Cathepsin D non-proteolytically induces proliferation and migration in human omental microvascular endothelial cell via activation of the ERK1/2 and PI3K/AKT pathways

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Abstract

Epithelial ovarian cancer (EOC) frequently metastasizes to the omentum, a process that requires pro-angiogenic activation of human omental microvascular endothelial cells (HOMECs) by tumour-secreted factors. We have previously shown that ovarian cancer cells secrete a range of factors that induce pro-angiogenic responses e.g. migration, in HOMECs including the lysosomal protease cathepsin D (CathD). However, the cellular mechanism by which CathD induces these cellular responses is not understood. The aim of this study was to further examine the pro-angiogenic effects of CathD in HOMECs i.e. proliferation and migration, to investigate whether these effects are dependent on CathD catalytic activity and to delineate the intracellular signalling kinases activated by CathD. We report, for the first time, that CathD significantly increases HOMEC proliferation and migration via a non-proteolytic mechanism resulting in activation of ERK1/2 and AKT. These data suggest that EOC cancer secreted CathD acts as an extracellular ligand and may play an important pro-angiogenic, and thus pro-metastatic, role by activating the omental microvasculature during EOC metastasis to the omentum.

Keywords: cathepsin D, non-proteolytic, proliferation, migration, angiogenesis

Abbreviation: EOC, epithelial ovarian cancer; CathD, cathepsin D; EC, endothelial cell; ERK1/2, extracellular signal regulated kinase; AKT, protein kinase B; CAM, chorioallontoic membrane model; ECM, extracellular matrix.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal of all gynaecological cancers. Annually, approximately 200,000 women are diagnosed with this malignancy worldwide, resulting in 125,000 disease related deaths each year [1]. Initial symptoms are often vague, frequently leading to advanced disease with widespread metastases at diagnosis which is therapeutically challenging [2]. As a result, 5 year survival is still only approximately 45% [3].

EOC primarily metastasises via the transcoelomic route, with cancer cells disseminating within the peritoneum to form secondary foci. The omentum is a common initial point of spread, with omental invasion facilitating more widespread metastasis [4]. In order to establish a secondary tumour within the omentum ovarian cancer cells must attach to the mesothelium, invade the omental tissue and then initiate angiogenesis i.e. sprouting of new blood vessels from the pre-existing vascular bed, to sustain secondary tumour growth. This process requires a complex interplay between tumour and resident cells, with secretion of growth factors and chemokines that ultimately leads to activation of a pro-angiogenic phenotype in the omental microvascular endothelial cells (ECs) and subsequently neovascularisation [5-9].

Vascular endothelial growth factor A (VEGFA) is known to be a major pro-angiogenic stimulator in many tumour types and both VEGFA expression and secretion are known to be upregulated in EOC [10, 11]. However, our previous studies have indicated that angiogenic changes in the omental ECs during metastasis of ovarian cancer to the omentum are primarily driven by alternative pro-angiogenic factors secreted by EOC cells [12]. These data are supported by the observation that anti-VEGFA therapy (bevacizumab) has shown limited efficacy in patients with ovarian cancer [13], highlighting the need for a clearer understanding of the pro-angiogenic pathways involved.

One of the alternative pro-angiogenic proteins we previously identified is cathepsin D (CathD), an aspartic endopeptidase, typically involved in degrading unfolded, dysfunctional self- or foreign- proteins in lysosomes and phagosomes [14]. Although CathD is a lysosomal enzyme and its enzyme activity is usually regulated within the acidic compartment of lysosomes, it has also been reported to be secreted via, an as yet, unknown mechanism. Physiologically, CathD has been found in human, bovine and rat milk and serum [15-18], and pathophysiologically overexpression and hypersecretion of CathD has been demonstrated in a variety of cancers including ovarian, breast, endometrial, lung and prostate, as well as malignant glioma and melanoma [12, 19-32]. Indeed, we have detected CathD in the secretome of EOC cells *in vitro* [12], in ascites of patients suffering from ovarian cancer (unpublished data) and have shown that there is a significantly higher expression of CathD in omentum of patients with metastasised serous ovarian carcinoma compared with omentum from patients with benign ovarian cystadenoma [33].

There is increasing evidence that extracellular CathD may play a role in tumour angiogenesis including in metastasis of ovarian cancer to the omentum. In the wider cancer field CathD increases tumour vascularity in tumour xenografts in mice [18] and enhances angiogenesis in a chick chorioallontoic membrane model (CAM) [34], induces pro-angiogenic changes in more than one type of EC [12,34], as well as inducing degradation of extracellular matrix (ECM) components and release of growth factors such as basic fibroblast growth factor (bFGF) [22], and proliferation of cancer cells [35-39] and fibroblasts [40]. In relation to omental metastasis exogenous CathD induces pro-angiogenic cellular effects on disease relevant human omental microvascular ECs (HOMECs) e.g. enhanced migration structures [12] and as mentioned above is overexpressed in the omentum of patients with metastasised EOC [33].

Interestingly, although lysosomal CathD has proteolytic functions, secreted CathD has been reported to be active in both a proteolytic and non-proteolytic manner. For instance, both wild-type and mutated (ASN 231, proteolytically inactive) CathD stimulate proliferation of 3Y1-Ad12 rat tumour cells in athymic nude mice [36]. Additionally, CathD can act as a protein ligand to induce proliferation of fibroblasts [40] and MCF7 breast cancer cells [22, 41]. A pro-angiogenic role for non-proteolytically active CathD has been demonstrated in studies using pepstatin A, a specific inhibitor of CathD-proteolytic activity, which inhibited CathD-induced migration and tube formation both in cultured human umbilical vein ECs and angiogenesis in a CAM model [34].

Despite the emerging role for CathD in tumour growth, and the data described above suggesting specific involvement of extracellular CathD in angiogenesis in secondary omental lesions of EOC, the downstream cell signalling pathways activated by the protein are still poorly understood and remain to be elucidated. Given the therapeutic challenges posed by EOC a fuller understanding of the processes involved in secondary tumour formation may aid development of treatment strategies. We have previously published a technique for isolating disease relevant HOMECs [42] and in this study we used this cell model to (a) investigate whether CathD exerts its migratory and/or proliferative effects through a proteolytic or non-proteolytic mechanism and (b) the downstream signalling cascades activated by CathD. We demonstrate, for the first time that CathD significantly increases HOMEC proliferation and migration via proteolytic-independent mechanisms. Importantly, we also show that exogenous CathD activates the intracellular kinases ERK1/2 and AKT (S473) as part of a signalling cascade that ultimately induces a proliferative and migratory phenotype in HOMECs.

