Investigating the Interaction between the Tetraspanin CD82 and Gangliosides

And

Investigating the Binding Partners of CLEC14A and the Function of the CLEC14A Extracellular Domain

By Puja Lodhia

A dissertation submitted to the University of Birmingham in partial fulfilment of the Degree of MRes Biomedical Research

College of Medical and Dental Sciences

University Of Birmingham

Submitted August 2012

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Investigating the Binding Partners of CLEC14A and the Function of the CLEC14A Extracellular Domain

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Abstract

CLEC14A is tumour endothelial marker (TEM). It is upregulated by reduced shear stress and facilitates angiogenesis. However, the biological activity of CLEC14A is poorly characterised. This study validates two binding partners of CLEC14A by co-immunoprecipitation and mass spectrometry. The activity of the CLEC14A extracellular domain (ECD) was also investigated. Flow cytometry revealed that CLEC14A-ECD is able to bind to the surface of human umbilical vein endothelial cells (HUVECS). However, treatment of HUVECS with 20 ug/ml CLEC14A-ECD did not affect cell motility in scratch wound assays. The cleavage site of CLEC14A by the intra-membrane serine protease rhomboid-like protein 2 (Rhbdl2) was also explored. These results will increase the understanding of the biological activity of CLEC14A and will aid the intelligent design of drugs targeting CLEC14A to inhibit tumour angiogenesis.

Acknowledgements

Many thanks to all the members of the Bicknell Lab for their help over the course of this project. Thank you to Dr P. Noy for his guidance and supervision and to Prof. R. Bicknell for his comments and input.

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1. Introduction

Angiogenesis is a process which occurs extensively during development. However, in the adult, growth of blood vessels is restricted to the female reproductive cycle, wound healing, and of particular interest, tumour related angiogenesis (1). Tumour vessels are physiologically distinct from normal vasculature leading to differential gene expression in tumour endothelial cells. These differences can be exploited by anti-cancer therapeutics targeting tumour endothelial specific biomarkers to inhibit tumour growth (2). There is a need to establish biomarkers to allow targeting of tumour vessels. CLEC14A was recently discovered as a tumour endothelial marker by bioinformatic data mining (3)(4). Its expression is associated with reduced shear stress and CLEC14A appears to be pro-angiogenic. CLEC14A is therefore a potential target for therapeutics.

Tumour vessels are known to differ from normal vessels in several ways. The process of angiogenesis is normally regulated by pro and anti-angiogenic factors such as VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor) and interleukin 8. Tumours are unable to grow to more than $2mm^3$ in volume without developing a blood supply, which they instigate by overproduction of angiogenic factors. However, this process is dysregulated and leads to the growth of new vessels which are disorganised and tortuous, without recognisable vessel hierarchy (1). Vessels are poorly covered by smooth muscle and may lack pericytes which are involved in endothelial cell maturation and support. Fewer tight junctions between cells results in tumour vessels becoming more permeable and prone to leakage. As a result, tumour vessels are unable to effectively deliver oxygen and remove waste from tumour tissues. These abnormal vessels also obstruct delivery of therapeutics to the tumour. However, differences between normal and tumour vessels are potential targets for cancer therapies.

1.1 CLEC14A

CLEC14A was highlighted as a tumour endothelial specific gene by bioinformatic data mining (2)(4). Its expression is highly elevated in tumour vessels compared to normal vessels and represents a target for anti-tumour therapeutics.

1.1.1 CLEC14A Structure

CLEC14A is a member of the C-type lectin family of proteins which includes more than 1000 members (5). CLEC14A consists of 490 amino acids. It has a single membrane spanning domain, a cytoplasmic tail which is 69 amino acids in length, and an extracellular domain containing the C-type lectin domain and an epidermal growth factor like domain (fig. 1). The C-type lectin domain has a double loop structure composed of an overall domain loop and a long loop (6). Four conserved cysteine residues create disulphide bonds between the two loops and are vital for maintaining protein conformation (6).

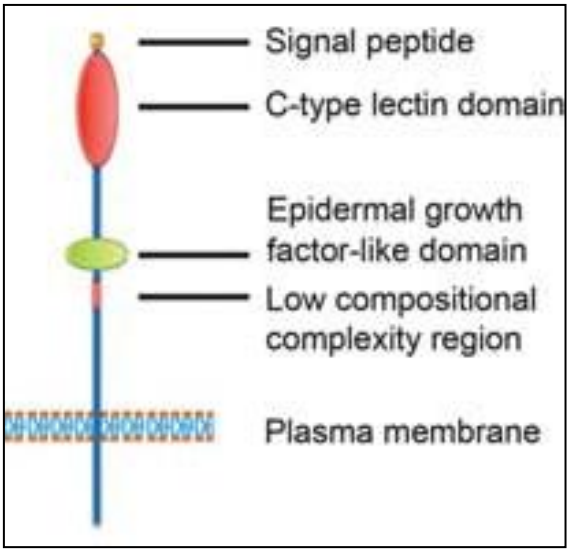

Figure 1. Structure of CLEC14A. CLEC14A consists of a C-type lectin domain, and epidermal growth factor like domain, a single pass transmembrane domain and a short cytoplasmic tail (3).

1.1.2 CLEC14A Function

CLEC14A is highly expressed on the tumour vascular endothelium of a number of solid tumours including ovarian, liver and prostate cancers. Knockdown of CLEC14A in zebrafish models revealed the protein to have a pro-angiogenic role in vascular development. It also increases cell migration and tubule formation. Expression of CLEC14A is also upregulated in conditions of reduced shear stress such as the disrupted blood flow that occurs in tumour vasculature (3)(4).

1.2 CLEC14A as a Target for Anti-Cancer Therapeutics

Specific expression of CLEC14A in tumour endothelium raises it as a prospective target for therapeutics. Monoclonal antibodies for CLEC14A could bind and inhibit the proteins pro-migratory and pro-angiogenic activities or deliver cytotoxic agents to the tumour vasculature. Of particular interest are the binding partners of CLEC14A, which if identified, could aid the development of a function blocking CLEC14A specific antibody.

1.3 CLEC14A Binding Partners

Previous work by K. Khan, using co-immunoprecipitation of HUVEC lysates and mass spectrometry analysis, shortlisted four potential binding partners for CLEC14A.

1.3.1 Metadherin

Metadherin (MTDH), also known as LYRIC or AEG-1 is upregulated in glioma, prostate and liver cancer among others (7). 40% of breast cancer cases feature elevated expression of MTDH, and its expression is associated with a poor clinical outcome (8). Expression of MTDH is increased by the oncogenes Ras and cMyc and MTDH is able to act as a transcriptional co-activator to promote expression of genes involved in proliferation, survival and invasion including NFκB (fig. 2). MTDH is believed to activate PI3K/Akt and wnt/β-catenin pathways to stimulate cell proliferation (9). It is

therefore extensively involved in oncogenic pathways. MTDH also features a lung homing domain which interacts with an unknown receptor on endothelial cells to facilitate extravasation and metastasis of tumour cells (7). It is possible that the unknown endothelial receptor is CLEC14A.

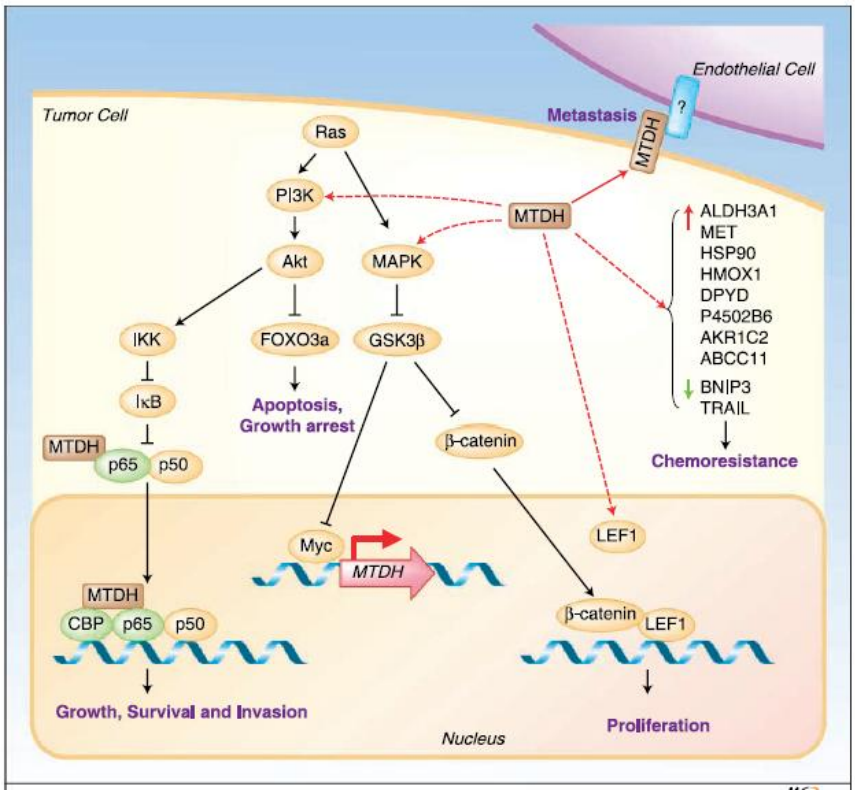

Figure 2. Roles of Metadherin in Oncogenic Pathways. Activation of MAPK by oncogenic Ras stabilises c-Myc which promotes MTDH expression. MTDH acts as a transcriptional co-activator with CBP, p65 and p60 to upregulate genes involved in growth, survival and invasion. MTDH also interacts with an endothelial cell surface receptor to mediate metastasis (7).

