BSA-treated control)
GM-CSF secretion (% of BSA- treated control)
IL-6 secretion (% of BSA- treated control)
ICAM-1 secretion (% of BSA- treated control)
Galectin-3 (µg/ml)
***

Fig 2

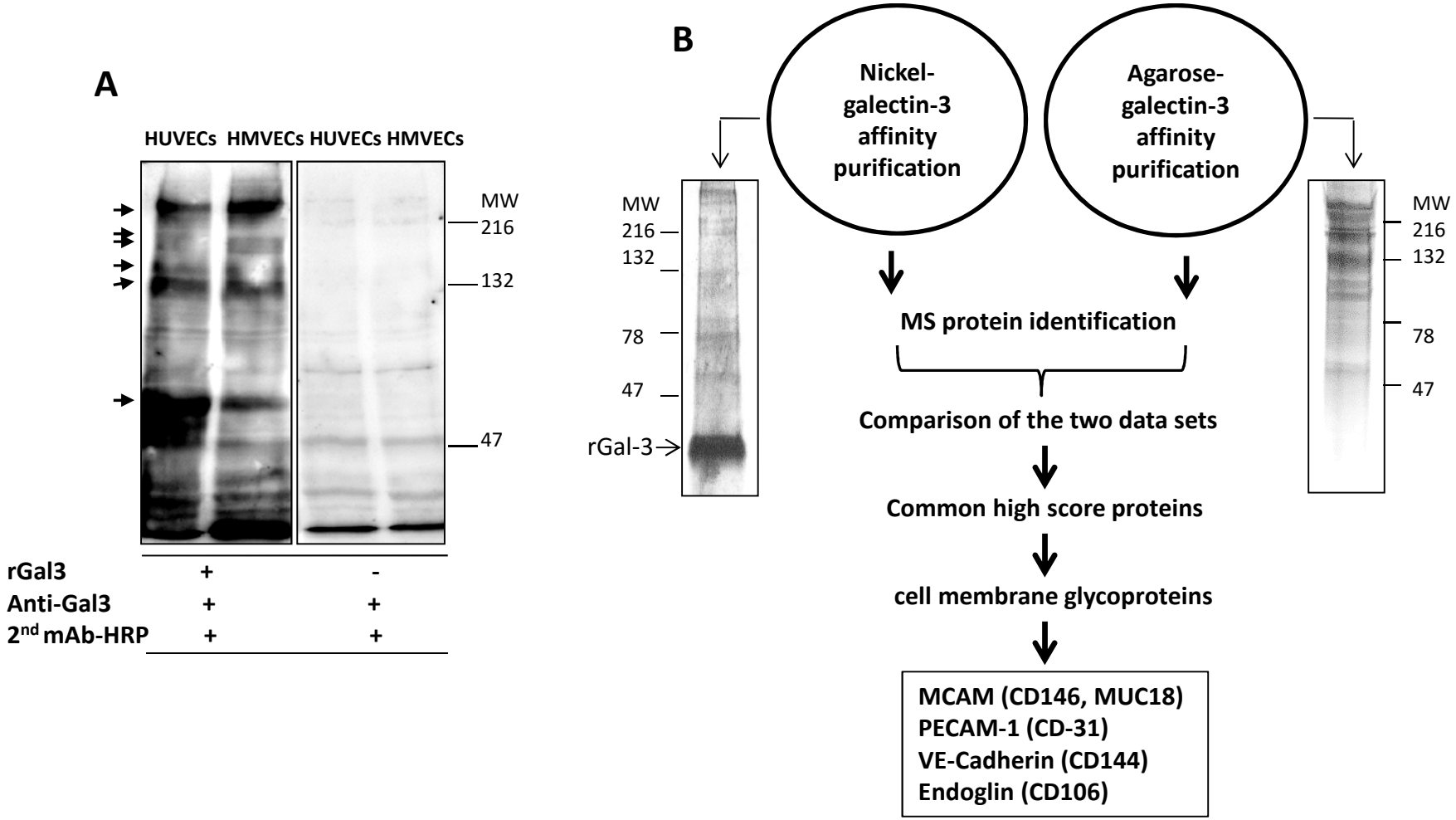

A
HUVECs HMVECs HUVECs HMVECs
MW
216
132
47
rGal3 + -
Anti-Gal3 + +
2nd mAb-HRP + +
B
Nickel-galectin-3 affinity purification
Agarose-galectin-3 affinity purification
MW
216
132
78
47
rGal-3
MS protein identification
Comparison of the two data sets
Common high score proteins
cell membrane glycoproteins
MCAM (CD146, MUC18)
PECAM-1 (CD-31)
VE-Cadherin (CD144)
Endoglin (CD106)