2. Materials and methods

2.1. Primary cell culture

Non-malignant omental tissue samples were collected from patients undergoing surgery at the Royal Devon and Exeter NHS Foundation Trust (Exeter, United Kingdom) with ethical approval and informed written consent. HOMECs were isolated, characterised and cultured as primary cells as previously described [42]. Briefly, HOMECs were cultured in endothelial cell growth media (MV2, PromoCell, Heidelberg, Germany) supplemented with supplied growth factors, 5% (v/v) foetal calf serum (FCS) and 0.1% (v/v) gentamycin (Sigma, Poole, UK). Cells were maintained at 37°C in a humidified atmosphere supplemented with 5 % CO_2.

2.2. Cell proliferation assay

HOMEC proliferation was assessed using the WST-1 water soluble tetrazolium salt-1 assay (Roche, Welwyn Garden City, UK). Cells were seeded at a density of $1x10^4$ cells per well in 2% (w/v) gelatin (Sigma, Poole, UK) coated 96-well plates (Greiner Bio One, Stonehouse, UK) and treated overnight in growth factor-deprived media containing 2% (v/v) FCS. After 24 hours, treatments (CathD, VEGF as positive control ± inhibitors) were added at the given concentrations (Table 1) and incubated for 72 hours. Subsequently, WST-1 reagent was added in a 1:10 dilution to the assay medium for 2 hours incubation and absorbance was measured at 450 nm against the blank in PHERAstar BMG plate-reader. Based on the data obtained 50ng/ml CathD was determined to be optimal for proliferation and this concentration was used for all subsequent experiments unless otherwise noted.

2.3. pH experiments

2.3.1. Measurement of pH of cell culture media during cell culture

HOMECs were seeded at a density of $3x10^5$ cells per well in 6 well plates, based on preliminary optimization experiments. After overnight incubation in growth factor depleted media as above, fresh media supplemented ± CathD (50ng/ml) was added. Culture media was collected and pH was measured after 24, 48 and 72 hours using an ABL80 FLEX blood-gas analyser (Radiometer, Crawley, UK). pH of medium-only was also measured at the beginning of incubation period.

2.3.2 Measurement of enzymatic activity of CathD at different pHs

CathD proteolytic activity was measured using a CathD-specific fluorogenic substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2, (100 nmol/l, Enzo Life Sciences, Exeter, UK,), in the presence or absence of pepA (1 µmol/l) in black opaque 96-well plates (Greiner Bio One, Stonehouse, UK). Prepared buffer solutions at specific pHs containing substrate ± pepA were added to the wells (100µl). Subsequently, 20µl of 50ng/ml CathD was added as required to make up the final volume of 120µl. Control wells contained substrate or substrate and pepA, and 20µl of corresponding pH buffer solution. The plate was shaken for 60 seconds in a plate-reader immediately prior to fluorescence reading at Ex/Em: 320/393. The experiment was performed away from direct light exposure. The pH buffer solutions were prepared by mixing citric acid monohydrate and Na_2HPO_4 solutions and 0.005% (v/v) Tween 20 (Sigma-Aldrich, Poole, UK) in the correct proportions to ensure a final pH of: 3, 3.6, 4, 4.6, 5, 5.6, 6, 6.6, 7 and 7.6 (supplementary data).

2.4. Detection of phosphorylation of intracellular signalling intermediates

2.4.1. Phosphokinase array

Upregulation of phosphorylation of intracellular kinases was detected using a Proteome Profiler Human Phospho-Kinase Array kit (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. Briefly, HOMECs were seeded in $10cm^2$

petri dishes and cultured as above until confluent. Cells were starved overnight and then treated ± 50ng/ml CathD, ± inhibitors as detailed, for 4 minutes and subsequently lysed. A BCA protein assay was performed to quantify the total protein levels in each lysate. Controls received carrier alone. 200µg of protein (lysate) was incubated with antibody coated membranes and levels of phosphorylated proteins were assessed by chemiluminescence detected on film. The relative expression of specific phosphorylated proteins was determined following quantification of spot density on scanned images by Image-J. The results are expressed as mean dot density (arbitrary units).

2.4.2. Cell based ELISA

Phosphorylation levels of ERK1/2 and AKT were measured using specific cell-based ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Cells were treated ± VEGF (20ng/ml, positive control) and CathD (50ng/ml) in the presence or absence of ERK1/2 and AKT inhibitors at their given concentrations (Table 1) for 4 and/or 10 minutes. Phosphorylation was examined at two time points since activation of intracellular kinases can be transient [43]. The shorter time point (4 minutes) for CathD was selected because previous reports suggest that MAPK/ERK1/2 and AKT phosphorylation are maximum at 4-5 minutes [44]. Fluorescence intensity was measured and the results are expressed as fold change in phospho-ERK1/2 or -AKT relative to total ERK or AKT levels (compared to control).

2.5. HOMEC migration

Cell migration was assessed using a Cultrex Cell 96 transwell migration assay (R&D Systems, Abingdon, UK) as per kit instructions. Briefly, cells were grown in growth factor-deprived media supplemented with 2% FCS for at least 24 hours. Cells were then seeded at $5x10^4$ per upper chamber and treated ± VEGF (20ng/ml, positive control) and/or CathD (50ng/ml) and in the presence or absence of ERK1/2 and/or AKT inhibitors at their given concentrations (Table 1). Negative controls received carrier alone. After 6 hours incubation at 37^oC, the bottom chambers were washed, followed by addition of cell dissociation solution/calcein AM for a further hour to label and detach migrated cells. Fluorescence in the bottom wells was read at 485 nm excitation and 520 nm emission.

2.6. Statistical analysis

Data are expressed as mean ± standard deviation (SD) and analysed using Mann-Whitney U test. A *p* value of less than 0.05 was considered statistically significant. For all data, *n* represents the number of wells or dishes tested under each condition and also the results from at least two primary cell populations.