1.3.2 Multimerin2

Multimerin2 (MMRN2), also known and Endoglyx-1 is an extracellular matrix glycoprotein expressed specifically in the endothelium (10)(11)(12). Although mostly associated with the extracellular matrix, it is known to be present in the cell membrane and also in the cytoplasm. It reduces endothelial cell motility and impairs tubule sprouting (12). Immunostaining revealed MMRN2 to be present in tumour endothelial cells and in particular in areas of neoangiogenesis

associated with cancer. In vivo studies demonstrated that MMRN2 binds to VEGF-A, sequestering the ligand and preventing activation of the VEGF receptor to inhibit tumour angiogenesis (12).

1.3.3 T-Cadherin

Cadherins are a family of transmembrane proteins associated with intercellular adhesion. T-Cadherin, also known as H cadherin or cadherin 13, differs from other members of the cadherin family in that it does not possess a transmembrane or cytosolic domain but is attached to the plasma membrane by a GPI (glycosylphosphotidylinositol) anchor (13). It is unclear whether T-Cadherin is able to mediate cell adhesion due to its unique structure. Instead, it is thought to function as a signalling receptor for various processes. It is expressed on most endothelial cells, blood vessel associated smooth muscle cells and pericytes while expression is downregulated in breast and lung cancer. Wound healing assays demonstrated that T-Cadherin promotes migration of endothelial cells and knockout mice exhibited reduced vascularisation of mammary tumours, suggesting a role for T-Cadherin in angiogenesis (14).

1.3.4 Vigilin

Vigilin is the least well studied of the four binding partner candidates. It is upregulated by increased cholesterol and has a proposed role in cholesterol trafficking, hence its alternative name, high density lipoprotein binding protein (HDLBP) (15). It is associated with atherosclerosis, although its involvement in angiogenesis is unknown. Vigilin also suppresses expression of the proto-oncogene c-fms which is known to be involved in breast cancer (16).

1.4 CLEC14A Cleavage Site

CLEC14A is known to be cleaved by Rhomboid-Like protein 2 (Rhbdl2), a membrane embedded serine protease (Swain et al. Unpublished). The cleavage site is of interest as a therapeutic anti-CLEC14A antibody may be sequestered by cleaved CLEC14A circulating in the blood stream. It is therefore important for antibodies to target the uncleaved portion of the molecule to be effective. A study by Strisovksy et al. 2009, aiming to identify the recognition motif of rhomboids

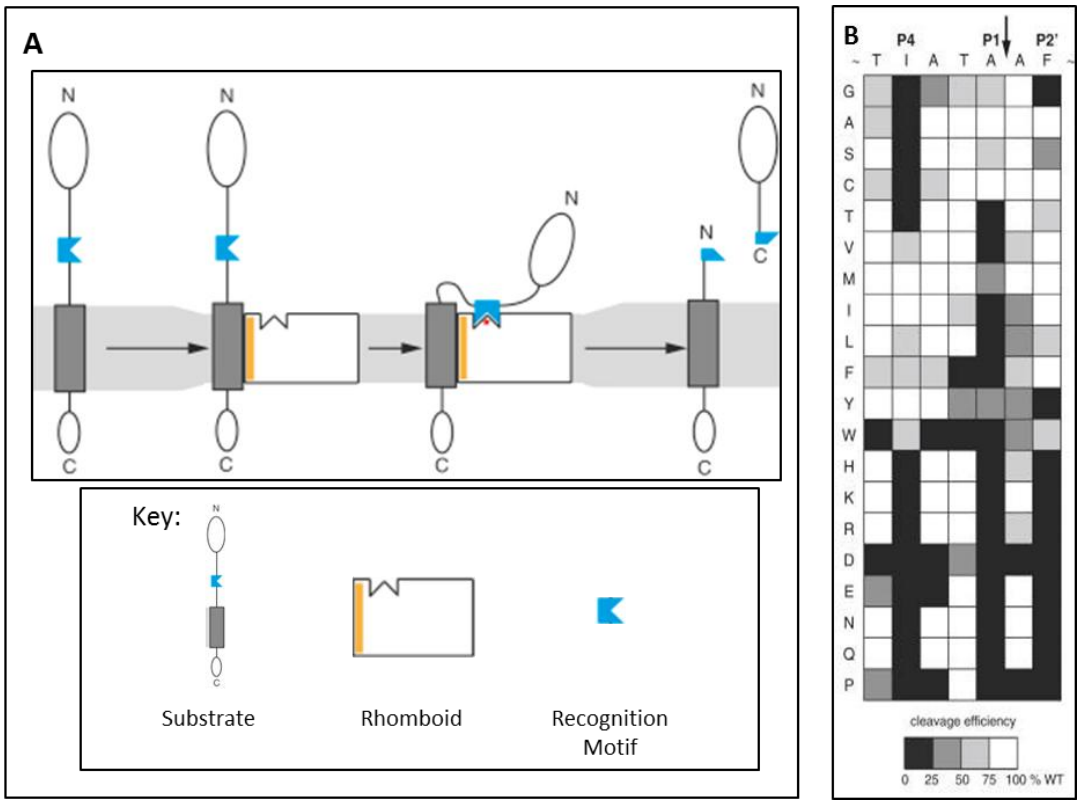

Figure 3. A. Proposed Cleavage Mechanism of Rhomboids. The rhomboid recognition motif is located in close proximity to the transmembrane domain. Structural flexibility of a linker region between the transmembrane domain and the recognition motif allow interaction with the rhomboid active site. **B. Effect of Amino Acid Mutation of Rhomboid Cleavage Motif on Cleavage Efficiency.** Substitution of amino acids surrounding the cleavage site (marked by arrow) effects rhomboid cleavage ability (17).

revealed characteristics of the cleavage site position, in particular its close proximity to the transmembrane domain (fig. 3) (17). Site directed mutagenesis of amino acids flanking the cleavage site also revealed how changes to the amino acid sequence alters the cleavage ability of rhomboids. This data allowed the identification of a prospective cleavage site in CLEC14A, namely two serine residues in the 395th and 396th positions close to the transmembrane domain. These will be mutated

to two aspartic acid residues which are not efficiently cleaved by rhomboids in order to confirm the cleavage site location.

1.5 Function of CLEC14A Extracellular Domain

The function of the CLEC14A extracellular domain (ECD) has not yet been found. It is unknown whether the CLEC14-ECD can perform functions similar to the full length protein. This will be investigated by performing scratch wound assays and observing cell migration when treated with CLEC14A-ECD.

1.6 Aims

The aims of this study are:

1. Determine the cleavage site of CLEC14A by Rhbdl2 by mass spectrometry and site directed mutagenesis.
2. Confirm potential binding partners of CLEC14A by co-immunoprecipitation
3. Investigate the function of CLEC14A-ECD using scratch wound assays

2. Materials and Methods

2.1 Antibodies and Reagents

Anti-CLEC14A polyclonal antibody was purchased from R and D Systems. Anti-CLEC14A CRT3 antibody was from Cancer Research Technologies. Anti-MMRN2 polyclonal antibody was purchased from Novus Biochemicals. Anti-MTDH monoclonal antibody was purchased from Cell Signalling Technology. Anti-CHD13 antibody was purchased from R and D Systems. Anti-Vigilin polyclonal antibody was purchased from Abcam Plc. Anti-sheep HRP antibody was from R and D Systems. Anti-mouse, rat and rabbit HRP antibodies were from Dako. Anti-human IgG HRP antibody was from Dako. Anti-human FC FITC antibody was purchased from Sigma.

2.2 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in M199 medium (Sigma Aldrich) supplemented with 10 % foetal calf serum (PAA Laboratories), 100 units/ml peniciliin and 100 µg/ml streptomycin and 1% L-glutamine (Gibco Life Technologies), 45 mg/ml heparin and 0.003% bovine brain extract. Plates were incubated with 0.1% gelatine for 30 minutes at 37°C prior to use to promote cell attachment. Human Embryonic Kidney cells (HEK293Ts) were cultured in DMEM (Gibco Life technologies) supplemented with 10% foetal calf serum and 100 units/ml penicillin, 100 µg/ml streptomycin. Cells were incubated at 37°C, 5% CO2.