Fig 3

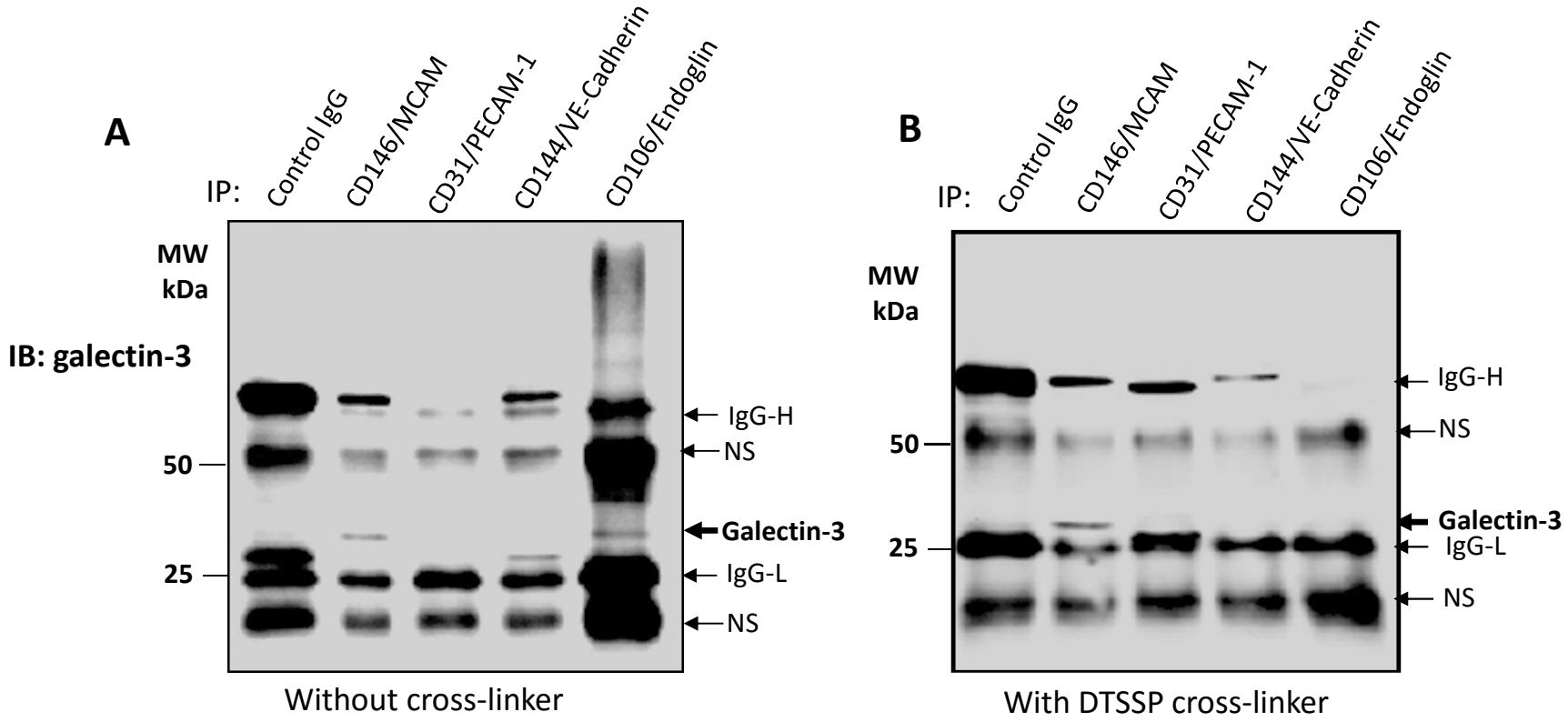

A
IP:
Control IgG
CD146/MCAM
CD31/PECAM-1
CD144/VE-Cadherin
CD106/Endoglin
MW
kDa
IB: galectin-3
50
25
IgG-H
NS
Galectin-3
IgG-L
NS
Without cross-linker
B
IP:
Control IgG
CD146/MCAM
CD31/PECAM-1
CD144/VE-Cadherin
CD106/Endoglin
MW
kDa
50
25
IgG-H
NS
Galectin-3
IgG-L
NS
With DTSSP cross-linker

