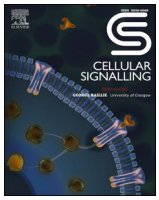
Cellular Signalling 121 (2024) 111261

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig



Short Communication

Calcitonin gene-related peptide and intermedin induce phosphorylation of p44/42 MAPK in primary human lymphatic endothelial cells *in vitro*

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ARTICLE INFO

Keywords:
Lymphatic endothelial cells
CGRP
Adrenomedullin 2
Intermedin
Adrenomedullin
p44/42 mitogen-activated kinases
Skin

ABSTRACT

Calcitonin gene-related peptide (CGRP) and adrenomedullin 2/intermedin (AM2/IMD) play important roles in several pathologies, including cardiovascular disease, migraine and cancer. The efficacy of drugs targeting CGRP signalling axis for the treatment of migraine patients is sometimes offset by side effects (*e.g.* inflammation and microvascular complications, including aberrant neovascularisation in the skin). Recent studies using animal models implicate CGRP in lymphangiogenesis and lymphatic vessel function. However, whether CGRP or AM2/IMD can act directly on lymphatic endothelial cells is unknown. Here, we found that CGRP and AM2/IMD induced p44/42 MAPK phosphorylation in a time- and dose-dependent manner in primary human dermal lymphatic endothelial cells (HDLEC) *in vitro*, and thus directly affected these cells. These new findings reveal CGRP and AM2/IMD as novel regulators of LEC biology and warrant further investigation of their roles in the context of pathologies associated with lymphatic function in the skin and other organs, and therapies targeting CGRP signalling axis.

1. Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide encoded by *CALCA* gene and belongs to the calcitonin family of peptides together with adrenomedullin (AM), adrenomedullin 2 (AM2), also known as intermedin (IMD), amylin (AMY) and calcitonin [1]. CGRP is a neuropeptide and a potent vasodilator that plays multiple roles in physiological and pathophysiological conditions and processes including cardiovascular disease, wound healing, inflammation, cancer, lymphoedema and migraine [2–9]. Recent reports demonstrated the efficacy of drugs targeting the CGRP signalling axis for the treatment of migraine patients, but also revealed significant side effects such as inflammation and microvascular complications, including aberrant neovascularisation in the skin [10–12], highlighting the importance of studying cellular targets of CGRP in human tissues.

Recently, several studies demonstrated that CGRP plays a role in lymphangiogenesis *in vivo* [13,14]. In *Calca* knockout mice ($Calca^{-/-}$), lymphatic capillary formation and macrophage numbers in the skin were reduced in postoperative lymphoedematous tail model [13]. It was proposed in this study that CGRP promotes accumulation of macrophages, which produce vascular endothelial growth factor-C (VEGF-C) that acts on vascular endothelial growth factor receptor-3 (VEGFR-3) in lymphatics, leading to lymphangiogenesis [13]. In addition, in sutured mouse cornea model, exogenous CGRP induced neovascularisation and formation of larger blood and lymphatic vessels [14]. Whilst these studies demonstrated that CGRP plays a role in lymphangiogenesis, whether this peptide has direct effect on lymphatic endothelial cells (LEC) remains unknown.

The mitogen-activated protein kinases (MAPK) p44/42 are essential for angiogenesis and lymphangiogenesis [15,16]. Previous studies demonstrated that CGRP, AM2/IMD and AM induce p44/42 MAPK phosphorylation in endothelial cells (EC) from blood vessels and endothelial progenitor cells (EPC) *in vitro* [17–21]. In contrast to AM [18,22,23], no studies have reported the effects of CGRP and AM2/IMD on LEC to date. To address this gap in knowledge, in the present study we investigated p44/42 MAPK phosphorylation in proliferating primary HDLEC (from two donors) cultured *in vitro* and stimulated with CGRP or AM2/IMD at different time points and concentrations, and compared and contrasted responses to AM. We found that both CGRP and AM2/IMD induce p44/42 MAPK phosphorylation in a time- and dose-

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https://doi.org/10.1016/j.cellsig.2024.111261
Received 10 May 2024; Received in revised form 7 June 2024; Accepted 12 June 2024
Available online 13 June 2024

dependent manner, indicating that these peptides have direct effects on LEC, and thus revealing their roles as novel regulators of LEC biology.

2. Materials and methods

2.1. Reagents

Synthetic human adrenomedullin (AM) trifluoroacetate salt (#4030315), human α-calcitonin gene-related peptide (CGRP) (#4013281) and human intermedin (IMD) (#4044529) were from Bachem. Primary and secondary antibodies were obtained from a range of manufacturers and used at dilutions and concentrations described below. Rabbit polyclonal anti-human CLR (LN-1436; 1:1000) was raised and characterised previously [24]. Primary mouse monoclonal CD31/platelet adhesion molecule-1 (PECAM-1 (#555444; 1:100), mouse IgG1 (#555746; 1:100) and mouse IgG2 (#555740; 1:100) all from BD Biosciences. Mouse monoclonal beta-actin (#ab6276; 1:1000) was from Abcam, rabbit polyclonal anti-phosphorylated p44/42 MAPK (#9101S; 1:1000) and anti-total 44/42 MAPK (#9102S; 1:1000) were from Cell Signalling Technology, goat polyclonal PROX1 (#AF2727; 1:100), rabbit IgG (#ab105C; 1:1000) and goat IgG (#ab108C; 1:200) were from R&D Systems. Secondary conjugated polyclonal donkey anti-mouse IgG Alexa 488 (#A-21202; 1:600), anti-goat IgG Alexa 488 (#A-11055; 1:600), anti-goat IgG Alexa 594 (#A-11058; 1:600) and phalloidin Alexa Fluor™ 635 (#A34054; 1:100) all from Invitrogen, goat horseradish peroxidase (HRP) anti-mouse IgG (#P0447; 1:1000) and anti-rabbit IgG (#P0448; 1:1000) were from Dako.