2.3 SDS PAGE and Western Blotting

Protein samples were analysed by SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) on 10% (w/v) gels. Proteins were transferred to Immobilon P PVDF methanol activated membrane (Millipore) by western blotting. Membranes were blocked with 5% semi-skimmed milk (Marvel) for 1 hour. Membranes were incubated in primary antibody for 1 hour at

room temperature and washed for 3 x 5 minute washes with PBST, followed by 1 hour incubation at room temperature in the relevant secondary antibody diluted to 1:5000 in 5% milk, PBST. Membranes were washed as before and developed using Chemiluminesence Detection Reagents 1 and 2 (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare).

2.4 Cell Lysis

Cells were lysed using standard RIPA buffer supplemented with 'Complete Protease Inhibitor Cocktail tablets' (Roche Diagnostics). Cell pellets were resuspended in RIPA buffer and sheared using 19g needles before incubation on ice for 15 minutes. Lysates were centrifuged at 4°C for 15 minutes at 13,000rpm to pellet cell debris. Protein concentration was measured using BioRad Protein Assay (BioRad Laboratories GmBH) and sample concentrations were normalised prior to gel loading.

2.5 Transfection

HEK293T cells were co-transfected with CLEC14A (HA tagged) and Rhbdl2 using PEI (Polyethylenimine) reagent as per Aricescu et al. 2006 (18). Conditioned media was collected five days after transfection and filtered using a 0.2 µM bottle top filter. Proteins were visualised on 10% (w/v) gels stained with Coomassie Blue. The presence of CLEC14A was confirmed by western blotting. CLEC14A was purified from the conditioned media by immunoprecipitation using anti-HA antibody.

2.6 Flow Cytometry

Cells were detached from the culture plates using cell dissociation solution (Sigma). 1 x 10^6 cells were resuspended in 300 µl PBS, 2.5% FCS. CLEC14A-ECD-Fc was added at various concentrations and the reactions were incubated for 1 hour at 4°C on a rotary mixer. Reactions were centrifuged at 1100 rpm for 5 minutes to pellet cells. The supernatant was removed and cells were was with PBS three times. Cells were resuspended in 300 µl PBS, 2.5% FCS. Goat anti-human FC FITC antibody was added to a dilution of 1:100. Reactions were incubated at 4°C for 1 hour on a rotary

mixer. Cells were then pelleted and washed 3 times before resuspension in 500 µl PBS, 2.5% FCS. Samples were then analysed by FACS Calibur Flow Cytometer using CellQuest data analysis software.

2.7 Co-immunoprecipitation of CLEC14A with Binding Partners

HUVEC cell pellets were reuspended in lysis buffer (10% glycerol, 1% Triton, 50mM Tris HCL, 100 nM NaCl, 20 mM NaF, 10 mM KH_2PO_4 and 0.1 mM DTT with phosphatase inhibitors (5 mM NaF, 1 mM Na3VO4, 10 mM Na beta-glycerophosphate, 1 mM EDTA and 5 mM Na pyrophosphate)) and 'Complete Protease Inhibitor Cocktail tablets' (Roche) for 30 minutes on ice. Lysates were centrifuged at 13,000rpm for 10 minutes. The supernatant was transferred to fresh eppendorfs and binding buffer was added (1 mg/ml BSA, 2.5% glycerol, 50 mM Tris HCl, 20 mM KCl, 0.1 mM EDTA with phosphatase inhibitors and 'Complete Protease Inhibitor Cocktail Tablets'). 4 µl of the relevant antibody or CLEC14A-ECD-Fc (20 µg/ml) was added and lysates were incubated at 4°C on a rotary wheel for 2 hours. 30 µl of protein G sepharose beads (Sigma Aldrich) were added to the lysates which were then incubated at 4°C on a rotary wheel overnight. Lysates were centrifuged at 13,000rpm for 10 seconds. Bead pellets were resuspended in wash buffer (100 mM TRIS HCl, 200 mM NaCl, 0.5% NP40 and 'Complete Protease Inhibitor Cocktail tablets'). The supernatant was removed and the wash step repeated twice more. 4x SDS loading buffer was added and samples were boiled for 2 minutes.

2.8 SiRNA knockdown

$1.75x10^5$ cells were seeded in 6 well culture plates. The following day, SiRNA duplexes were added to Opti-Mem serum free media, (Gibco, Life technologies). RNAiMAX lipofectamine (Invitrogen) was mixed with Opti-Mem media to a final concentration of 0.3% lipofectamine per reaction. The lipofectamine mixture was incubated at room temperature for 10 minutes and was then added to the duplexes. The mixture was incubated at room temperature for 10 minutes. Cells were washed with PBS and Opti-Mem and siRNA duplex mixtures were added for a final siRNA

duplex concentration of 50nM. Cells were incubated with siRNA duplexes for 4 hours. The media was removed and replaced with antibiotic free M199 media. Assays were performed two days after transection. As controls, cells were transfected with negative control siRNA duplexes. Mock transfections without siRNA were also carried out. CLEC14A siRNA duplexes were D1-GAACAAGACAATTCAGTAA and D2-CAATCAGGGTCGACGAGAA (Euro-Gentec, Liege, Belgium). MMRN2 siRNA duplexes were D1-CUUACUAGCUCUUUGCAAA and D2-GAGACUUUCGAUCAGAUU (Ambion, Applied Biosystems International). MTDH siRNA duplexes were D1-CCGAAGUACUCGUCAAAAA and D2-GAAUCUCCCAAACAAAUAA (Ambion, Applied Biosystems International).

2.9 Scratch Wound Assay

HUVECs were seeded on 6 well plates pre-treated with 0.1% gelatine. SiRNA knockdown of CLEC14A was carried out on the following day. On the fourth day after seeding, scratches were created in the cell monolayer using a plastic pipette tip. The media was removed and replaced with fresh media or media containing 20 μg/ml CLEC14A ECD. Images were taken at 2 hour time intervals using a Leica DM IL microscope (Leica, Milton Keynes, UK) and USB 2.0 2M Xli camera (XL Imaging LLC, Carrollton, TX, USA). Scratch areas were quantified using Image J software. Cells were harvested and lysed to confirm knockdown by western blotting.

2.10 QPCR

RNA isolation from HUVECs maintained in normoxic and hypoxic conditions was carried out by Dr. P. Noy. cDNA was prepared using Multiscribe Reverse Transcriptase (Applied Biosystems, Life Technologies). Methods were as per manufacturer's instructions. CLEC14A primers were forward 5'-CTGGGACCGAGGTGAGTG-3' and reverse 5'-CGCGATGCAAGTAACTGAGA-3' with universal probe number 24. Flotillin-2 primers were forward 5'-TGTTGTGGTTCCGACTATAAACAG-3' and reverse 5'-GGGCTGCAACGTCATAATCT-3' with universal probe number 28. MMRN primers were forward 5'-

GGACCAGTCAGCTTCAAACC-3’ and reverse 5’-CGGTGCACATCATTCTGG-3’ with universal probe number 3. MTDH primers were forward 5’-ACTGGAGATGCTAATACAAATGGA-3’ and reverse 5’-GAAACTGGCTCAGCAGTAGACC-3’ with universal probe number 12. CDH13 primers were forward 5’-GAGAACTCCGCTCGTTCTGT-3’ and reverse 5’-AGTGCAGTCCAAATCTTCTGC-3’ with universal probe number 2. Vigilin primers were forward 5’-CGCATCAAGAAGATTTATGAGGA-3’ and reverse 5’-GGGCCCAATGACATACTTGT-3’ with universal probe number 86. All primers were from Euro-Gentec, Leige, Belgium. All probes were from Roche. QPCRs were carried out using Sensimix PCR reagent, Bioline INC, as per manufacturer’s instructions.

2.11 Mutagenesis

Site directed mutagenesis of CLEC14A was carried out using Quickchange II Site Directed Mutagenesis Kit (Agilent Technologies). Mutant primers were purchased from Eurogentec. Primer sequences were sense 5’-CACTCCTCAGGCTTTCGACTCCGACGATGCCGTGGTCTTCATATTTGTG-3’ antisense 5’-CACAAATATGAAGACCACGGCATCGTCGGAGTCGAAAGCCTGAGGAGTG-3’. PCR products were analysed with 1% agarose gel electrophoresis.