Fig 4

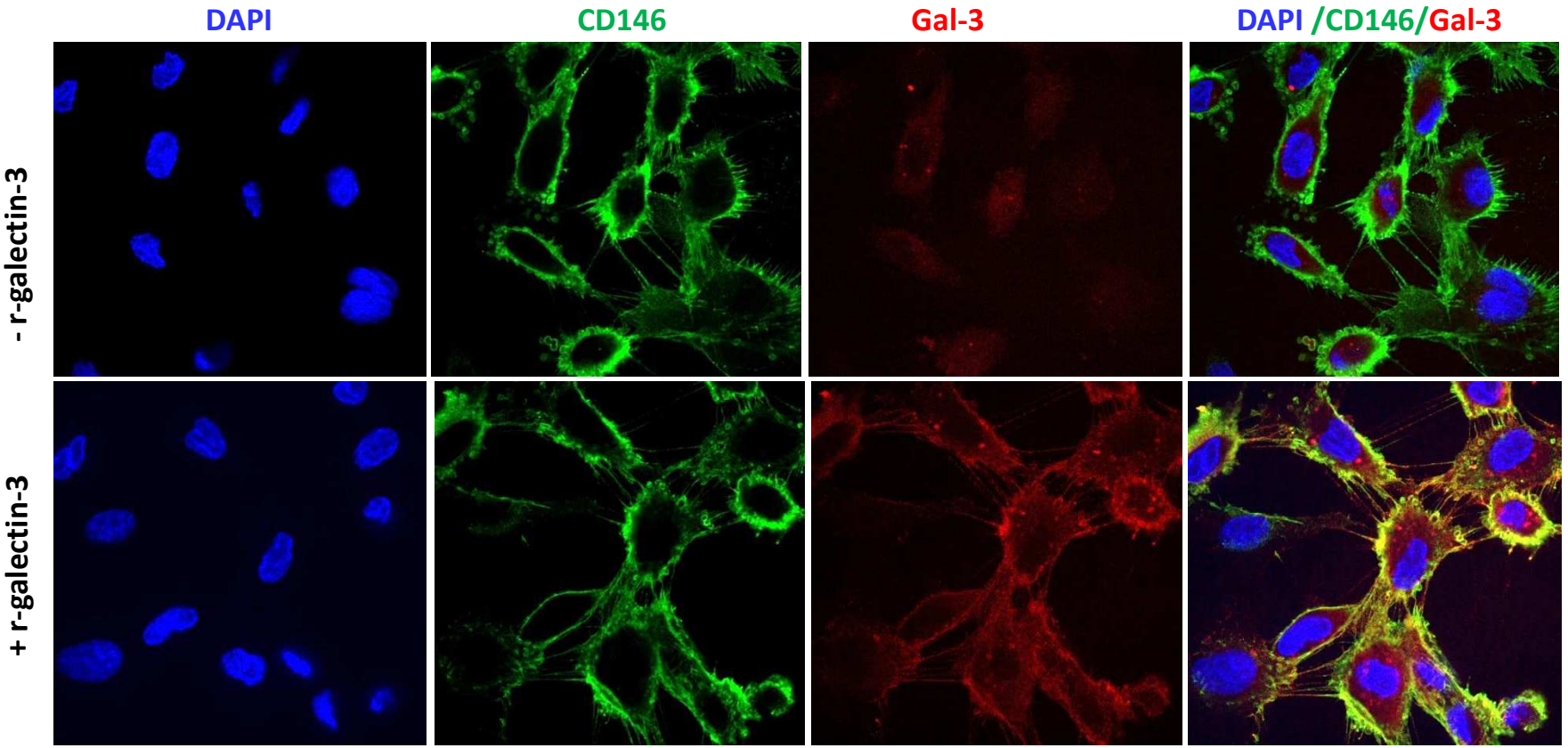


Fig 5

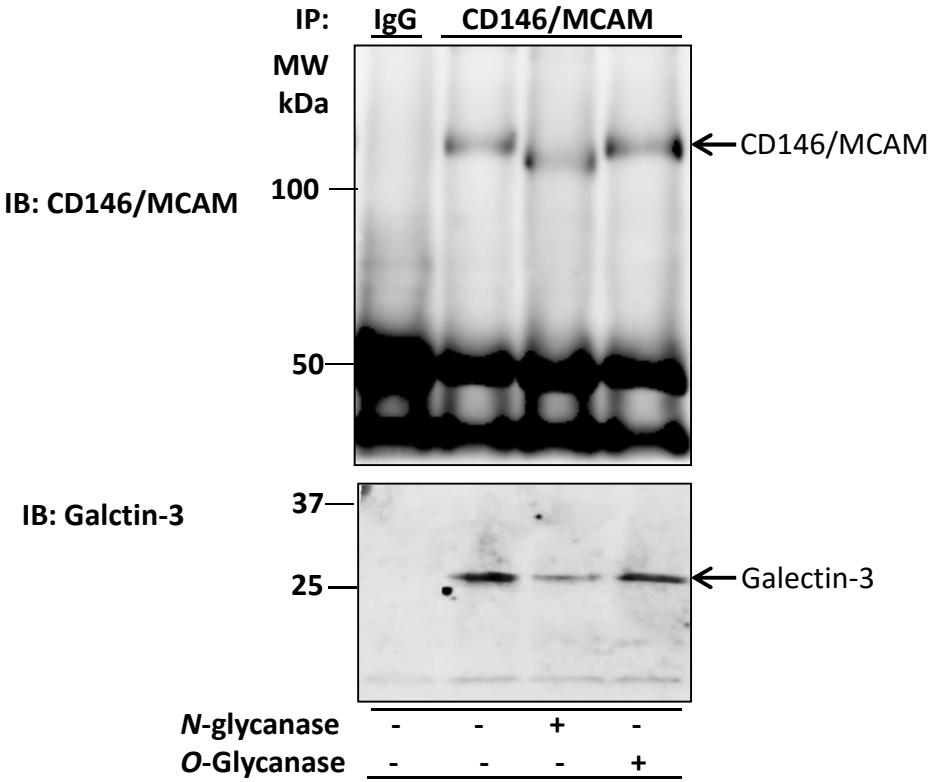

IP:
IgG
CD146/MCAM
MW
kDa
IB: CD146/MCAM
100
50
CD146/MCAM
IB: Galctin-3
37
25
Galectin-3
N-glycanase - - + -
O-Glycanase - - - +