3. Results

3.1. CathD induces proliferation of HOMECs

It is now recognised that during growth tumours secrete growth factors into their microenvironment that activate normally relatively quiescent ECs, inducing proliferation, migration and ultimately new vessel formation which ensures a nutrient supply for the growing tumour. We have previously reported that CathD is secreted by EOC cells and since CathD has been reported to be involved in angiogenesis [45], initial studies examined the dose dependent (20, 50 and 80 ng/ml) proliferative effects of CathD on HOMECs. The range of concentration was selected based on a concentration 58ng/ml that was detected in the peritoneal fluid of women with endometriosis [46]. Additionally, previously a publication from our laboratory suggested that the EOC cell line SKOV3 secretes 14ng/ml in to the conditioned media. 50ng/ml CathD induced a significant increase in HOMEC proliferation after 72 hours (147.0±25.8% vs control, [100%], p=<0.001, n = 20; Fig. 1), and thus this concentration was used for all further experiments.

3.2. CathD induces HOMEC proliferation via a non-proteolytic mechanism

We next performed a series of experiments to investigate whether mature CathD enhances HOMEC proliferation in a manner dependent on its proteolytic activity. Initial studies examined whether CathD was proteolytically active in the cell culture conditions studied. Preliminary studies confirmed that the pH of cell growth media in both the presence and absence of CathD remained at between pH 7.12 and 7.34 even over 72 hours of cell culture (Table 2). Using a fluorogenic substrate (Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2) CathD enzymatic activity was then tested over a range of pHs from 3 to 7.6 (Supplementary data and Fig. 2a) which included the published optimum pH for proteolytic activity (pH 3.5-4) and the pH neutral conditions confirmed above in cell culture (pH 7-7.6). CathD activity peaked at pH4 with no activity above control levels observed at pH 7-7.6. Indeed, substrate hydrolysis was approximately 10 fold greater at pH4 vs pH7 (Fig. 2a), confirming that CathD is proteolytically active at its optimum pH. When co-incubated with pepA (1 μmol/l), a well-known inhibitor of CathD-proteolytic activity, CathD-mediated cleavage of the substrate was completely inhibited at pH 4 confirming the effectiveness of the inhibitor (Fig. 2a)

Fig. 2b demonstrates that CathD-induced proliferation of HOMECs was not inhibited by pepA over a range of concentrations including 1 μmol/l which fully inhibited the enzymatic activity of CathD above (Fig. 2a). The data presented in Fig. 2c confirm that pepA was not cytotoxic to HOMECs at any of the concentrations used i.e. between 0.1μmol/l and 10 μmol/l.

These data combined indicate that CathD is not proteolytically active in the assay conditions studied and that the proliferative effect of CathD in HOMECs is not the result of its proteolytic activity but rather via a non-proteolytic mitogenic mechanism.

3.3. CathD activates proliferative kinase ERK1/2 and AKT (S473)

If CathD exerts its mitogenic in HOMECs via a non-proteolytic mechanism this raises the possibility that the protein acts as an extracellular ligand, interacting with an, as yet unknown, receptor to activate intracellular signalling pathways. This was initially investigated using a proteome-profiler phosphokinase array as a screening tool. Several kinases were identified to be phosphorylated in HOMECs during CathD treatment for 4 minutes. These included the known cell proliferative kinases ERK1/2 and AKT which demonstrated a 3-fold and 2.5-fold increase in phosphorylation respectively, during CathD treatment compared to control (Fig. 3a).

In order to confirm this initial screen, a cell-based ELISA was carried out with 4 and 10 minutes incubation with CathD and VEGF, where VEGF was a positive control.

After 4 minutes treatment with CathD, there was a ~1.8-fold and ~1.5-fold increase in ERK1/2 and AKT phosphorylation relative to the total ERK1/2 and AKT levels respectively and compared to control (untreated) (Fig. 3b, d). However, after 10 minutes incubation, phosphorylated levels of both ERK1/2 and AKT reduced to the basal level observed in untreated cells (Fig. 3c, e). Interestingly, although CathD-induced ERK1/2 phosphorylation was transient, VEGF-induced ERK1/2 phosphorylation was maintained for at least 10 minutes.

The validity of the cell based ELISA kit was verified using known inhibitors of ERK1/2 and PI3K/AKT. Pre-incubation with non-toxic concentrations (determined during preliminary investigations and based on cell morphology, data not shown) of MEK1/2 inhibitors U0126 (10 μmol/l) and PD 98059 (25 μmol/l) totally abolished the CathD (and VEGF) induced increase in phosphorylation (Fig. 4a, b). Similar results were observed in the Akt ELISA using LY294002, a PI3K inhibitor and MK2206, a selective AKT inhibitor (Fig. 4c, d). For instance, in the presence of LY294002, CathD-induced levels of phosphorylated AKT reduced from ~1.7-fold to 0.4-fold (Fig. 4c). In the case of MK2206, levels of phosphorylated AKT decreased from 1.6-fold to 0.5-fold (Fig. 4d). This indicates that both drugs inhibit the PI3K/AKT pathway in HOMECs.

3.4. CathD-induced HOMEC proliferation is mediated via ERK1/2 pathway, and not AKT

The data presented above raise the possibility that CathD-induced HOMEC proliferation involves the activation of ERK 1/2 and Akt. Indeed, both ERK 1/2 inhibitors, U0126 and PD 98059, significantly reduced CathD-stimulated proliferation to levels equal to or below control levels. For example, at 10 μM of U0126 and 25 μM of PD98059, cell proliferation decreased to 103.1±8.8% (n=8) and 74.2±4.8% compared to CathD (140.6±17.9%, $p<0.001$, n=20; Fig. 5a, b), all normalised to control. A similar observation was made in HOMECs treated with CathD in the presence of the PI3K inhibitor LY294002 (108.9±7.6% vs 146.9±9.9% CathD-only treatment, both normalised to control; Fig. 5c) but not the AKT inhibitor MK2206 (Fig. 5d; discussed later). Together, these data suggest that CathD induces HOMEC proliferation via a non-proteolytic mechanism that involves activation of intracellular pathways downstream of ERK1/2 phosphorylation and possibly PI3K.