2.12 In-Gel Protein Digestion

All eppendorfs, glass plates and tools were rinsed with 0.1% formic acid, 50% acetonitrile. Gels were placed on glass plates and bands were excised with a sterile scalpel. Bands were washed with 50% acetonitrile, 50 mM ammonium bicarbonate for 45 minutes at 37°C with agitation. Gels were dried by speedvac. Gel bands were incubated in 50 mM DTT in 10% acetonitrile, 50 mM ammonium bicarbonate for 1 hour at 56°C. The supernatant was removed and gels were incubated in the dark at room temperature in 100 mM iodoacteamide in 10% acetonitrile, 50 mM ammonium bicarbonate for 30 minutes. Gel bands were washed three times with 10% acetonitrile, 40 mM ammonium bicarbonate for 15 minutes with agitation. Gels were dried by speedvac and then rehydrated in Promega Sequence Grade Modified Trypsin (12 µg/ml) for 1 hour at room temperature. 10% acentonitrile in 40 mM ammonium bicarbonate was added and gels were left to

incubate at 37°C overnight. The supernatant was collected. 3% formic acid was added to the gel bands and incubated at 37°C for 1 hour. The supernatant was collected and stored at -20°C for analysis by mass spectrometry.

3. Results

3.1 Production of Rhbdl2 Cleaved CLEC14A

HEK293t cells were co-transfected with Rhbdl2 and CLEC14A so that cleaved CLEC14A could be purified and analysed by mass spectrometry to determine the cleavage site.

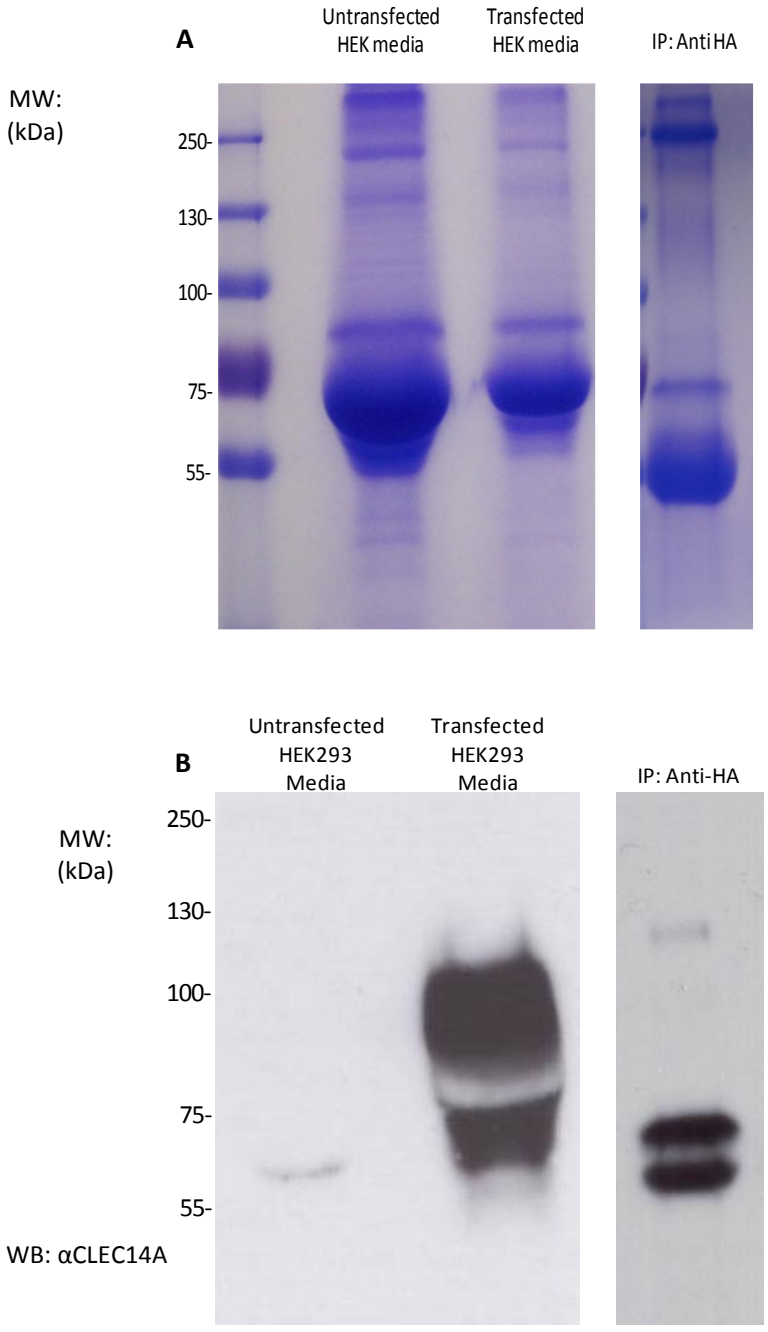

Figure 4. A. Coomasie Blue stained gels of conditioned media from CLEC14A and Rhbdl2 transfected HEK293T cells and CLEC14A immunoprecipitated using anti-HA antibody from conditioned media. Conditioned media and control untransfected media appear almost identical on coomassie gels. CLEC14A immunoprecipitated with anti-HA antibody appears as a band at 75kDa. **B. Western Blots of CLEC14A and Rhbdl2 transfected and untransfected HEK293t cells as well as CLEC14A immunoprecipitated with anti-HA antibody.** Western blots confirmed the presence of CLEC14A in the media of transfected HEK239t cells but not in untransfected cells. CLEC14A appears as a band at 75kDa when immunoprecipiated with anti-HA antibody.

Although coomassie stained gels of media from CLEC14A and Rhbdl2 transfected and control untransfected cells appeared very similar, western blotting confirmed that CLEC14A was present in transfected cell media while being absent from control media (fig. 4). CLEC14A was immunoprecitated using anti-HA antibody to purify it and remove serum proteins. After immunoprecipitation, CLEC14A appeared as a band at 75kDa on the western blot. Bands corresponding to CLEC14A were excised, digested and sent for analysis by mass spectrometry to determine the rhomboid cleavage site. Results are currently being awaited.

3.2 Site-Directed Mutagenesis of Proposed CLEC14A Cleavage Site

Oligonucleotide primers were designed to mutate S395 and S396 to DD. However, PCR reactions to incorporate the mutant primers into the CLEC14A plasmid were unsuccessful.

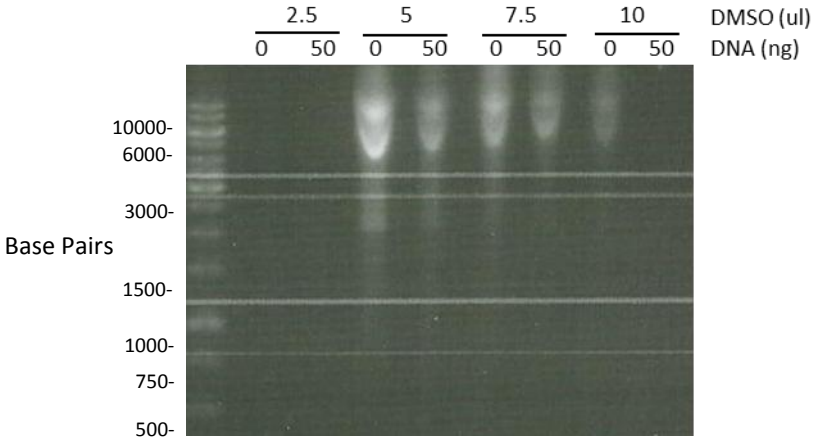

Figure 5. Site directed mutagenesis PCR. Optimisation of PCR reactions was attempted by increasing DMSO concentrations. DNA bands indicate supercoiled DNA suggesting the mutant primer was not incorporated into the plasmid.

Initial PCRs produced no PCR product detectable by agarose gel analysis. Optimisation of the PCR reaction was attempted by increasing the DMSO concentration to reduce formation of primer dimers (fig. 5). However, PCR products were supercoiled indicating failure of the primer to incorporate into the plasmid. Efforts to optimise the PCR reaction are on-going.