2.2. Ethical statement and cell culture

2.2.1. Ethical statement

Primary human dermal lymphatic endothelial cells (HDLEC, passage 1) from breast skin of two healthy female donors (46-year-old-D1 and 39-year-old-D2) were purchased commercially from PromoCell® (lot #431Z012.3 and #406Z043.4 respectively). These cells have been tested by the manufacturer for the absence of HIV-1, HIV-2, HBV, HCV, HTLV-1, HTLV-2 and microbial contaminants and by us for the absence of mycoplasma using EZ-PCR Mycoplasma Test kit (Biological Industries; #20–700-20) and the HyperLadder™ 1 kb (Bioline/Meridian Bioscience; # BIO-33026).

2.2.2. Cell culture

Throughout this study HDLEC from D1 and D2 were used in sub-confluent (proliferating) conditions. Cells were seeded onto T-75 (Greiner Bio-One; #658950) pre-coated flask in microvascular (MV2) EC full growth medium (PromoCell®; #C-22121). Recombinant human vascular endothelial growth factor-C (VEGF-C) (R&D Systems; #9199-VC; 7.5 ng/mL) was also added to the MV2 full growth medium. Cultures were incubated at 37 °C in a 5% CO_2 humidified atmosphere, and the medium was replaced every 24 h. Cells were passaged 1:3 at confluence (~80%) by release with trypsin/EDTA (ethylenediaminetetraacetic acid) and sub-cultured using the same method.

2.3. Immunofluorescence

HDLEC characterisation and the analysis of proliferation were done by using immunofluorescence as previously described [24]. HDLEC from D1 and D2 were seeded (5000 cells per well) into 8-well slide chambers (Fisher Scientific; #16250681). Once cells reached 80% confluency, HDLEC were starved in MV2 basal growth medium containing 0.5% foetal bovine serum (FBS, Gibco; #10500064) and no growth factors. After 24 h, cells were fixed using either 4% paraformaldehyde (PFA) or acetone/methanol (2:3 ratio) solutions. Prior to 4% PFA fixation for seven minutes, the culture medium was removed, and cells were gently washed with phosphate-buffered saline (PBS) (Fisher Scientific; #10209252). Next, the PFA solution was removed and cells were washed with PBS once and stored in PBS at 4 °C until further experimentation. For fixation using acetone/methanol, the culture medium was removed, and cells were gently washed with PBS once and incubated with the fixative for three minutes. Next, the fixative was removed and the plates were left to air dry for 20–30 min, then slides were wrapped in cling film and stored at −20 °C. *Prior* to primary antibody incubation, a pre-blocking step, using 10% donkey serum (Bio-Rad; #C06SB, diluted in PBS containing 0.1% Triton-X100) for 30 min at room temperature (RT) was performed. Primary antibodies were diluted at appropriate concentrations in 2% donkey serum and added to 8-well slides, wrapped in cling film and incubated overnight at 4 °C. The next day, cells were placed on ice and washed with PBS/Triton three times for three minutes before the incubation with secondary antibodies in 2% donkey serum solutions. Incubation with secondary fluorophore-conjugated antibodies was performed under light protection at RT for 45 min. Next, the secondary antibody solution was removed and cells were washed with PBS/Triton-X100 thrice. When required, incubation with phalloidin (Alexa Fluor™ 635) was performed for 40 min at RT, followed by mounting using an antifade mounting medium 4′,6-diamidino-2-phenylindole (DAPI, Vectashield®, Vibrance; #H15800–2). Cells were imaged using the confocal microscope (ZEISS LSM 710) and x20 objective. Image analysis was performed using the Zen Blue 3.0 (ZEISS) software.

2.4. Cell viability assay

For cell viability analysis, HDLEC from D1 and D2 were sub-seeded (3500 cells) into 96-well plates (SARSTEDT; #83.3920) in MV2 full growth medium, and a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed (at 6, 24, 48 and 72 h). For cell viability analysis after CGRP, AM2/IMD or AM stimulation, at 24 h post sub-seeding HDLEC from D1 and D2 were starved in MV2 basal medium containing 0.5% FBS and no growth factors for further 24 h. Then HDLEC were stimulated with individual peptides at different concentrations (10^{-12}-10^{-6} M) and incubated for further 24 h, and MTS assay was performed according to the manufacturer protocol. Briefly, 20 μL of CellTiter-96®Aqueous and One Solution MTS assay (Promega; #G3580) was added to each well using a multichannel pipette (Transferpette, SLS Labs) and plates were incubated for 120 min at 37 °C and 5% CO_2. The measurements of absorbance at a wavelength of 490.0 nm were taken using a Tecan Infinite M200 Plate Reader.

2.5. Stimulation with peptides

Peptide stimulation of HDLEC from D1 and D2 was performed as previously described [24]. The cells were sub-seeded (~75,000 cells per well) into 6-well plates (SARSTEDT; #83.3924). The next day, cells were starved in MV2 basal growth medium containing 0.5% FBS and no growth factors. After 24 h HDLEC from D1 and D2 were treated with peptides (AM, AM2/IMD or CGRP, diluted in filtered PBS) at 10^{-6} M or vehicle (PBS) at different time points (0–30 min), or at different concentrations (10^{-12}-10^{-6} M) for 10 min. Next, 6-well plates were placed at 37 °C in a 5% CO_2 humidified atmosphere for the indicated time point and then processed for cell lysis.

2.6. Cell lysis

HDLEC (D1 and D2) lysis was performed as previously described [24]. All steps were performed on ice. Cells were washed with ice-cold filtered PBS and lysates were harvested using cell scrapers in radioimmunoprecipitation assay (RIPA) lysis buffer solution, in which phosphatase inhibitor (phosSTOP™, Roche; #4906845001) was added. Samples were processed in 1.5 mL tubes aspirating up and down and repeating three times at 10-min intervals. Insoluble material was pelleted by centrifugation at 13,000*g* for 10 min at 4 °C, and the

supernatant was stored at −20 °C prior to the determination of total protein concentration.