3.5. HOMEC migration is induced by CathD treatment via both the ERK1/2 and AKT pathways

Endothelial cell migration is another key step in tumour-angiogenesis. In an initial experiment, CathD significantly increased HOMEC migration by 174.9±52.9% ($p>0.001$, n = 10; data not shown) compared to control (100%). This prompted an investigation into the downstream signalling cascades. Inhibitors of both ERK1/2 and AKT completely abolished CathD-induced HOMEC migration to basal levels observed in control, untreated wells. For instance, in the presence of U0126 and PD98059, CathD-induced HOMEC migration reduced to 91.8±7.9% (n=7) and 99.2±9.9% (n=7; Fig. 6a) respectively, compared to CathD treatment alone (135.7±26.4%, $p<0.001$, n=12), all expressed as percentage of control (100%). In the presence of the PI3K/AKT inhibitors LY294002 and MK2206, CathD-induced HOMEC migration was reduced to 92.9±46.3% (n=6) and 105.6±45.8 (n=6; Fig. 6b.) respectively, compared to CathD treatment (180.0±65.6%, $p<0.001$, n=12), all expressed as percentage of control (100%). These data combined with the ELISA data (Figure 3.9), suggest that CathD induces HOMEC migration via a pathway that requires activation of both the ERK1/2 and AKT(S473) pathways.

4. Discussion

Treatment of ovarian cancer remains a significant clinical challenge due to late diagnosis and limited effective treatment options for advanced metastatic disease. Anti-angiogenic treatment strategies targeting VEGF have proved disappointing and indeed we have previously reported that angiogenesis occurring in the omentum during metastasis of EOC may occur independently of VEGF signalling. Additionally, we showed that CathD is secreted from EOC cells and has pro-angiogenic effects on disease relevant omental ECs i.e. induced migration. These observations raised the possibility that the pro-angiogenic effects of CathD may contribute to the robust angiogenesis observed during EOC metastasis to the omentum. However, the full effect of CathD on HOMECs and the mechanisms by which it acts to induce these cellular changes is unknown. Here we demonstrate for the first time that CathD induces significant proliferation and migration in human microvascular endothelial cells and that these effects are not dependent on the proteolytic activity of the enzyme. Further to this we also demonstrate that CathD induced HOMEC proliferation via activation of the ERK1/2 pathway and migration via both ERK1/2 and AKT pathways. These data support the hypothesis that CathD secreted from EOC metastasising to the omentum contributes to angiogenesis in the growing secondary omental lesion.

CathD is an aspartic endopeptidase involved in degrading unfolded, dysfunctional self- or foreign- proteins in lysosomes and phagosomes [11]. The protein is synthesised in rough endoplasmic reticulum as preprocathepsin D and is cleaved and further modified into procathepsin D (pCathD) that is targeted and transported to intracellular vesicles such as lysosomes and phagosomes by both mannose-6-phosphate receptor (M6PR) dependent and independent pathways [14]. pCathD is converted to its active mature form CathD in the acidic environment of lysosomes. Although CathD is a lysosomal enzyme and its enzyme activity is usually regulated within the acidic compartment of lysosomes, it has also been shown to be enzymatically active extra-lysosomally under normal physiological conditions at neutral pH e.g. CathD has been shown to be involved as a key mediator of induced apoptosis [47-51]. We therefore examined whether CathD is acting via a proteolytic- or non-proteolytic mechanism to induce proliferation in HOMECs. Investigation of the pH of cell culture media throughout the course of the proliferation assays confirmed that the pH was consistently above 7 i.e. outside of the optimum published range of CathD activity; suggesting that proliferation was not due to the enzymatic activity of the enzyme. This was further confirmed using a known inhibitor of CathD proteolytic activity, pepA. Cell proliferation was assessed with or without CathD in the presence or absence of increasing concentrations of pepA. The data indicate that in the presence of a range of concentrations of pepA, CathD was still able to induce proliferation in HOMECs. Next, to confirm the inhibitory effects of pepA we examined CathD activity using a CathD-specific substrate at an array of pHs. At pHs where CathD was active i.e. pH4 pepA completely inhibited its proteolytic activity. The enzyme was not proteolytically active at pH7. Thus, our data indicate that not only is CathD not proteolytically active at the pH observed in cell culture media, but also that the inhibitor used i.e. pepA fully abolishes CathD activity in conditions where the enzyme is active. Taken together these data indicate, for the first time, that CathD acts as a mitogen via a non-proteolytic mechanism in HOMECs i.e. acts as an extracellular ligand.

It is well known that several downstream signalling pathways are activated following treatments with mitogenic factors. Since it appears that CathD induces cell proliferation as a protein receptor ligand for HOMECs, we hypothesised that proliferative intracellular downstream signalling pathways may be activated in HOMECs following CathD treatment. Initially, we investigated activation of possible downstream proliferative kinases in these cells using a human proteome profiler. This kit identifies the phosphorylation status of 43 intracellular kinases and showed that ERK1/2 and AKT phosphorylation levels were upregulated in HOMECS following CathD treatment, compared to control. These data were confirmed using live cell-based ELISAs. The relationship between activation of these pathways and the proliferative effects of CathD were confirmed using well-known MEK1/2 (upstream of ERK1/2) inhibitors (U0126 and PD98059) and PI3K/AKT kinase inhibitors (LY294002 and MK2206). ELISA data confirmed the effectiveness of the inhibitors at significantly reducing ERK1/2 and AKT phosphorylation respectively to baseline levels. Next, we showed that the two MEK1/2 inhibitors reduced or abolished CathD induced HOMEC proliferation over 72 hours. A similar observation was also made in HOMECs treated with the PI3K inhibitor LY294002 but not with the selective AKT inhibitor MK2206. However, the PI3K/AKT pathway inhibitor used, LY294002, is known to cross-react with the ERK1/2 pathway where it inhibits ERK1/2 phosphorylation [52]. Thus, it is possible that LY294002 in fact inhibited the ERK1/2 pathway and reduced HOMEC proliferation, and that AKT is not involved in the induction of cell proliferation as the specific AKT inhibitor failed to reduce the proangiogenic effect in HOMECs. Taken together, these data suggest that the ERK1/2 pathway is involved in the induction of HOMEC proliferation by exogenous CathD.