3.3 MMRN2 and MTDH are Upregulated by Hypoxia

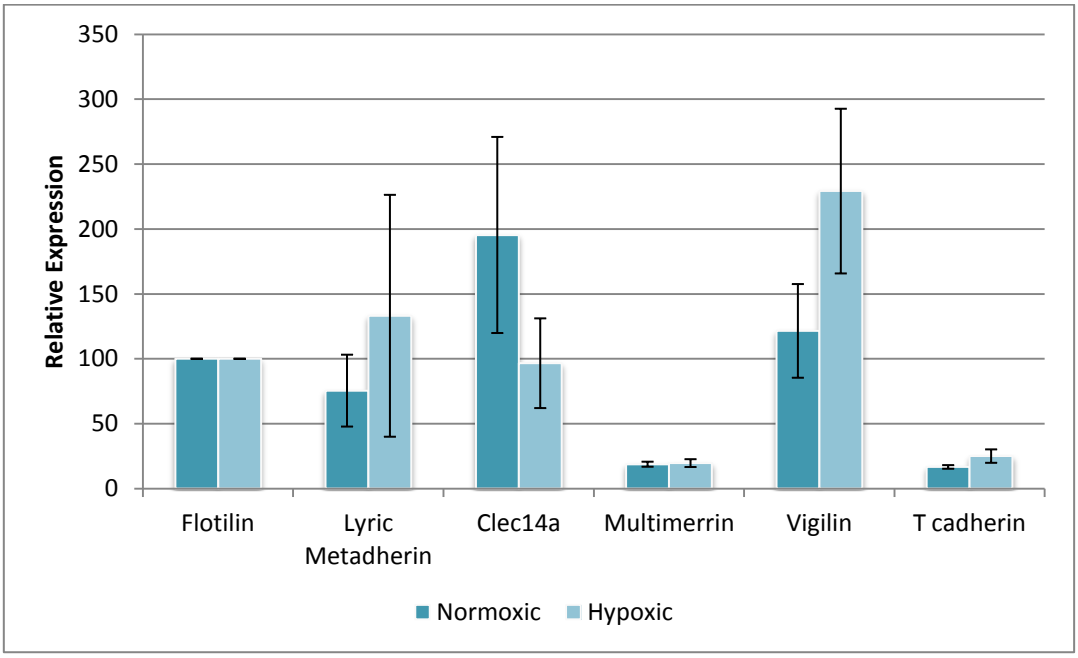

Figure 6. Relative expression of MTDH, MMRN2, Vigilin, T Cadherin and CLEC14A compared to Flotilin2 in normoxic and hypoxic conditions. MTDH and Vigilin are upregulated by hypoxia. CLEC14A was downregulated by hypoxia. Expression of MMRN2 and T Cadherin did not change. Error bars represent standard error of the mean.

Expression of the binding partner candidates in HUVEC cells maintained in normoxic and hypoxic conditions was quantified by QPCR relative to the expression of the housekeeping gene Flotilin2 (fig. 6). MTDH and Vigilin were both upregulated in hypoxic conditions although the increase was not statistically significant. This supports previously published data indicating both genes to be induced by hypoxia (19)(20). There was no significant change in expression of MMRN2 or T Cadherin in normoxic and hypoxic conditions. CLEC14A was downregulated by hypoxia although this has not been observed in previous studies.

3.4 Multimerin2 and Metadherin Co-Immunoprecipitate with CLEC14A

CLEC14A was pulled down from HUVEC lysate by αCLEC14A antibody (fig. 7). A second antibody against CLEC14A, CRT3 was less effective. CLEC14A-FC added to the lysate was also detected in the western blot. α-human IgG coated beads did not pull down CLEC14A, confirming specific interaction of CLEC14A with the αCLEC14A antibodies.

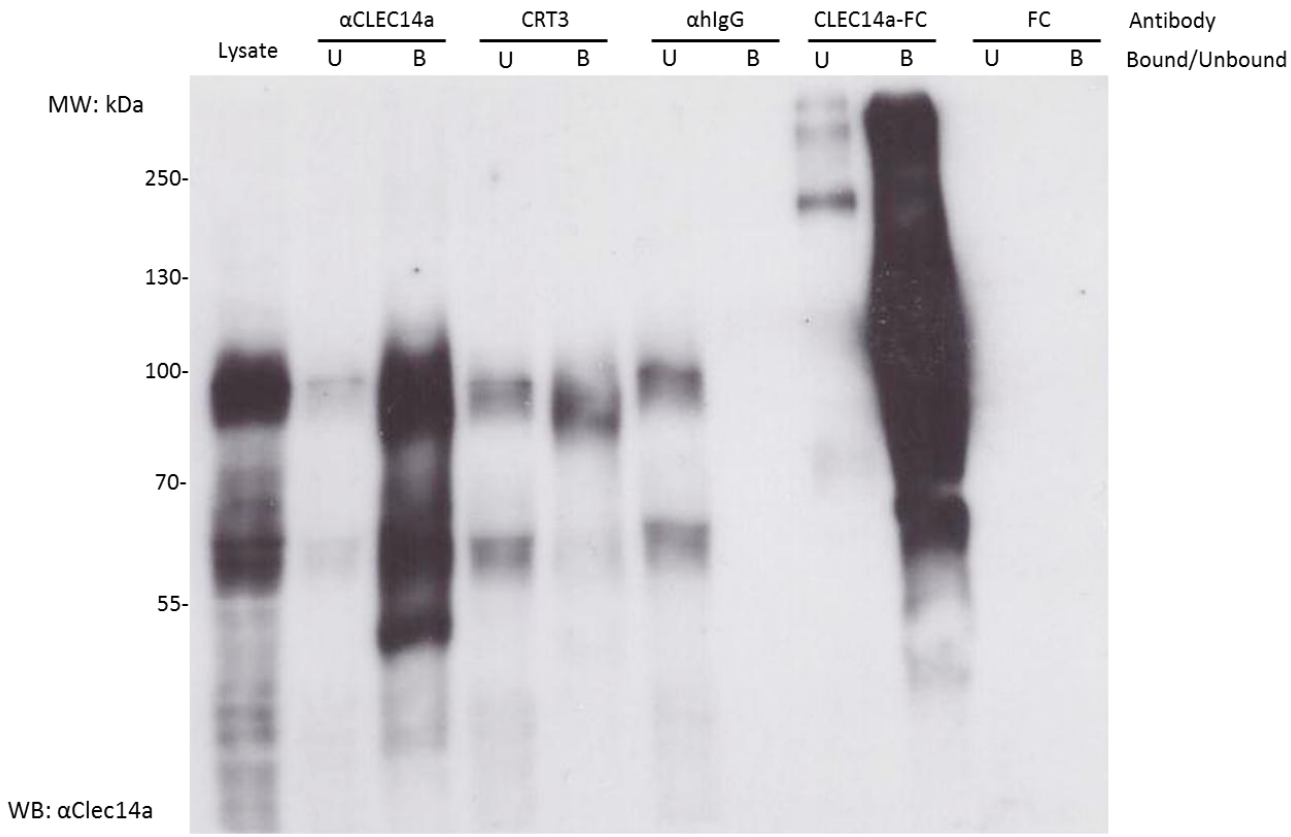

Figure 7. Immunoprecipitation of CLEC14A from HUVEC lysate. CLEC14A was immunoprecipitated with αCLEC14A antibody and with a second CLEC14A antibody, CRT3. αhIgG, anti-human IgG antibody. CLEC14A appears as a band at around 100kDa. Bound, fraction bound to beads. Unbound, supernatant containing unbound molecules after pelleting of beads.

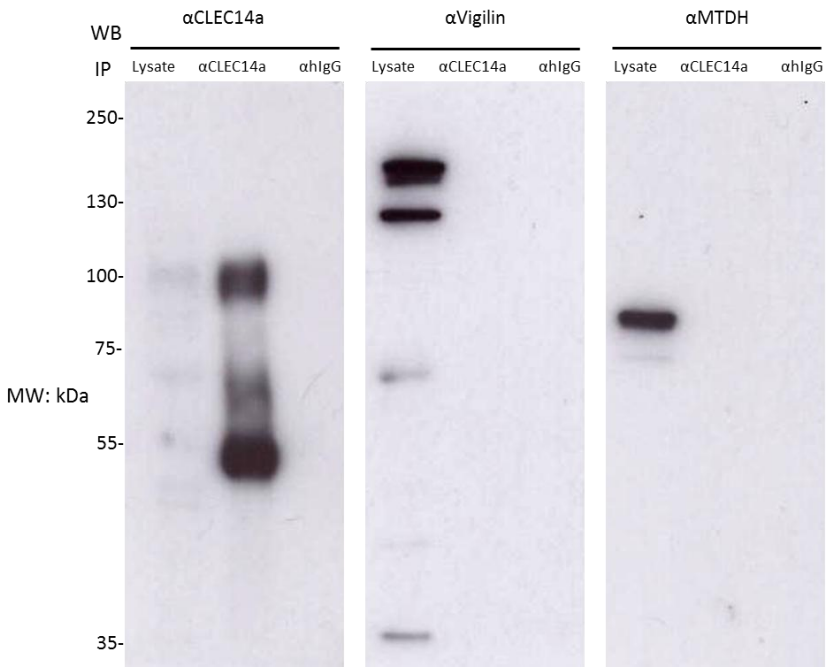

Figure 8. CLEC14A pulled down with αCLEC14A antibody did not co-immunoprecipitate with Vigilin or MTDH. Vigilin and MTDH were not detected in the αCLEC14A antibody pull-downs.