Fig 6

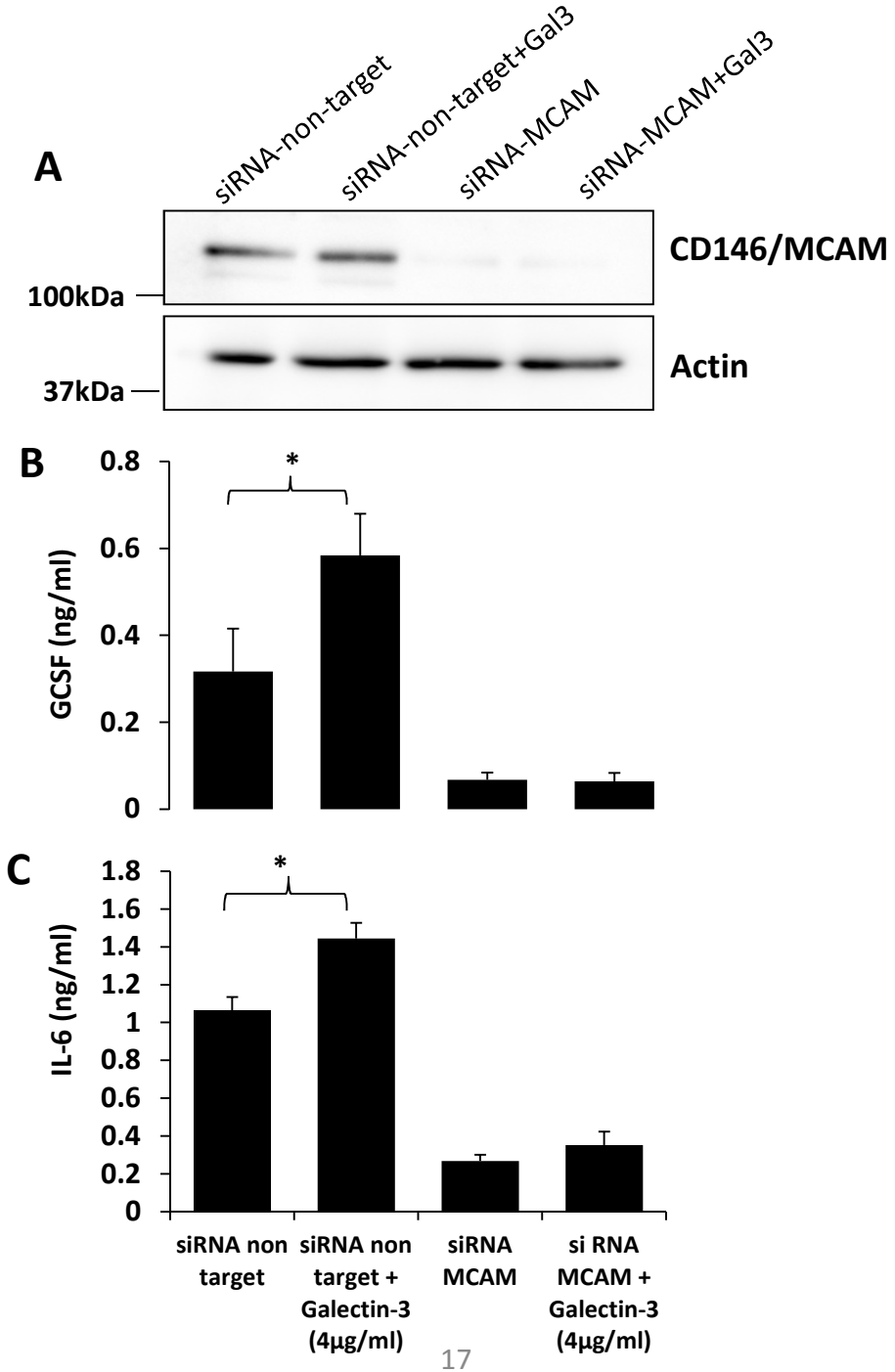