2.7. Quantification of total protein concentration

Bicinchoninic acid (BCA) assay (Pierce™; #10678484) was used for the determination of total protein concentration of cell lysates according to the manufacturer's instructions. The measurements of absorbance at a wavelength of 562.0 nm were taken using a Tecan Infinite M200 Plate Reader.

2.8. SDS-PAGE and immunoblotting

Protein lysates from HDLEC D1 and D2 were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described [24]. Samples were electrophoretically separated on 10% polyacrylamide-based gel (acrylamide/methylene bisacrylamide solution at 37.5:1 ratio, 375 mM Tris-pH 8.8, 0.1% SDS, 0.1% ammonium persulfate (APS) and 0.04% tetramethylethylenediamine (TEMED) set with 5% stacking gel (acrylamide/methylene bisacrylamide solution at 37.5:1 ratio, 126 mM Tris-pH 6.8, 0.1% SDS, 0.1% APS and 0.01% TEMED). Electrophoresis was run using Tris/Glycine/SDS-based (0.25 M Tris, 1.92 M Glycine, 800 mL double distilled water, 1%SDS, pH 8.3) running buffer at 100 V for 120 min or until optimal resolution of proteins was obtained at 4 °C. Transfer to polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Science, Amersham; #10600021) was performed using Tris/Glycine-based (0.25 M Tris, 1.92 M Glycine, 800 mL double distilled water, pH 8.3) transfer buffer at 60 V for three hours at 4 °C. The membranes were incubated in a blocking solution (5% non-fat milk in Tris-buffered saline containing 0.5% Tween-20 (TBS/T) for 60 min prior to primary antibody incubation. For primary antibody incubation, the membranes were placed in a 50 mL falcon tube (SARSTEDT; #62.547.254) containing the primary antibody solution and incubated overnight on a tube roller at 4 °C. For secondary HRP-conjugated antibody incubation, membranes were washed with TBS/T three times for five minutes and added to a tube containing the relevant secondary antibody and incubated at RT for 45 min. HRP activity was then detected using an enhanced chemiluminescence (ECL) kit (Bio-Rad; #170–5061). After detection, the membranes were stripped by using a stripping buffer (Fisher Scientific; #10016433) and re-probed or stored at −20 °C. Anti-human β-actin was used as a control to monitor and confirm equal loading of total protein in samples. Imaging and densitometry were performed using Bio-Rad ChemiDoc XRS+ System (BioRad Laboratories, Herefordshire) and Bio-Rad ImageLab software (version 6.0) respectively. Exposure times were varied and relied on the quality and intensity of the obtained signal.

2.9. Statistical analysis

All results were presented as box plots with median values with maximum and minimum whiskers. Immunoblotting images for p44/42 MAPK were analysed in ImageLab software using the raw images obtained from the Chemidoc™ XRS + System. The relevant bands were identified/selected (phosphor-p44/42MAPK and total p44/42 MAPK) by drawing a box around the appropriate area and the signal within that area was quantified by using the software (version 6.0). Phospho-p44/42 band intensity data were normalized to the intensity of the total p44/42 MAPK, then normalized throughout to the untreated "0" minute/concentration of p44/42 MAPK band. The Shapiro-Wilk normality test was used to test the normal distribution of the data set. The non-parametric Kruskal Wallis test was performed, followed by the uncorrected Dunn's test, which compares each group to the control group, without any corrections for multiple comparisons. Results were deemed significant if $p < 0.05$. GraphPad Prism 8 software (San Diego, CA, USA) was used for statistical analysis unless stated otherwise. The different statistical tests used for relevant experiments are described in individual figure legends.

3. Results

3.1. HDLEC from two donors are viable and proliferative

First, we characterised HDLEC from two female donors (D1 and D2) by using lymphatic-specific and pan-EC markers (Fig. 1A, Fig. S1A, B). The immunofluorescence (IF) analysis showed that HDLEC from D1 and D2 were positive for the lymphatic-specific prospero homeobox protein 1 (PROX1) and the pan-EC cluster of differentiation 31 (CD31) markers (Fig. 1A, Fig. S1A, B). Next, the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay results revealed that *in vitro* cultured HDLEC from both donors were viable, with viability of HDLEC D1 being higher than HDLEC D2 (whilst both cells were seeded at similar cell density) over 24–72 h (Fig. 1B). Furthermore, IF analysis demonstrated that the proliferation marker Ki67 was expressed in HDLEC from both donors in comparable percentage of cells (Fig. 1C, D). In summary, these findings indicate that HDLEC from D1 and D2 were positive for PROX1 and CD31, viable and proliferating *in vitro*.

3.2. CGRP and AM2/IMD induced p44/42 MAPK phosphorylation in proliferating HDLEC in a time-dependent manner

Next, we investigated whether stimulation of serum- and growth factor-depleted proliferating HDLEC D1 and D2 with CGRP, AM2/AM or AM (all at 10^{-6} M) at different time points (0–30 min) could induce p44/42 MAPK phosphorylation (Fig. 2A, B). In HDLEC D1 an increase in p44/42 MAPK phosphorylation was observed after exposure to each of three peptides (Fig. 2A), compared to control (vehicle-treated cells) (Fig. S3 A, B). CGRP treatment of HDLEC D1 induced p44/42 MAPK phosphorylation in a time-dependent manner, starting as early as 5 min and with significant phosphorylation observed at 10–30 min ($p < 0.05$) (Fig. 2A). Also, significant p44/42 MAPK phosphorylation at 5–15 min ($p < 0.05$) was observed upon AM2/IMD and AM stimulation. Overall, results for CGRP treatment were comparable to AM2/IMD and AM (Fig. 2A).