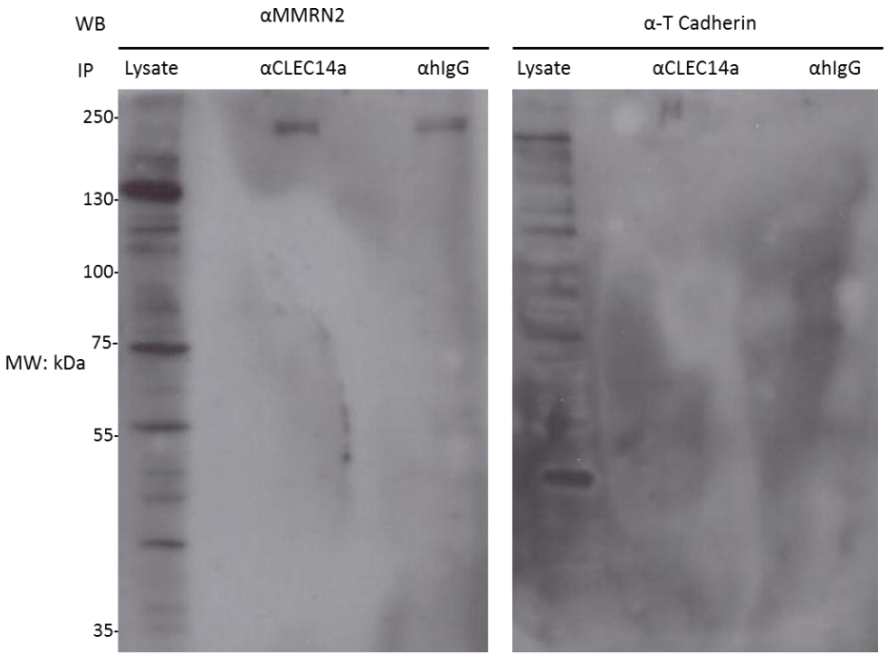

Figure 9. CLEC14A pulled down with αCLEC14A antibody did not co-immunoprecipitate with MMRN2 or T-Cadherin. MMRN2 and T-Cadherin were not detected in the αCLEC14A antibody pull-downs.

MTDH, MMRN2 Vigilin and T-Cadherin did not appear to co-immunoprecipitate with CLEC14A pulled down with αCLEC14A antibody (figs. 8 and 9). However, MMRN2 and MTDH were

pulled down from HUVEC lysate by CLEC14A-Fc indicating that MMRN2 and MTDH are binding partners for CLEC14A (fig. 10). CLEC14A Fc did not pull down Vigilin or T-Cadherin.

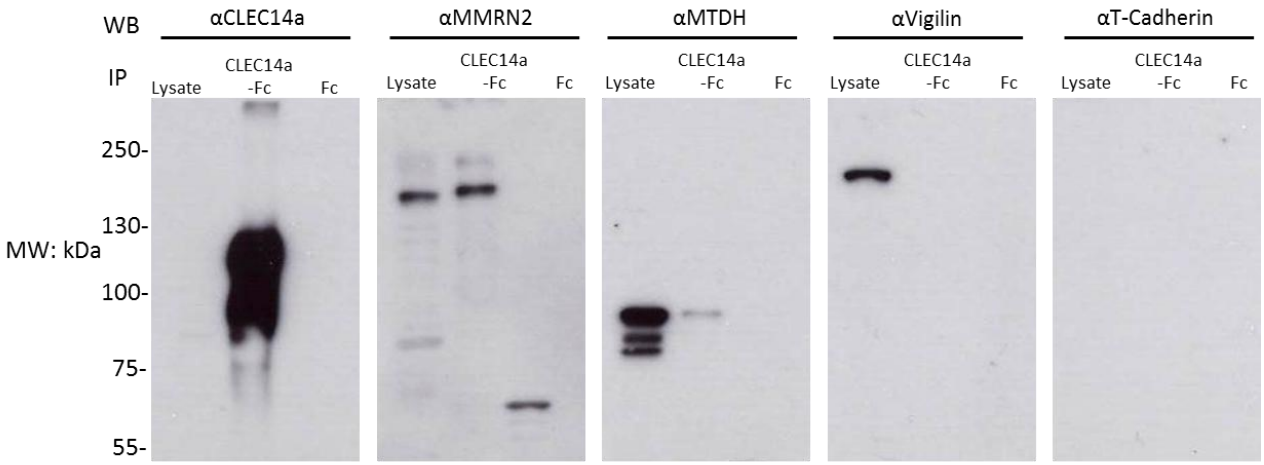

Figure 10. CLEC14A co-immunoprecipitates with MMRN2 and MTDH. MMRN2 and MTDH were pulled down from HUVEC lysate by CLEC14A-Fc. Vigilin and T-Cadherin were not pulled down by CLEC14A-Fc.

3.5 CLEC14A-ECD-FC Binds to the Surface of HUVECS

To further investigate the interaction of CLEC14A with its binding partners, HUVECS were incubated with various concentrations of CLEC14A-ECD-Fc and binding was detected by flow cytometry, with the aim of knocking down binding partner candidates and examining the effects on CLEC14A binding to the cell surface. HUVECS incubated with increasing concentrations of CLEC14-ECD-Fc displayed increased FITC fluorescence intensity, indicating binding of CLEC14A-ECD-Fc to the HUVEC cell surface (fig. 11). Binding increased with increasing concentrations of CLEC14A-ECD-Fc (fig. 12).

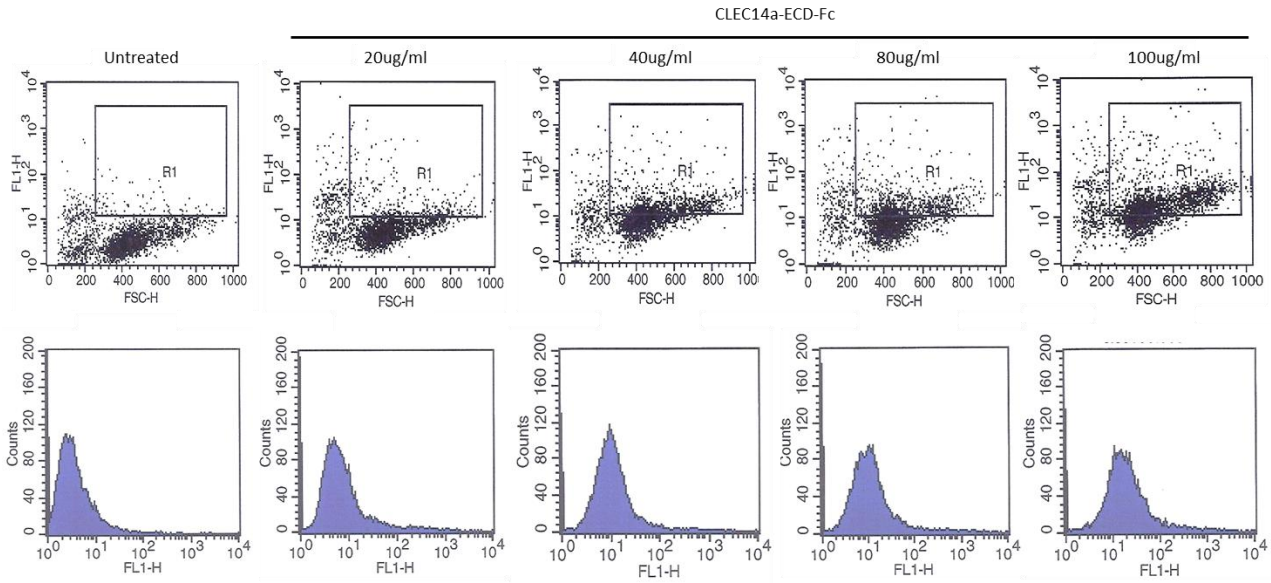

Figure 11. **Flow Cytometry with HUVECs incubated with CLEC14A-ECD-Fc.** HUVECS incubated with increasing concentrations of CLEC14A-ECD-Fc exhibited increased FITC fluorescence indicating binding of CLEC14A-ECD-Fc to the cell surface.