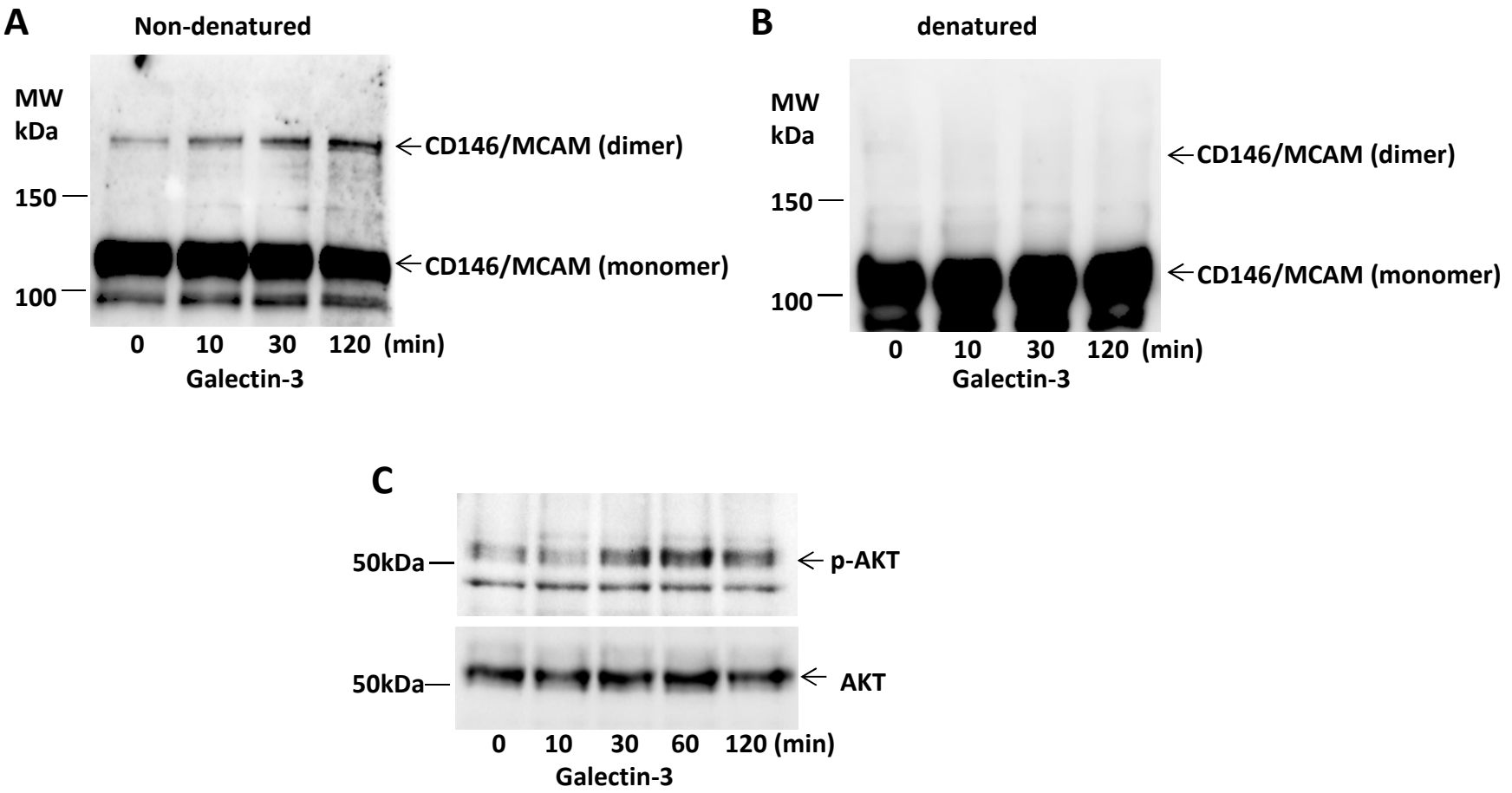