Furthermore, CGRP, AM2/IMD and AM also induced p44/42 MAPK phosphorylation in HDLEC D2 and these effects were similar to HDLEC D1 (Fig. 2B).

p44/42 MAPK phosphorylation was induced by all three peptides (10^{-6} M) at 5–15 min ($p < 0.05$), but only CGRP-stimulated effect was sustained at 30 min. In summary, our results revealed that CGRP and AM2/IMD, similar to AM, induce p44/42 MAPK phosphorylation in a time-dependent manner in HDLEC from both donors.

3.3. CGRP and AM2/IMD induced p44/42 MAPK phosphorylation in proliferating HDLEC in a dose-dependent manner

CGRP, AM2/IMD and AM at 10^{-6} M significantly induced p44/42 MAPK phosphorylation at 10 min in HDLEC D1 and D2 (Fig. 2A, B). Therefore, next we investigated p44/42MAPK phosphorylation at this time point in serum- and growth factor-depleted proliferating HDLEC stimulated with CGRP, AM2/IMD or AM at various concentrations (10^{-12}-10^{-6} M) (Fig. 3A, B). The immunoblotting analysis revealed that CGRP consistently induced p44/42 MAPK phosphorylation at 10^{-7}-10^{-6} M in HDLEC D1 ($p < 0.05$) and D2 ($p < 0.05$) (Fig. 3A, B).

These effects of CGRP were comparable to AM2/IMD (10^{-7}-10^{-6} M) and AM (10^{-7}-10^{-6} M) stimulated HDLEC D1 (Fig. 3A). In HDLEC D2, AM2/IMD and AM induced p44/42 MAPK phosphorylation at 10^{-8}-10^{-6} M and 10^{-9}-10^{-6} M, respectively (Fig. 3B). CGRP consistently induced p44/42 MAPK phosphorylation at 10^{-7}-10^{-6} M ($p < 0.05$) in HDLEC from both donors, whilst responses to AM2/IMD and AM varied. Although HDLEC D1 had increased cell viability when compared to D2 (Fig. 1B), all three peptides had similar effects on p44/42 MAPK