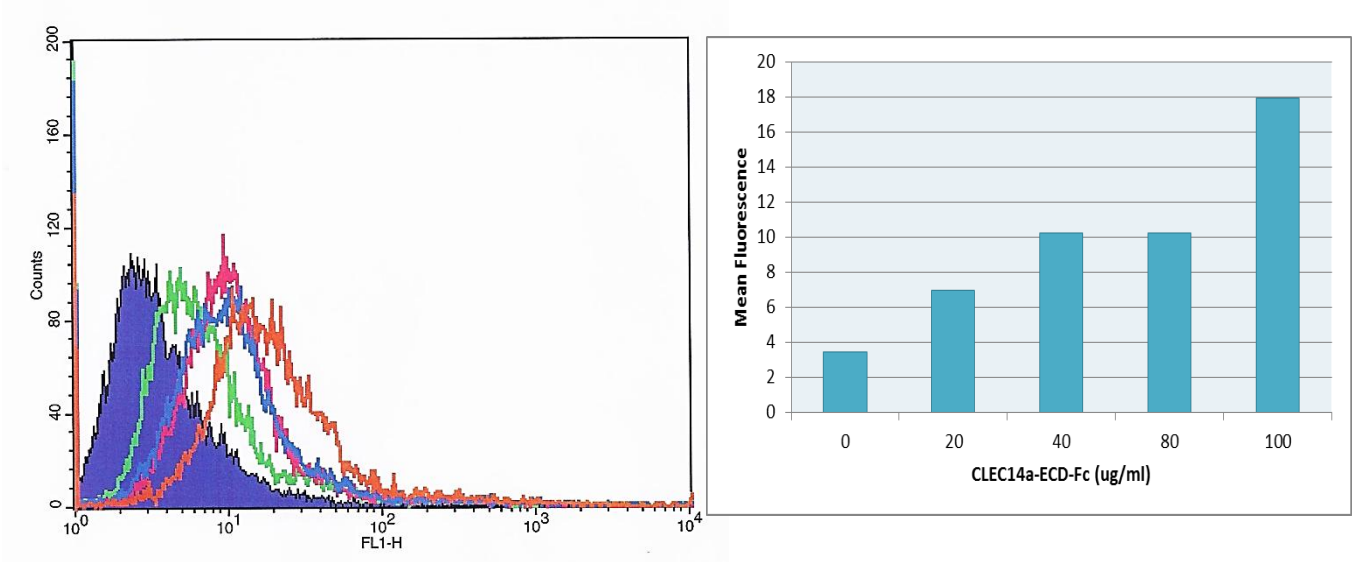

Figure 12. Left, Overlay of Fluorescence Intensity Histograms of HUVECS incubated with CLEC14A-ECD-Fc. Increasing concentrations of CLEC14A-ECD-Fc result in increased fluorescence. Blue, 0 µg/ml CLEC14A-ECD-Fc. Green, 20 µg/ml CLEC14A-ECD-Fc. Magenta, 40 µg/ml CLEC14A-ECD-Fc. Purple 80 µg/ml CLEC14A-ECD-Fc. Orange, 100 µg/ml CLEC14A-ECD-Fc. **Right, Mean Fluorescence of HUVECs Incubated with Increasing Concentrations of CLEC14A-ECD-Fc.** Fluorescence increased with increasing concentrations of CLEC14A-ECD-Fc.

SiRNA was used to knockdown MMRN2 and MTDH in HUVECs so that changes in binding of CLEC14A with reduced expression of binding partners could be observed. However, knockdown for both MMRN2 and MTDH was unsuccessful (fig. 13), so flow cytometry experiments could not be carried out.

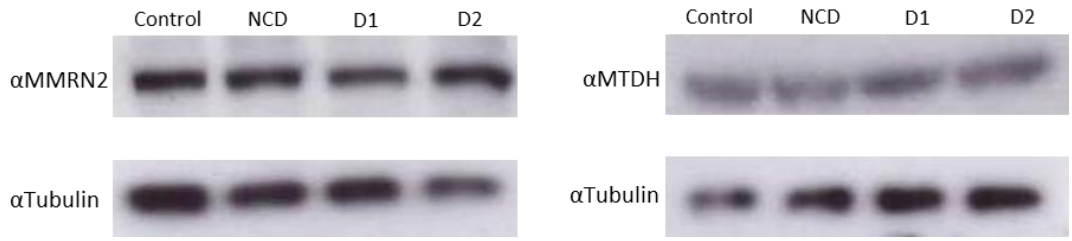

Figure 13. SiRNA knockdown of MMRN2 and MTDH was unsuccessful. MMRN2 and MTDH were not knocked down by 2 different SiRNA duplexes for each binding partner candidate.

3.6 Treatment of HUVECS with CLEC14A-ECD-Fc Does Not Affect Cell Migration

HUVECS were treated with CLEC14A-ECD-Fc or CLEC14A was knocked down by siRNA and the effects on cell migration were assessed by scratch wound assays (figs.14 and 15).

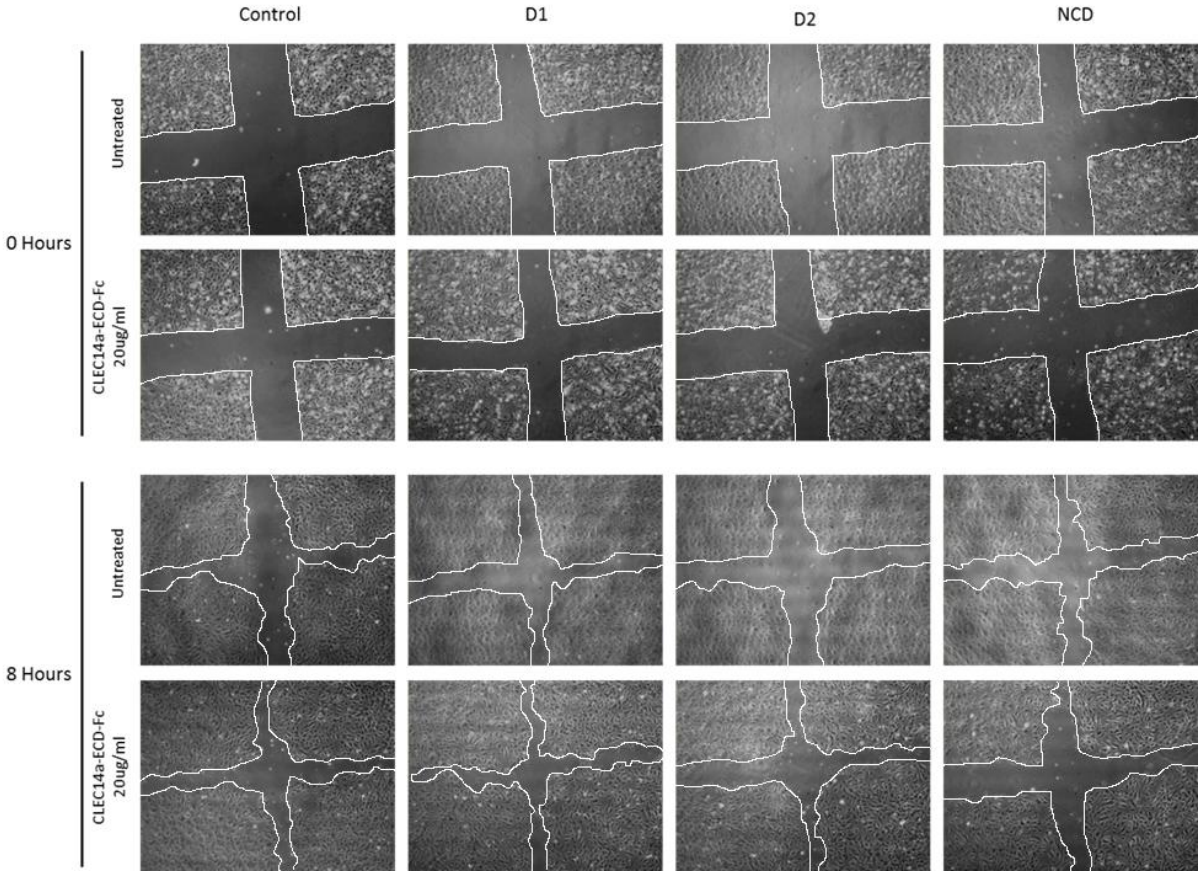

Figure 14. HUVEC Scratch Wounds at 0 and 8 hours. There was no difference in the rate of cell migration between control and knockdown cells or between untreated cells and cells treated with 20 µg/ml CLEC14A-ECD-Fc.

Treatment of HUVECS with 20 µg/ml CLEC14A-ECD-Fc did not affect cell migration. There was no significant difference in scratch wound areas of untreated and treated HUVECS at 8 hours

suggesting that the CLEC14A-ECD-Fc may not function as the full length CLEC14A protein does. However, the concentration of CLEC14A-ECD-Fc used may be too low to elicit a response. Flow cytometry analysis of CLEC14A-ECD-Fc binding to HUVECS as described above revealed that much higher concentrations of CLEC14A are able to bind to the cell surface and that binding is not saturated at 100 µg/ml. Repetition of the scratch wound assays with higher concentrations of CLEC14A-ECD-Fc would reveal whether the ECD can affect cell motility.

