Further characterisation of CLR interactions in primary human dermal lymphatic endothelial cells

Student: Sarah Bishop; Supervisors: Mr Dimitrios Manolis and Dr Leonid L Nikitenko, Centre for Biomedicine, Hull York Medical School, University of Hull, Hull, UK.

Background

The lymphatic vasculature is lined by lymphatic endothelial cells (LEC), which have distinct structural and functional characteristics. In human endothelial cells, the calcitonin receptor-like receptor (CLR) is a class B G protein-coupled receptor (GPCR) that mediates essential functions and plays a role in cardiovascular and skin diseases, migraine and cancer (Nikitenko et al., 2008; Nikitenko et al., 2013; Barwell et al., 2012). Recently, endogenous CLR has been shown to interact with 37 proteins ("CLR interactome") in human dermal lymphatic endothelial cells (HDLEC) (Manolis et al., 2024). These novel members of CLR interactome belong to several protein classes including kinases, transcription factors, and trafficking regulators. Further characterisation of these CLR interactions is an essential step that could contribute to/advance our understanding of their roles in regulating the properties of this GPCR, including signalling, post-translational modification, and trafficking (Manolis et al., 2024).

Aims of the project

The key aim of this project was to further characterise some of these CLR interactions in HDLEC. To fulfil this aim, the following research objectives were addressed:

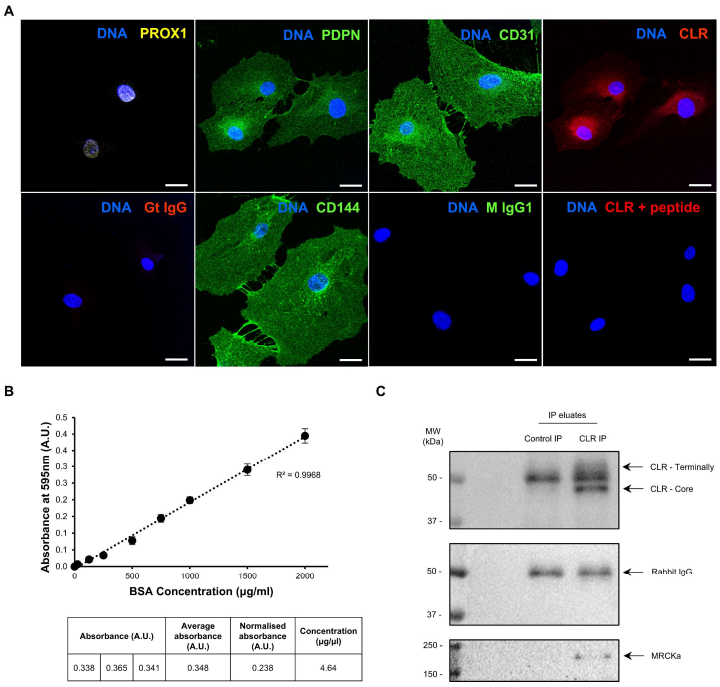
1. To characterise HDLEC by using immunofluorescence (IF) and confocal microscopy for detecting the expression of LEC-specific markers.
2. To validate that newly discovered proteins belong to CLR interactome by using immunoprecipitation (IP) and immunoblotting (IB).

Methods

Primary HDLEC acquired from the labia of a 29-year-old female donor were purchased from PromoCell (Cat# C-12217). HDLEC were cultured as previously described (Nikitenko et al., 2013; Manolis et al., 2024). Briefly, cells were seeded onto a T-75 precoated flask and supplemented with PromoCell EC growth medium MV2 (Cat# C-22121) with the addition of recombinant human vascular endothelial growth factor C (VEGF-C; R&D Systems; Cat# 9199-VC; 7.5 ng/ml). At the confluence of ~80%, HDLEC were sub-seeded in an 8-well slide for HDLEC characterisation by IF, or a T-75 for further culture, cell lysis, IP and WB (further CLR interactome characterisation. HDLEC were fixed in acetone/methanol (2:3 ratio) solution and characterised/authenticated for the expression of specific pan-endothelial (CD31 and CD144) and LEC-specific (PROX1 and podoplanin) markers by IF and confocal microscopy. Incubation with primary antibodies (as above or anti-human CLR/immune serum LN-1436, Nikitenko et al., 2006) was followed by incubation with secondary antibodies conjugated with either Alexa 488 or Alexa 594 fluorophores. Human CLR immune serum with 10 µg/ml of the immunizing peptide, or isotype-matching mouse and goat immunoglobulins (IgG) were used as a negative control for immunostaining (Nikitenko et al., 2006). Mounting was done using 4′,6-diamidino-2-phenylindole (DAPI; VECTASHIELD Vibrance Antifade Mounting Medium with DAPI, Vector Laboratories, Cat# H-1800). Confocal microscopy was performed on a ZEISS LSM 710 with an AXIO Observer Z1 microscope as previously described (Manolis et al., 2024). Prior to IP, HDLEC were lysed in radioimmunoprecipitation assay lysis buffer solution containing protease (Thermo Fisher Scientific, Cat# A32965) and phosphatase (Roche, Cat# 4906845001) inhibitors. Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Cat# 23227) was used to determine total protein concentration in cell lysates, according to the manufacturer's instructions. The measurements of absorbance at a wavelength of 562.0 nm. IP was performed using protein G magnetic beads (Invitrogen, Cat# 10007D) as previously described (Manolis et al., 2024). Briefly, equal amounts of protein (600 µg; 2.0 µg/µl) were mixed with 2.7 µg of either rabbit anti-human CLR/immune serum LN-1436 (Nikitenko et al., 2006) or preimmune serum (as a negative control) and incubated by head-over-tail rotation for 90 min at 4°C before coupling to 4.2 mg of protein G magnetic beads and incubation by further rotation for 60 min at 4 °C. To further characterise CLR interactome, protein expression of specific members of CLR interactome in IP eluates and HLDEC lysates were analysed by IB as previously described (Manolis et al., 2024). In brief, samples were electrophoretically separated on 10% polyacrylamide-based gel, transferred to polyvinylidene difluoride membrane and protein expression was detected by chemiluminescence.