Since CathD was also shown to induce migration in these cells, the downstream signalling cascades activated during this pro-angiogenic response were also investigated. Since, it has been shown in several studies that activated ERK1/2 and AKT pathways are involved in cell migration [53-55] the involvement of these kinases in CathD induced HOMEC migration was examined. Interestingly, we show for the first time that both kinases are involved in the CathD-induced migration in HOMECs.

Taken together our data suggest that CathD may be an important pro-angiogenic factor in in the omental metastasis of EOC. Indeed, it is now well recognised that pCathD/CathD is overexpressed and hyper-secreted in several different cancer types, including ovarian cancer. Over-expression of CathD has been established as a poor prognostic marker in breast cancer patients and was shown to induce tumour invasion into surrounding tissue [19]. It has been reported that pCathD is overexpressed by, and hyper-secreted from oestrogen-treated MCF7 breast cancer cells and that the secreted protein acts as a protein ligand to stimulate MCF7 cell growth via an autocrine mechanism [41]. While secreted-pCathD is generally considered to be proteolytically inactive, it has been proposed that the acidic pH in the tumour microenvironment promotes the conversion pCathD into mature, biologically active CathD [56]. This was supported by data indicating that pCathD, collected from tumour-conditioned media, became auto-activated if the pH was lowered and was subsequently able to degrade ECM proteins and release growth factors such as bFGF, steps important for cancer cells to invade surrounding tissue [22, 57].

Evidence for a role of CathD in angiogenesis has been increasing. An early study performed in 3Y1-Ad12 tumour xenografts mice using both catalytically active and inactive (mutated) forms of CathD suggested both a proteolytic and mitogenic role for CathD [36]. The underlying mechanism of CathD induced angiogenesis, however, was not elucidated. CathD has also been shown to induce blood vessel formation in the CAM model and a role for CathD in angiogenesis was further suggested by the observation that migration of HUVECs and *in vitro* angiogenic tube formation were increased when cells were treated with active pure CathD [34]. CathD was proteolytically active in these experiments as complete inhibition of angiogenesis, tube formation and migration was achieved by addition of pepA [34]. Proteolytically active CathD has also been suggested to induce angiogenesis in breast cancer by cleaving and releasing ECM-bound pro-angiogenic bFGF [22]. In contrast it has also been suggested that CathD activity may be anti-angiogenic. For instance, pCathD secreted by prostate cancer cells was shown to have a possible role in generating angiostatin via proteolysis—a specific inhibitor of angiogenesis *in vitro* as well as *in vivo* [25].

Due to the heterogeneity of EC morphology, proteomic properties and functionalities in different vascular beds it is important to study cellular responses in disease relevant ECs. Using disease-relevant HOMECs we show for the first time that exogenous CathD induces EC proliferation and migration in a proteolytic-independent manner. The proliferative effect is downstream of activation of the ERK1/2 pathway and the migratory effect is via activation of both the ERK1/2 and AKT pathways. Given the array of angiogenesis-associated proteins secreted by tumour cells and the complexity of the angiogenic process it is likely that EOC-induced omental angiogenesis is driven by the interplay of a range of proangiogenic factors, of which CathD is only one element. However, a greater understanding of the mode of action of each individual pro-angiogenic factor is required to fully dissect this process. We believe that our data highlight CathD as one of these proangiogenic factors which may be a promising target in anti-angiogenic therapy in the treatment of ovarian cancer metastasis.

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Conflicts of interest

The authors declare no conflict of interest.

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Table 1. Concentrations of treatments added to cell proliferation assay.

Treatments	Purpose	Concentration(s)	Source
VEGF	Positive control	20 ng/ml	Peprotech (London, UK)
CathD	Treatment	20, 50, 80 ng/ml	Sigma-Aldrich (Poole, UK)
pepA	CathD inhibitor	0.1, 1, 2.5, 5 and 10 µmol/l	Sigma-Aldrich (Poole, UK)
U0126	MEK/ERK1/2 inhibitor	10 µmol/l	Stratech (Suffolk, UK)
PD 98059	MEK/ERK1/2 inhibitor	25 µmol/l	Stratech (Suffolk, UK)
LY294002	PI3K inhibitor	25 µmol/l	Stratech (Suffolk, UK)
MK2206	AKT inhibitor	5 µmol/l	Stratech (Suffolk, UK)

Table 2: **pH of cell culture media and supernatant during CathD treatment.** Cells were seeded in 6 well plates and treated with or without CathD (50ng/ml) for 24, 48 and 72 hours. Media were collected and their pH was measured. n.d. denotes not determined.

	pH			
	0h	**24h**	**48h**	**72h**
Basal mv2	7.34	n.d.	n.d.	n.d.
Untreated	n.d.	7.19	7.13	7.12
CathD	n.d.	7.21	7.13	7.12

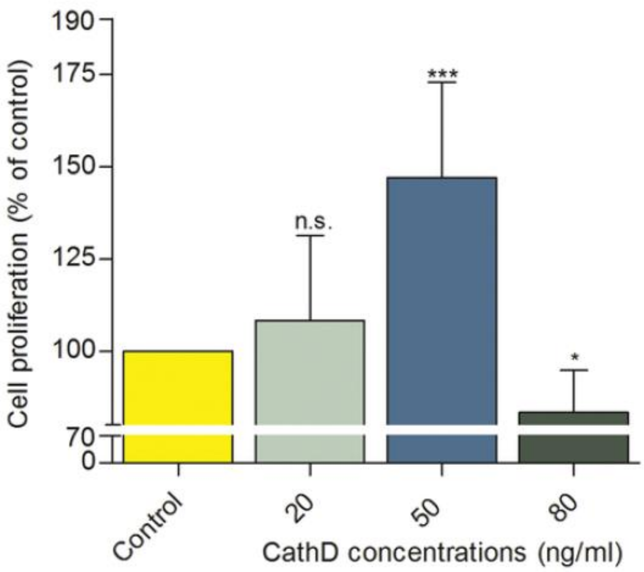

Fig. 1. **Increased proliferation of HOMECs in media supplemented with CathD (WST-1 assay).** Cells were seeded in 2% gelatin pre-coated 96 well plates at a density of 10,000cells/well in starvation media containing 2% FCS. After overnight incubation, cells were treated with or without various concentrations of CathD and incubated for 72 hours. A commercially available WST-1 kit was used to assess cellular proliferation based on absorbance using a PHERAstar BMG plate-reader at 450nm. Results are mean ± SD and shown as percentage of the control, *p<0.05, ***p<0.001 vs control (100%), n=5-20. n.s. denotes not significant.