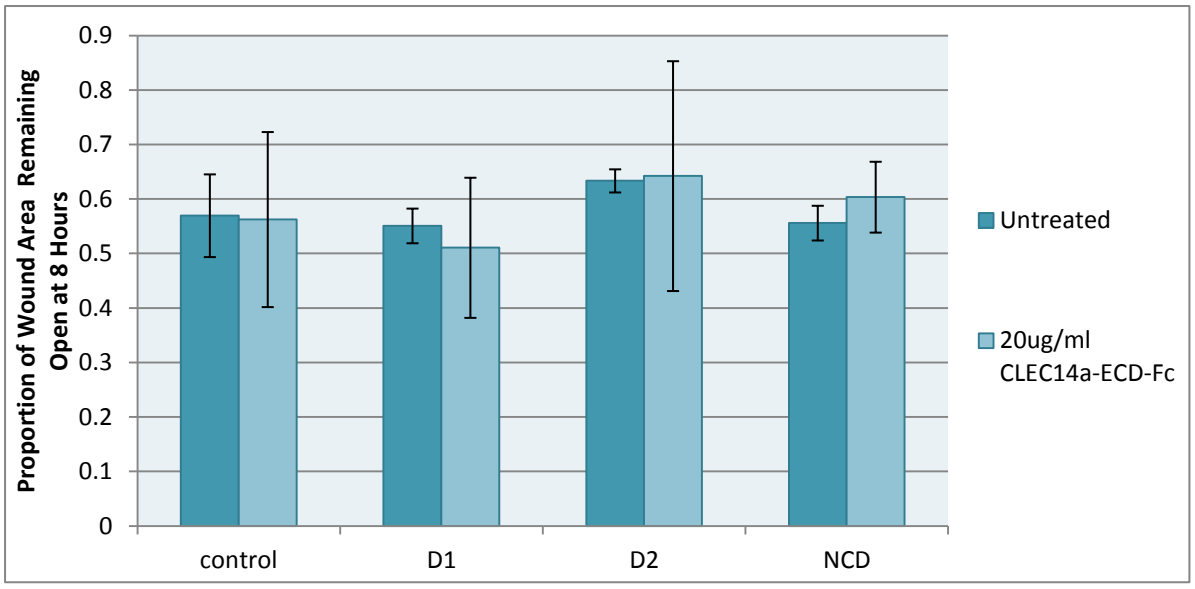

Figure 15. SiRNA knockdown of CLEC14A and treatment of HUVECs with CLEC14A-ECD-Fc did not affect cell migration. There was no significant difference in wound closure between cells treated with 20 µg/ml CLEC14A-ECD-Fc and untreated cells or between siRNA knockdown cells and control cells. Error bars represent standard deviation. Data shown represents wound areas at 8 hours.

SiRNA knockdown of CLEC14A did not appear to affect cell migration. There was no significant difference in scratch wound areas between the control or NCD cells and those treated with siRNA duplexes. Knockdown of CLEC14A was confirmed by western blotting, ruling out the possibility that the knockdowns were unsuccessful (fig.16). These results are in contrast to previous data showing that knockdown of CLEC14A reduced cell migration in scratch wound assays. It may be that variations in scratch sizes could have impacted upon the results although achieving consistent scratches proved to be difficult. Alternative cell migration assays, for example using well inserts to create wound areas of identical size rather that a pipette tip may give more accurate results.

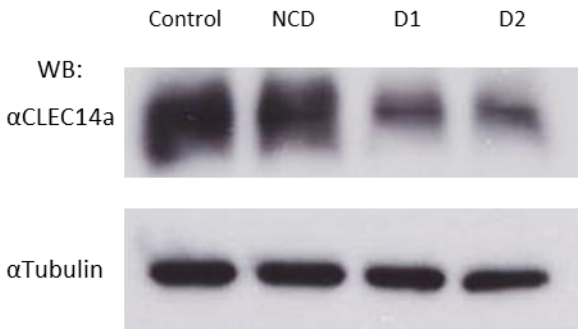

Figure 16. SiRNA knockdown of CLEC14A. Knockdown of CLEC14A in HUVECs used in scratch wound assays was confirmed by western blotting. There is some recovery of protein production as lysates were taken after scratch wound assays were carried out, four days after transfection. NCD, negative control duplex.

4. Discussion

CLEC14A is a specific marker for tumour vessels. This study demonstrated the interaction of purified CLEC14A-ECD-Fc with MMRN2 and MTDH by co-immunoprecipitation using HUVEC lysates. Binding of endogenous CLEC14A and MMRN2 or MTDH was not observed when pulled down with αCLEC14A antibody. It is possible that the interaction between endogenous CLEC14A and the candidate binding partners occurs at levels too low to detect by immunoprecipitation or is too unstable, while higher concentrations of CLEC14A-ECD-Fc enabled the interaction to be observed. It is also important to consider the orientation of CLEC14A and the candidate binding partners. CLEC14A may not interact laterally or in cis with its binding partners preventing co-immunoprecipitation with endogenous CLEC14A. However, CLEC14a may have trans interactions with binding partners present on the surface of another cell, resulting in co-immunoprecipitation of binding partners when exogenous CLEC14A is added. Interaction of CLEC14A with T-Cadherin and Vigilin was not observed by immunoprecipitation. While it is possible that Vigilin and T-Cadherin do not bind to CLEC14A, these negative results do not rule them out as binding partners. Interaction of Vigilin or T-Cadherin with CLEC14A could obscure or block antibody binding sites and prevent co-immunoprecipitation. Activity of the T-Cadherin antibody is also questionable as it failed to detect T-Cadherin from cell lysates in several instances.

Interaction of CLEC14A-ECD-Fc with the surface of HUVECS was observed by flow cytometry and fluorescence was dependant on CLEC14A-ECD-Fc concentration. To discover whether CLEC14A-ECD-Fc had bound to one of the candidate binding partners, siRNA knockdown of MMRN2 and MTDH was attempted but was unsuccessful. This experiment must be repeated and the transfection process refined to determine the target of CLEC14A-ECD-Fc. It may also be useful to knockdown CLEC14A and incubate cells with CLEC14A-ECD-Fc to confirm that CLEC14A-ECD-Fc is not binding to itself.

The ability of CLEC14A-ECD-Fc to bind to the cell surface was confirmed. However, its functionality is unknown. Treatment of HUVECs with CLEC14A-ECD-Fc did not affect wound healing or cell migration. It is possible that 20 µg/ml of CLEC14A-ECD-Fc is too low a concentration to elicit a response although interaction of CLEC14A-ECD-Fc with the cell surface was observed by flow cytometry at 20 µg/ml. It will be interesting to see whether increasing CLEC14A-ECD-Fc concentration affects cell motility in further scratch wound assays to determine how the ECD functions compared to the full length protein.

SiRNA knockdown of CLEC14A did not affect cell migration in scratch wound assays. This is in contrast to previously published data demonstrating that CLEC14A knockdown reduced cell motility. Inconsistencies in scratch wound areas may have impacted upon the results although achieving scratches of constant size proved to be difficult. A more accurate approach may be to use commercially available well inserts to obstruct cell growth. These inserts can then be removed leaving wound areas of consistent width, reducing variation in results. It may also be helpful to perform scratch wound assays sooner after knockdown rather than two days after transfection as indicated in the protocol to avoid recovery of protein production during the experiment.

MTDH is known to interact with an unidentified receptor on endothelial cells which facilitates extravasation of tumour cells and mediates metastasis. These results indicate that this unknown receptor is possibly CLEC14A and that CLEC14A not only has a role in angiogenesis, but is also involved in metastasis. If this is the case, a drug designed to inhibit CLEC14A may be able to inhibit tumour metastasis. The role of the CLEC14A-MTDH interaction in metastasis could be explored using transendothelial migration assays where HUVECs are grown over a porous membrane in a well insert. Tumour cells expressing MTDH are added to the insert and cells migrating across the membrane are counted to reveal whether the CLEC14A-MTDH interaction promotes metastasis.

The possible role of the interaction between CLEC14A and MMRN2 is less clear as CLEC14A is pro-angiogenic while MMRN2 inhibits angiogenesis. MMRN2 may inhibit CLEC14A or vice versa to regulate angiogenic activity.

As discussed, experiments to determine the cleavage site of CLEC14A are on-going while scratch wound assays and flow cytometry experiments with siRNA knockdowns require repetition. Further experiments to explore the interaction of CLEC14A with its binding partners includes the use of surface plasmon resonance (BiaCore) to observe direct interaction of the proteins without the presence of any other cellular components This may also reveal if T-Cadherin or Vigilin interact with CLEC14A as this could not be observed by immunoprecipitation.

X-ray crystallography of CLEC14A bound to MMRN2 or MTDH would provide key structural data which would be useful for designing a drug targeting CLEC14A. Intracellular signalling of CLEC14A remains largely unstudied and is an area which could be explored to determine its mechanism of action.

There is a great deal of interest surrounding CLEC14A and its role in angiogenesis. It is a highly promising biomarker for tumour angiogenesis and could be targeted by monoclonal antibodies to create an effective anti-cancer therapy. With the discovery of its binding partners, structure based design using x-ray crystallography could enable a highly specific drug targeting CLEC14A to be designed.

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