Results and outcomes

IF analysis demonstrated that a pure population of HDLEC expressing pan-endothelial and LEC-specific markers was used in this study (**Figure 1A**). Endogenous CLR expression was observed in perinuclear space and at the cell surface of HDLEC, confirming previous findings (Nikitenko et al., 2006; Manolis et al., 2024). Total protein concentration determined by BCA assay showed that HDLEC lysate contained the amount of protein required for IP and WB analysis (**Figure 1B**). IB analysis revealed that both core-glycosylated and terminally glycosylated forms of CLR were immunoprecipitated from HDLEC lysate with high specificity and that serine/threonine-protein kinase MRCK alpha (MRCKa) co-immunoprecipitated only with anti-human CLR/immune serum (**Figure 1C**).



Absorbance (A.U.)			Average absorbance (A.U.)	Normalised absorbance (A.U.)	Concentration (µg/µl)
0.338	0.365	0.341	0.348	0.238	4.64

Figure 1. Further characterisation of endogenous CLR interactome in human dermal lymphatic endothelial cells. (**A**) Human dermal lymphatic endothelial cells (HDLEC) were fixed with acetone-methanol. HLDEC were characterised by IF analysis using pan-endothelial markers calcitonin receptor-like receptor (CLR; top row-right; red colour), the cluster of differentiation 144 (CD144; bottom row-middle left; green colour), the cluster of differentiation 31 (CD31; top row-middle right; green colour) and LEC-specific markers prospero homeobox 1 (PROX1; top row-left; yellow colour) and podoplanin (PDPN; top row-middle left; green colour). CLR immune serum incubated with immunizing peptide was used as a negative control for immunostaining of CLR (bottom row-right). Mouse (M) or goat (Gt) immunoglobulins (IgG) were used as isotype controls (bottom row - left and middle right). Nuclei were counterstained with DAPI (blue). The scale bars represent 20µm. (**B**) A standard curve for bicinchoninic acid (BCA) assay was obtained using bovine serum albumin (BSA) standard solutions. The unknown concentration of HDLEC lysate was calculated using the equation from the line of best fit which had a strong coefficient of determination (R^2 = 0.9968). Each point represents the mean of three technical replicates. (**C**) Immunoblotting of sample eluates acquired upon immunoprecipitation (IP) of CLR (CLR IP) from total HDLEC lysate using anti-human CLR antibody/immune serum LN-1436. Pre-immune serum served as a negative control (control IP) and detection was done using an anti-rabbit IgG light chain antibody. The expression of serine/threonine-protein kinase MRCK alpha (MRCKA) was detected using primary mouse monoclonal antibody (Santa Cruz Biotechnology; Cat# sc-374568). Imaging and densitometry were performed using Bio-Rad ChemiDoc XRS+ and Bio-Rad Image Lab 6.0 software. The molecular weight of the bands is mentioned on the left in kilodaltons (kDa).

Future directions for the project

This work further characterises physiologically relevant CLR interactome in human cells and has the potential to promote further studies on the mechanistic roles of MRCKa implicated in CLR function (e.g. phosphorylation) and subcellular localization of this interaction (e.g. cell membrane) under specific conditions (e.g. agonist stimulation). The co-immunoprecipitation of endogenous CLR with this kinase further advances the fundamental understanding of the biology of this GPCR.

Departures from the original project plan

Learning new techniques took longer than anticipated due to their complexity. Hence, the completion of the project and achieving the aim and objectives took a bit longer than six weeks.

The value of the studentship and contribution to future career plans

To the student: This project has been an incredibly valuable experience, I have learnt many new technical skills such as immunofluorescence, immunoprecipitation and confocal microscopy, that I would not have performed during my undergraduate degree, had I not had the summer studentship opportunity. Previous skills, such as BCA assay and immunoblotting have been improved and further understood, which will contribute to becoming a more conversant biomedical scientist, and therefore more thorough in my third-year independent research project and my future education and career, enabling me to contribute a better quality of biomedical research.

To the research group: This studentship project has validated previous findings of CLR interactions with a newly discovered protein MRCKa. This provides a basis for further studies of this interaction in human cells in health and disease.

Photo of student, supervisor and research group

Reference List

Barwell, J., et al. *British Journal of Pharmacology*, [online] 166(1), 51–65 (2012).
Manolis, D., et al. *Journal of Biological Chemistry*, [online] 300(6), 107399–107399 (2024).
Nikitenko L.L., et al. *Journal of Cell Science*.,119(Pt 5):910-22 (2006).
Nikitenko, L.L. *Cell and Tissue Research*, 335(1), 223–240 (2008).
Nikitenko, L.L., et al. *Journal of Investigative Dermatology*, 133(7), 1768–1776 (2013).