A
siRNA-non-target
siRNA-non-target+Gal3
siRNA-MCAM
siRNA-MCAM+Gal3
CD146/MCAM
100kDa
Actin
37kDa
B
GCSF (ng/ml)
0.8
0.6
0.4
0.2
0
*
C
IL-6 (ng/ml)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
siRNA non target
siRNA non target + Galectin-3 (4µg/ml)
siRNA MCAM
si RNA MCAM + Galectin-3 (4µg/ml)

Fig 7


A
Non-denatured
MW
kDa
150
100
CD146/MCAM (dimer)
CD146/MCAM (monomer)
0 10 30 120 (min)
Galectin-3
B
denatured
MW
kDa
150
100
CD146/MCAM (dimer)
CD146/MCAM (monomer)
0 10 30 120 (min)
Galectin-3
C
50kDa
p-AKT
50kDa
AKT
0 10 30 60 120 (min)
Galectin-3

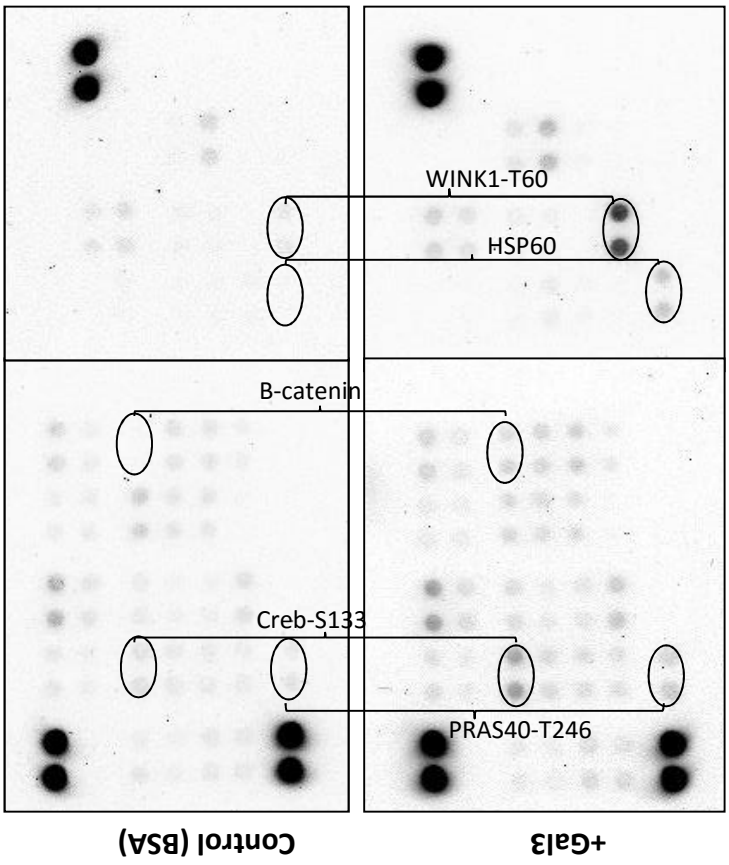

WINK1-T60
HSP60
B-catenin
Creb-S133
PRAS40-T246
Control (BSA)
+Gal3

Fig 8