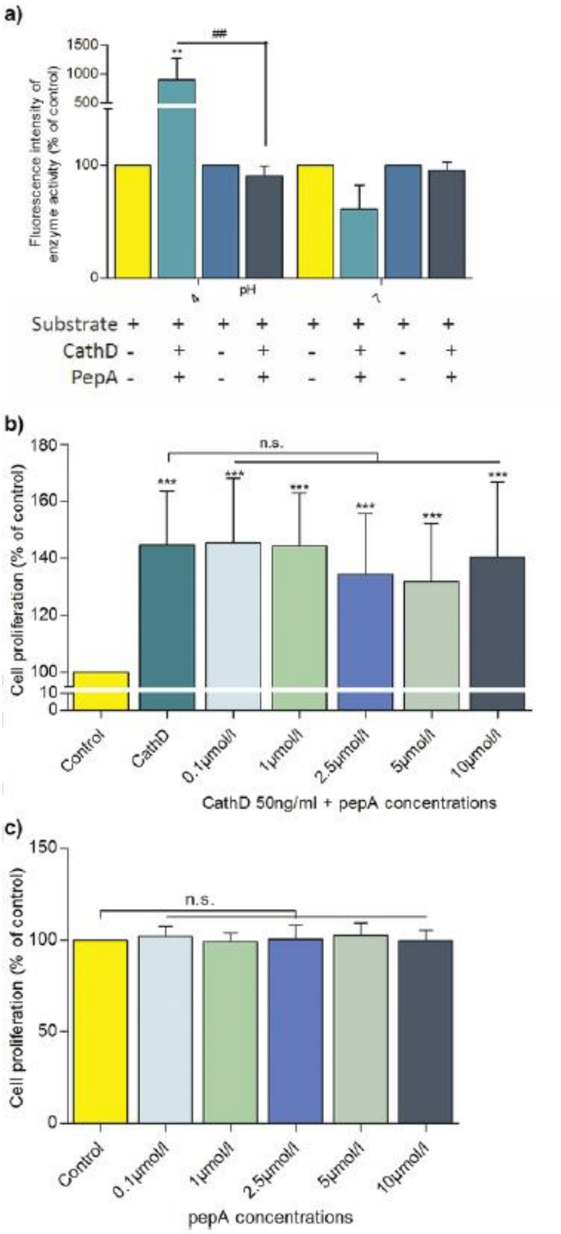

Fig. 2: **PepA, an inhibitor of CathD proteolytic activity, does not inhibit CathD-induced HOMEC proliferation.** a) CathD proteolytic activity is not observed at pH 7. A specific fluorogenic substrate (100 nmol/l) was incubated with or without CathD (50ng/ml) and in the absence or presence of pepA (1 μmol/l) at pH 4 and 7. Fluorescence signals were measured immediately using a SpectraMax plate reader at Ex/Em: 320/393. Control wells contained pH buffer and substrate and/or inhibitor. The data are represented as percentage of control. **p<0.01 vs control (substrate) (100%); ##p<0.01 vs CathD + substrate (expressed as % of control), n=3. b + c) Cells were seeded in 2% gelatin pre-coated 96 well plates at a density of 10,000cells/well in starvation media containing 2% FCS. After overnight incubation, cells were treated with or without CathD (50ng/ml) in the presence or absence of various concentrations of pepA (as shown above- b) CathD + pepA or c) pepA alone) and incubated for 72 hours. WST-1 assay was used to assess cellular proliferation based on absorbance using a PHERAstar BMG plate-reader at 450nm. Control wells contained 0.1% DMSO (carrier only). Results are mean ± SD and shown as percentage of the control, ***p<0.001 vs control (100%); n=8-16. n.s. denotes not significant.