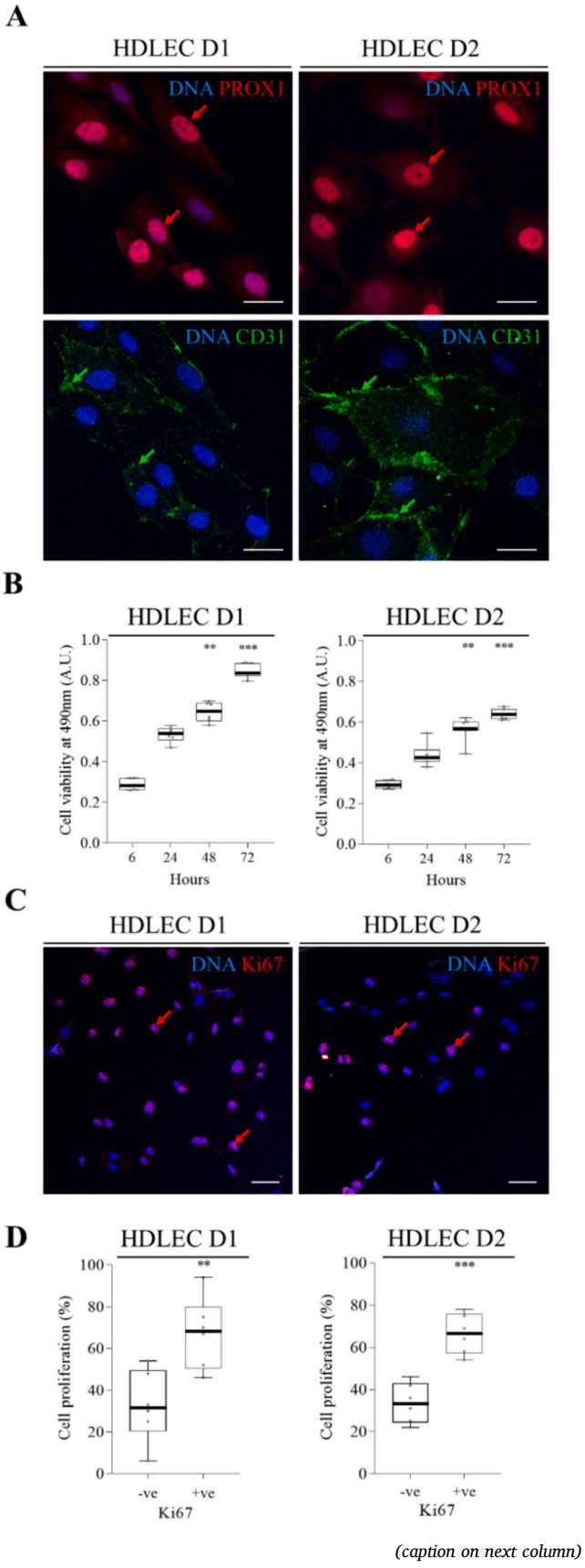

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Fig. 1. Expression of lymphatic- and pan-EC markers, cell viability and proliferation of primary HDLEC. Primary human dermal lymphatic endothelial cells (HDLEC) from breast skin, from two female donors - D1 and D2 (see Materials and Methods) were cultured in MV2 basal medium containing 0.5% foetal bovine serum (FBS) and no growth factors for 24 h. (A) HDLEC characterisation was done by immunofluorescence (IF) using lymphatic- and pan-EC markers, prospero homeobox protein 1 (PROX1) (*red colour*) and cluster of differentiation 31 (CD31) (*green colour*) respectively. Nuclei were counterstained with DAPI (*blue colour*). PROX1 expression in the nuclei is shown using red arrows and CD31 expression at cell-cell contacts using green arrows. Scale bars represent 20 μm. Full datasets can be found in the Supplementary Data (Fig. S1). (B) Cell viability of HDLEC D1 and D2 was assessed at 6, 24, 48 and 72 h after sub-seeding (without changing culture media) by using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. (C) Proliferation of HDLEC D1 and D2 was assessed by IF using mouse monoclonal antibody raised against proliferation marker Ki67 and positive staining for the marker was indicated with red arrows. Nuclei were counterstained with Hoechst (*blue colour*). Scale bars represent 50 μm. Full datasets can be found in the Supplementary Data (Fig. S2). (B, D) Box and whiskers plots overlaid with dots represent the results of quantification analysis of relative cell viability and proliferation. The data represents median values ($n = 3$ independent experiments for both D1 and D2), the box contains the 25th and 75th percentiles and whiskers are the minimum and maximum values of each dataset. The statistical analysis was performed using Kruskal Wallis test (based on Shapiro-Wilk normality test) followed by uncorrected Dunn's comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phosphorylation in HDLEC from both D1 and D2 at different time points and different concentrations (Figs. 2, 3). In summary, our results revealed that CGRP and AM2/IMD, similar to AM, induce p44/42 MAPK phosphorylation in a dose-dependent manner.

3.4. CGRP and AM2/IMD induced cell viability of proliferating HDLEC

Finally, we investigated whether CGRP- and AM2/IMD -induced p44/42 MAPK phosphorylation in serum- and growth factor-depleted proliferating HDLEC D1 and D2 translates into cell function by analysing cell viability after stimulation with individual peptides at different concentrations (10^{-12}-10^{-6} M) (Supplementary data, Fig. S4A, B). MTS assay data analysis showed that all three peptides significantly increased viability of proliferating HDLEC from both donors: CGRP - D1 and D2 at 10^{-6} M and 10^{-9}-10^{-6} M respectively, AM2/IMD - both D1 and D2 at 10^{-11}-10^{-6} M, and AM - D1 and D2 at 10^{-12}-10^{-6} M and 10^{-9}–10^{-6} M respectively. These findings revealed that the potency of CGRP for increasing viability of cells cultured under condition of serum- and growth factor-depletion at 24 h post-stimulation was higher for HDLEC D2, when compared to HDLEC D1, which had higher viability when cultured in complete/full (*i.e.* containing serum and growth factors) medium (Fig. 1B) but very similar pharmacological profile of p44/42 MAPK phosphorylation in response to CGRP at different time points (5–30 min) and concentrations (Figs. 2, 3). In summary, these results and comparative analysis revealed that CGRP and AM2/IMD, similar to AM, increase viability of proliferating HDLEC from both donors, confirming that induced by these peptides p44/42 MAPK phosphorylation (a short-term effect) is associated with cell function (a long-term effect).

4. Discussion

CGRP is involved in a variety of biological processes such as cardiovascular homeostasis [25], immune regulation [26], pain modulation [27], and most recently, lymphangiogenesis [13,14]. In the present study, we found that CGRP induced p44/42 MAPK phosphorylation in a time- and dose-dependent manner in proliferating primary HDLEC from two donors. To our knowledge, this is the first of its kind report about the direct effect of CGRP on LEC biology. This novel finding warrants further