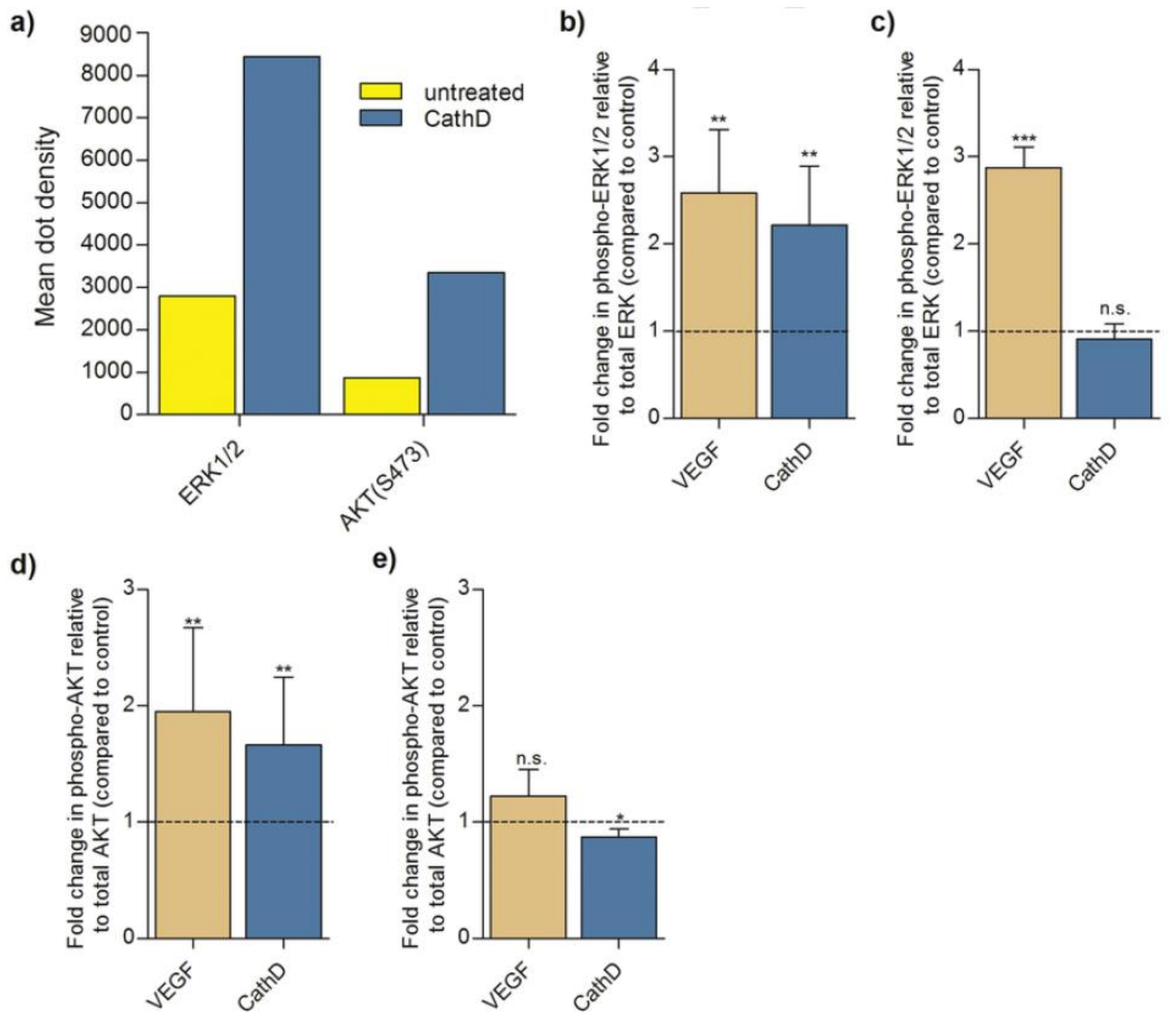

Fig. 3. **CathD-induced activation of intracellular kinases.** a) CathD induces phosphorylation of ERK1/2 and AKT(S473) in HOMECs. Phosphorylation status of the intracellular kinases was assessed in cell lysates from cells treated with or without CathD for 4 minutes. The results of 1 minute exposure are expressed as mean dot density (arbitrary units). The relative expression of specific phosphorylated proteins was determined following quantification of scanned images. b-e) CathD induces phosphorylation of ERK1/2 and AKT in HOMECs. Cells were seeded in 2% gelatin pre-coated 96 well plates at a density of 10,000cells/well in starvation media containing 2% FCS. After overnight incubation, cells were treated with or without 50ng/ml of CathD or 20ng/ml of VEGF (positive control) and incubated for 4 or 10 minutes. ERK1/2 (b, c) and AKT (d, e) phosphorylation was examined after 4 minutes (b, d) and 10 minutes (c, e) treatments. Commercially available cell-based ELISAs were used for the determination ERK1/2 and AKT(S473) phosphorylation level. The ELISA experiments were carried out on two cell batches. The data is represented by fold change in phosho-ERK1/2/AKT relative to total ERK1/2/AKT (compared to control). Results are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs control (dotted lines); n=4-6. n.s. denotes not significant.