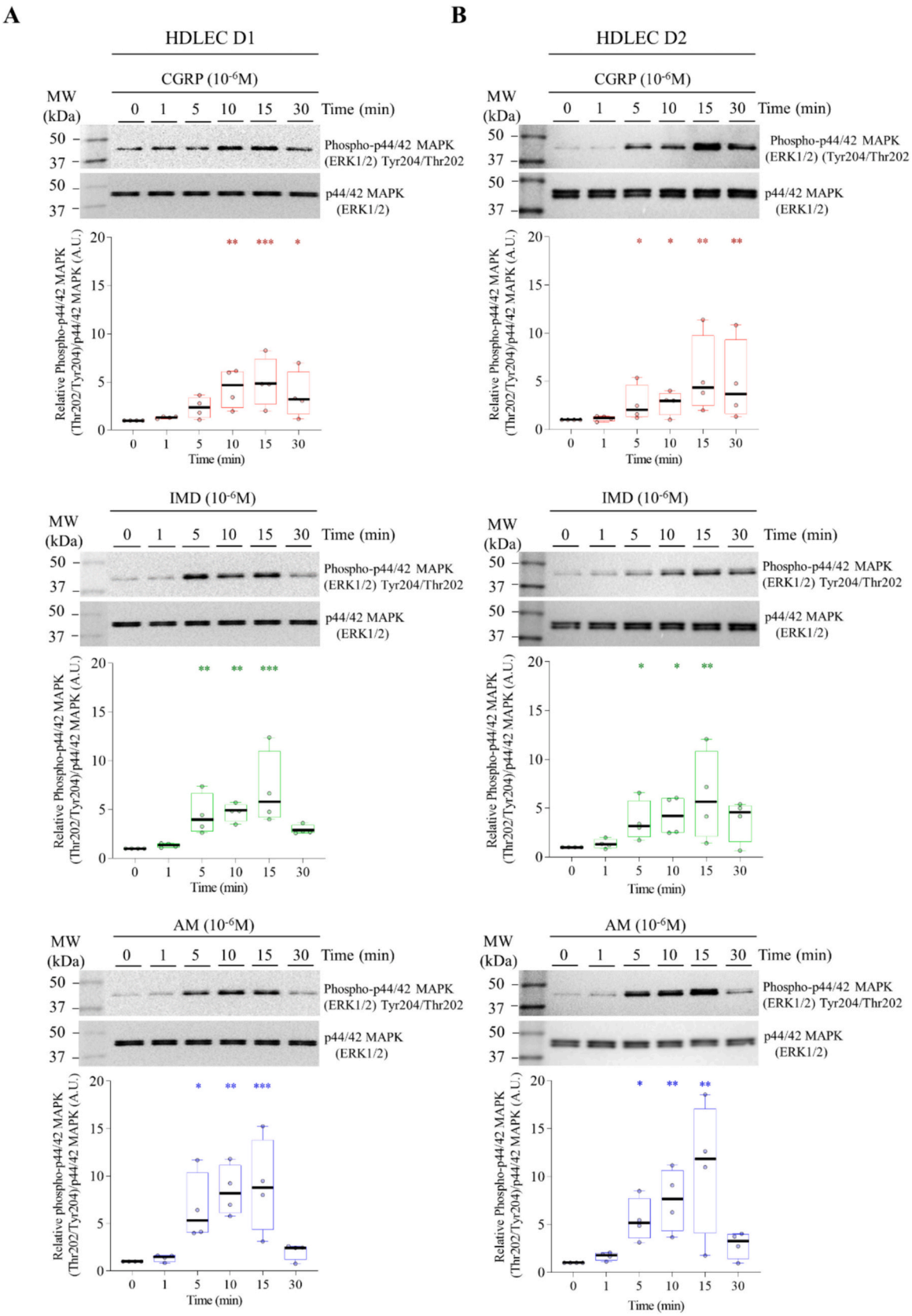

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Fig. 2. Phosphorylation of p44/42 MAPK in primary HDLEC upon stimulation with CGRP, AM2/IMD or AM at different time points. (A, B) Dynamics of p44/42 MAPK phosphorylation upon stimulation of primary human dermal lymphatic endothelial cells (HDLEC) from breast skin, from two female donors - D1 and D2 (see Materials and Methods) with calcitonin gene-related peptide (CGRP), adrenomedullin2/intermedin (AM2/IMD) or adrenomedullin (AM). Prior to cell lysis for protein extraction, HDLEC were cultured in MV2 basal medium containing 0.5% foetal bovine serum (FBS) and no growth factors for 24 h and then stimulated at different time points (0–30 min) at 10^{-6} M concentration with peptides. Phosphate-buffered saline (PBS) was used as a vehicle and control (Supplementary Data, Fig. S3). Bands of interest were quantified by densitometry analysis using Bio-Rad Image Lab 6.0 software. The phospho-p44/42 MAPK expression was first normalized to total p44/42 MAPK, and then to time point '0' for each peptide, and the results were plotted under each blot. Box and whiskers plots overlaid with dots represent the results of the quantification analysis of phospho-p44/42 MAPK relative to total p44/42 MAPK. CGRP (*red*), IMD (*green*) and AM (*blue*). The data represents median values ($n = 4$ independent experiments for both D1 and D2), the box contains the 25th and 75th percentiles and whiskers are the minimum and maximum values of each dataset. The statistical analysis was performed using Kruskal Wallis (based on Shapiro-Wilk normality test) followed by uncorrected Dunn's comparison test to determine differences compared to time point"0". *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

investigation of CGRP role in these cells in the context of conditions and pathologies associated with lymphatic function and therapies targeting CGRP signalling axis.

In animal models, CGRP is proangiogenic during hind limb ischaemia [28], tumour growth [29] and wound healing [30], and prolymphangiogenic in the sutured cornea [14] and postoperative tail models [13]. In the present study, we found that CGRP and AM2/IMD have direct effects on proliferating primary HDLEC (from two donors) cultured *in vitro*. Besides embryogenesis, LEC proliferation also takes place in adulthood in some physiological processes (wound healing and inflammation) and pathological conditions (cancer and lymphoedema) [13,14,29–31]. Under these processes and conditions, the expression and role of CGRP has been also reported based on findings from *in vivo* models [13,14,29,30]. For example, in *Calca*$^{-/-}$ knockout mice microvessel density and area, and M2 macrophage numbers were reduced during wound healing in the skin, lung cancer and postoperative lymphoedema [13,29,30]. In addition, in a laser-induced choroidal neovascularization model, inflammation was suppressed in *Calca*$^{-/-}$ knockout mice [31]. Several studies established the association of inflammation with lymphangiogenesis, lymphatic vessel remodelling and [32,33]. Therefore, since CGRP has been implicated in neovascularisation and action on microvasculature under conditions where lymphangiogenesis takes place, the findings from our *in vitro* study suggest that CGRP might have direct effects on proliferating LEC *in vivo* too.

Phosphorylation of p44/42 MAPK in EC is crucial during angiogenesis and lymphangiogenesis and EC survival [16,17]. Previous studies established a link between the MAPK signalling pathway and members of the calcitonin family of peptides, including CGRP, in several cell types, such as human umbilical vascular endothelial cells (HUVEC) and EPC, cardiomyocytes [20,21], Schwann cells [34] and smooth muscle cells [35]. In the present study, we found that CGRP and AM2/IMD, comparably to AM, induce p44/42 MAPK phosphorylation and survival in proliferating serum-starved primary HDLEC cultured *in vitro*. Our findings revealed that CGRP consistently induced phosphorylation of p44/42 MAPK at 10^{-7}-10^{-6} M in HDLEC obtained from breast skin from two adult female donors. These results are in agreement with other reports, which demonstrated that p44/42 MAPK phosphorylation was induced by CGRP at 10^{-7}-10^{-5} M in EC of embryonic origin (HUVEC and EPC) [20,21]. Furthermore, CGRP induces other signalling pathways, including protein kinase B (Akt) in human dermal microvascular EC [24], endothelial nitric oxide (NO) in keratinocytes [36], cAMP, NO and calcium (Ca^{2+}) mobilisation in HUVEC [21]. Therefore, CGRP and AM2/IMD effects on various signalling pathways and intermediates in proliferating primary human LEC from adult tissues (both male and female donors) require further investigation and validation of findings in other relevant pre-clinical *in vivo* and *in vitro* models.

Proliferating LEC acquire quiescence once the lymphatic network is established [37,38]. In the quiescent state, LEC are involved in immune cell transportation and other functions of lymphatic vessels [37,38]. Findings from several studies suggest that CGRP plays a role in quiescent lymphatic vessels [39–42]. For example, CGRP-containing nerve fibres are located in close proximity to lymphatic capillaries in rat skin [39]. In addition, exogenous CGRP stimulation leads to inhibition of NO-dependent vasomotion in perfused mesenteric lymphatic vessels of guinea pigs [40]. Also, *in vitro* studies demonstrated that by acting on primary murine dermal microvascular EC, CGRP facilitates the immune response and regulates the outcome of antigen presentation by Langerhans cells to T cells [41]. Furthermore, studies using *Adm2*$^{-/-}$ knockout mice, implicated AM2/IMD in the proliferation of quiescent CD31-positive vessels in the retina [42]. Therefore, it cannot be excluded that observed in these studies effects of CGRP and AM2/IMD may be due to direct action of these peptides on quiescent LEC in lymphatic vessels, thus warranting further investigation.

CGRP effects on cells are mediated by G-protein coupled receptors (GPCRs) calcitonin receptor-like receptor (CLR) or calcitonin receptor (CTR) upon their co-expression with receptor activity-modifying proteins 1, 2 and 3 (RAMPs 1–3) [43]. CLR and RAMPs 1–3 expression was previously shown in primary human LEC in tissues (skin, uterus and lung) and *in vitro* [22,23,44–47]. CGRP and AM compete for the same receptor in human dermal microvascular EC, involving different downstream mechanisms [24]. Furthermore, our recent study revealed that endogenously expressed in HDLEC CLR interacts with 37 novel proteins, which are involved in signalling, post-translational modifications, and trafficking of other GPCRs [47]. Altogether, these findings suggest that further studies are needed to dissect the role of CLR (or CTR) in mediating CGRP- and AM2/IMD-induced direct effects in primary LEC that were revealed in our study.

Recent reports demonstrated the use of drugs targeting the CGRP signalling axis, such as antibodies against CGRP and its receptors, for the effective treatment of migraine [2,48–50]. Since CGRP is implicated in both lymphangiogenesis and angiogenesis [13,14,29], it was proposed that prolonged treatment with these agents could lead to impaired neovascularisation in some patients [49]. Recent studies demonstrated that the use of CGRP monoclonal antibody (erenumab) resulted in skin disturbance during wound healing in a migraine patient, with skin biopsy demonstrating a deep perivascular and interstitial lymphohistiocytic infiltrate with admixed eosinophils, ulceration of the epithelium, heavy oedema of the papillary dermis and focally thrombosed vessels [10]. In addition, eight migraine patients treated with CGRP monoclonal antibodies (erenumab, fremanezumab or galcanezumab) developed inflammatory complications [11,51]. These complications include Susac's syndrome (linked to endotheliopathy, a disorder that develops due to functional changes in the endothelium) [51], granulomatosis with polyangiitis (a rare disorder that causes inflammation of the blood vessels in the nose, sinuses, throat, lungs and kidneys), drug reaction with eosinophilia and systemic symptoms (clinically presents, as an extensive mucocutaneous rash, accompanied by fever, lymphadenopathy, hepatitis, haematologic abnormalities with eosinophilia and atypical lymphocytes), autoimmune-hepatitis, poly-arthralgia, psoriasis and urticarial eczema [11]. All these effects were *de novo*, with a clear temporal relationship between exposure and symptom-onset [11]. Moreover, in a retrospective cohort study of 169 migraine patients treated with CGRP monoclonal antibodies (erenumab, fremanezumab or galcanezumab), nine patients exhibited microvascular complications [12]. In the context of these reports, the findings from our study suggest

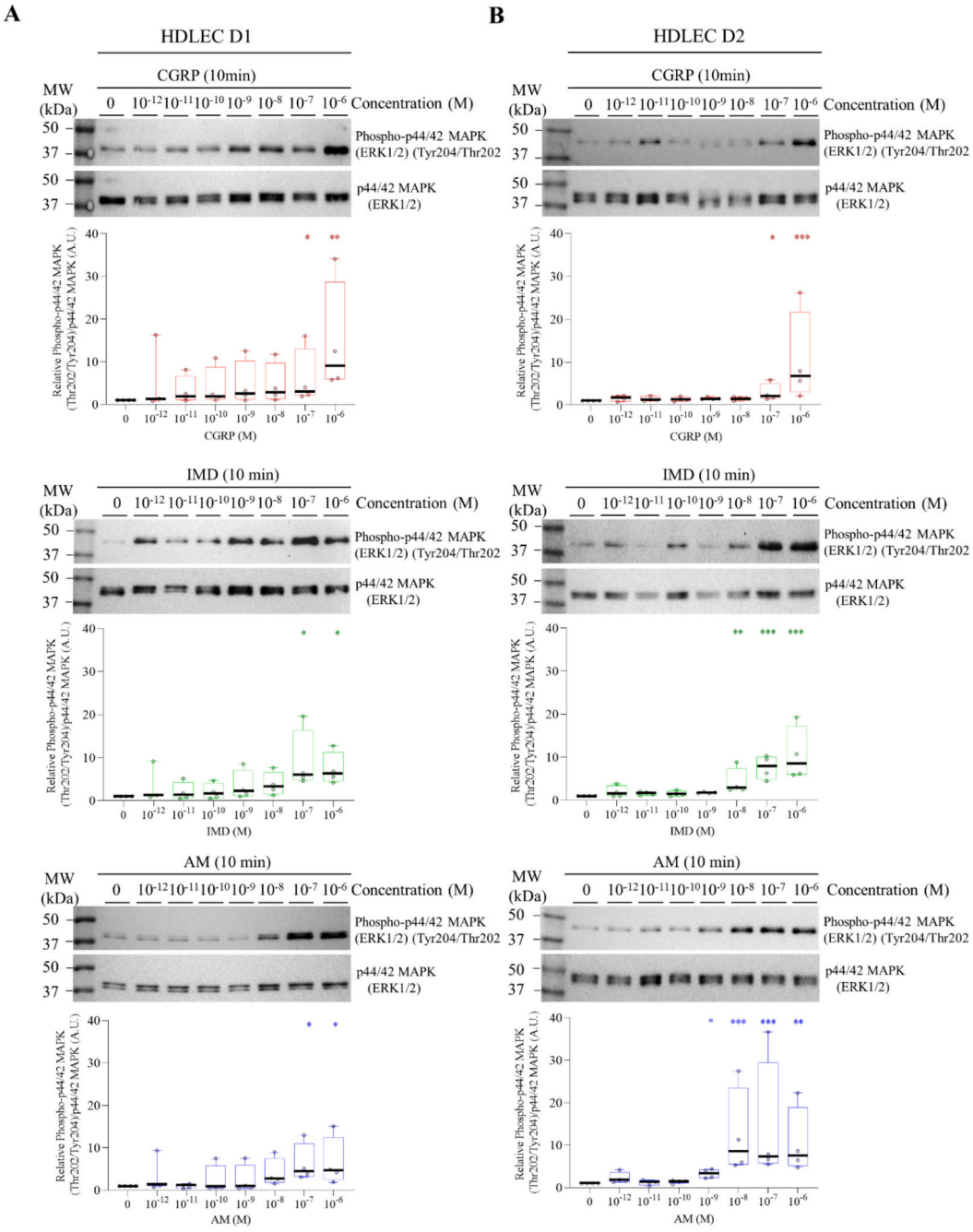

Fig. 3. Phosphorylation of p44/42 MAPK in primary HDLEC upon stimulation with CGRP, AM2/IMD or AM at different concentrations. (A, B) Dynamics of p44/42 MAPK phosphorylation upon stimulation of primary human dermal lymphatic endothelial cells (HDLEC) from breast skin, from two female donors - D1 and D2 (see Materials and Methods) with calcitonin gene-related peptide (CGRP), adrenomedullin2/intermedin (AM2/IMD) or adrenomedullin (AM). Prior to cell lysis for protein extraction, HDLEC were cultured in MV2 basal medium containing 0.5% foetal bovine serum (FBS) and no growth factors for 24 h and then stimulated with peptides at various concentrations (10^{-12}-10^{-6} M) for 10 min. Phosphate-buffered saline (PBS) was used as a vehicle. Bands of interest were quantified by densitometry analysis using Bio-Rad Image Lab 6.0 software. The phospho-p44/42 MAPK expression was first normalized to total p44/42 MAPK, and then to vehicle (PBS) for each peptide, and results were plotted under each blot. Box and whiskers plots overlaid with dots represent the results of the quantification analysis of phospho-p44/42 MAPK relative to total p44/42 MAPK. CGRP (*red*), IMD (*green*) and AM (*blue*). The data represents median values ($n = 4$ independent experiments for both D1 and D2), the box contains the 25th and 75th percentiles and whiskers are the minimum and maximum values of each dataset. The statistical analysis was performed using Kruskal Wallis (based on Shapiro-Wilk normality test) followed by uncorrected Dunn's comparison test to determine differences between stimulated and unstimulated cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that adverse/side effects of drugs targeting the CGRP signalling axis could be attributed (at least partially) to CGRP signalling and functions in human LEC and lymphatic vessels.

In summary, our first of its kind study reveals that CGRP and AM2/IMD induce phosphorylation of p44/42 MAPK in proliferating primary HDLEC, and thus have direct effects on these cells. These new findings suggest that CGRP and AM2/IMD are novel regulators of LEC biology. Our study opens up new avenues and serves as a springboard for further investigations of CGRP- and AM2/IMD-induced effects in both proliferating and quiescent LEC (and also other cellular targets for these

peptides in human tissues) under physiological conditions and pathologies associated with lymphatic function and beyond.

CRediT authorship contribution statement

Shirin R. Hasan: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **Dimitrios Manolis:** Visualization, Software, Methodology. **Ewan Stephenson:** Visualization, Methodology. **Oktawia A. Ryskiewicz-Sokalska:** Visualization, Methodology. **Anthony Maraveyas:** Writing – review & editing, Supervision, Funding acquisition. **Leonid L. Nikitenko:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Leonid Nikitenko reports financial support was provided by Welcome Trust Biomedical Vacation Scholarship. Dimitrios Manolis reports financial support was provided by Welcome Trust Biomedical Vacation Scholarship. Leonid Nikitenko reports financial support was provided by Biochemical Society UK Summer Vacation Studentship. Oktawia A. Ryskiewicz-Sokalska reports financial support was provided by Biochemical Society UK Summer Vacation Studentship. Leonid Nikitenko reports financial support was provided by Castle Hill Hospital Melanoma Charitable Fund. Shirin Hasan reports financial support was provided by Castle Hill Hospital Melanoma Charitable Fund. Anthony Maraveyas reports financial support was provided by Castle Hill Hospital Melanoma Charitable Fund. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Professors Roland Ennos and Tim Palmer, Drs. Camille Ettelaie, Eamon Faulkner, Matthew Morfitt, Magnus Johnson and Chao Huang (all - University of Hull) and Tatsuo Shimosawa (International University of Health and Welfare, Japan) for their constructive feedback on this manuscript. Funding: University of Hull Research Support Fund (E.S. and L.L.N.); Welcome Trust Biomedical Vacation Scholarship (Reference: 218420/Z/19/Z; D.M. and L.L.N.); Biochemical Society UK Summer Vacation Studentship (O.A.R-S and L.L.N.) and Castle Hill Hospital Melanoma Charitable Fund (S.R.H., L.L.N. and A.M.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2024.111261.

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