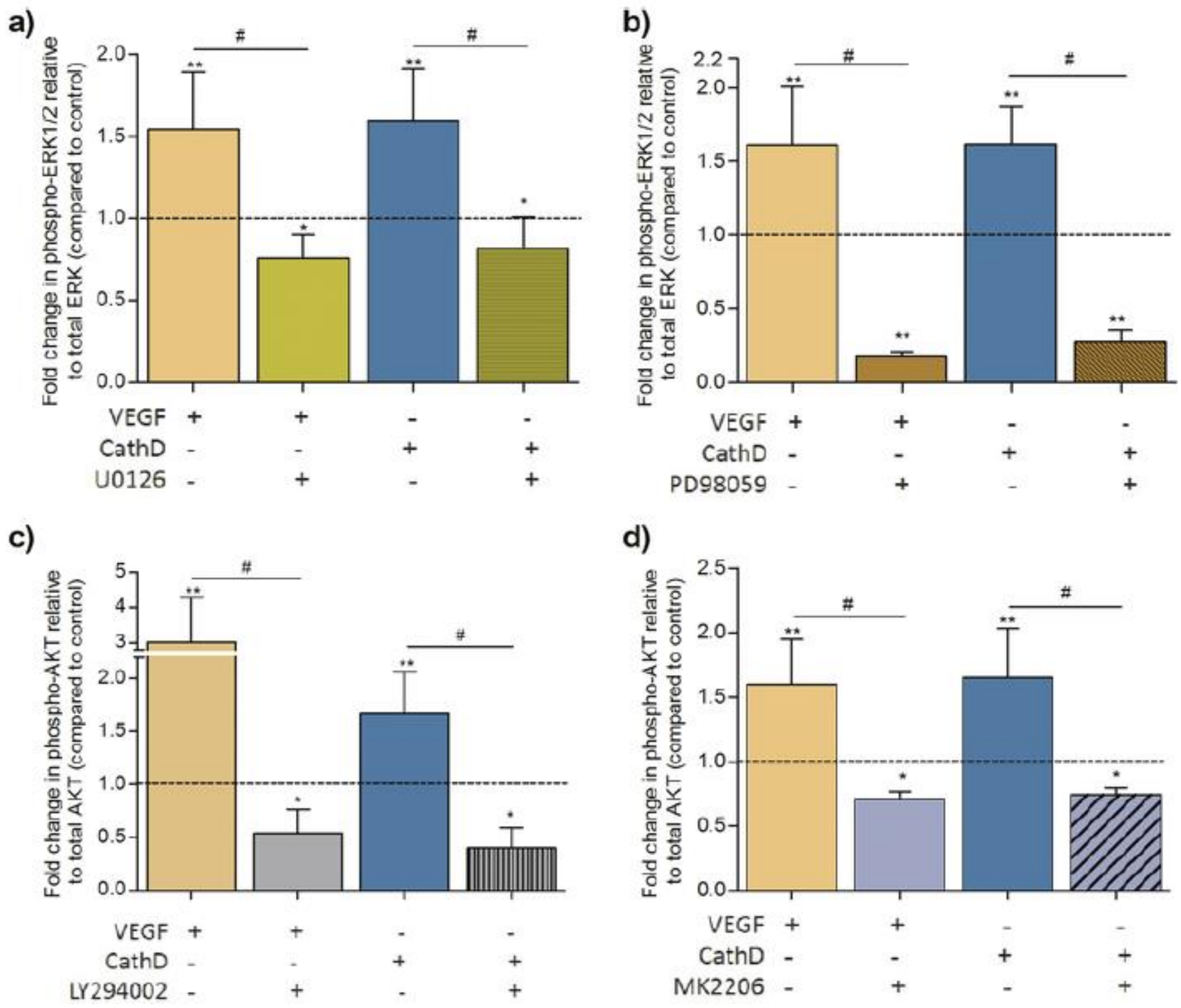

Fig. 4. **ERK1/2 and AKT inhibitors reduce ERK1/2 and AKT phosphorylation respectively in intact HOMECs.** After overnight starvation in media supplemented with 2% FCS, cells were pre-incubated with the ERK1/2 inhibitors a) U0126 (10 µmol/l) and b) PD98059 (25 µmol/l) or PI3K/AKT inhibitors c) LY294002 (25 µmol/l) and d) MK2206 (5 µmol/l) for (a + b) 20-30 minutes or (c + d) 2.5 hours, and then co-treated with or without 50ng/ml of CathD or 20ng/ml of VEGF for 4 minutes. Commercially available cell-based ELISAs were used for determination of ERK1/2 phosphorylation level. The data is represented as fold change in phosho-ERK1/2 relative to total ERK1/2 (compared to control). Results are mean ± SD, *p<0.05, **p<0.01 vs control (1-fold), #p<0.05 vs VEGF/CathD (normalised to control), n=4. The dotted lines represent basal level (control) of phosphorylation status in untreated HOMECs.

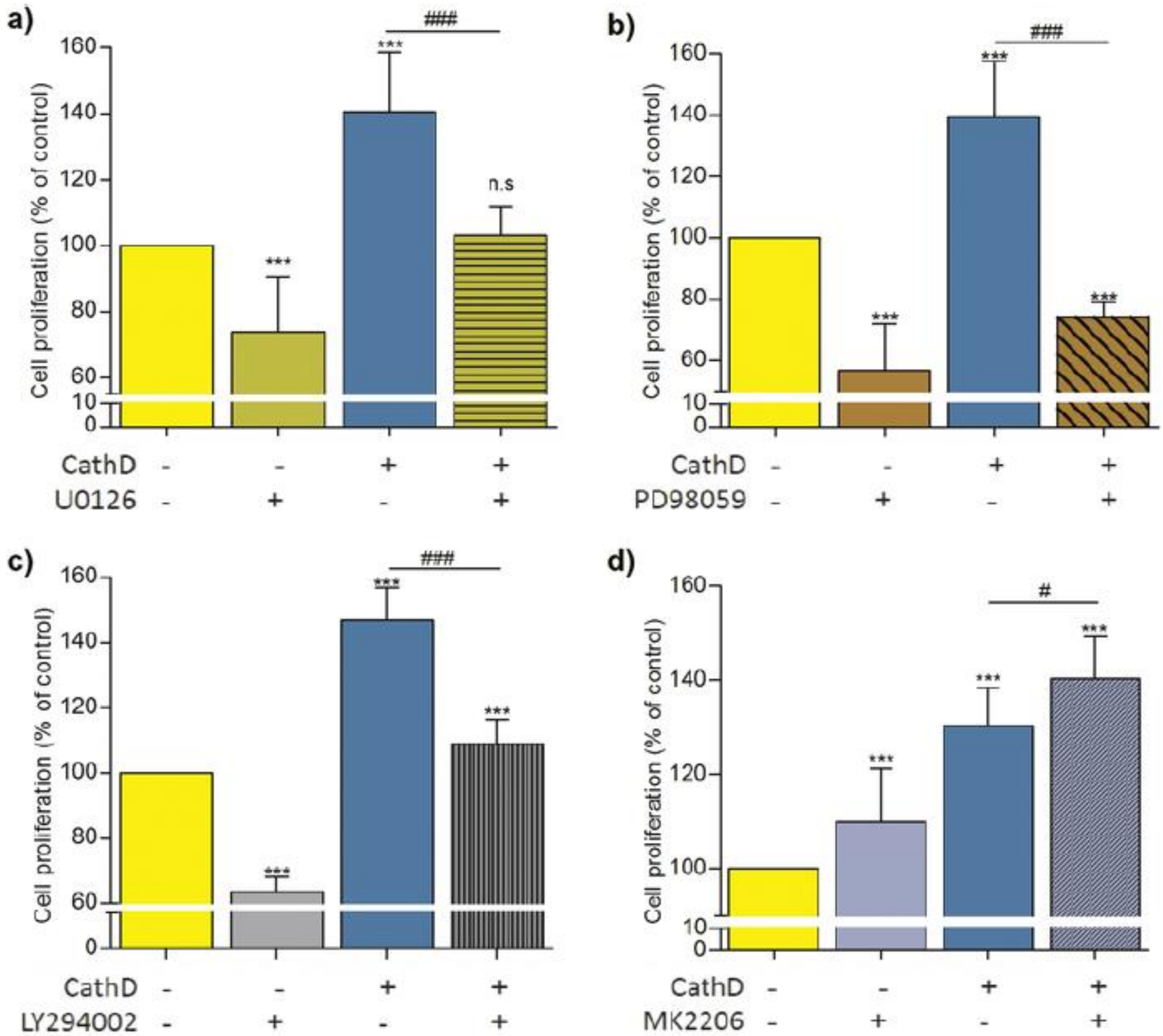

Fig. 5. **CathD-induced HOMEC proliferation is mediated via activation of the ERK1/2 and PI3K pathways, but not AKT pathway.** After overnight starvation in media supplemented with 2% FCS, cells were treated with or without CathD (50ng/ml) and in the absence or presence of a) U0126 (10 µmol/l), b) PD98059 (25 µmol/l), c) LY294002 (25 µmol/l) and d) MK2206 (5 µmol/l) and incubated for 72 hours. WST-1 assay was used to assess cellular proliferation. Results are mean ± SD and shown as percentage of the control, n.s., ***p<0.001 vs control (100%), ###p<0.001 vs CathD (normalised to control 100%), n=8-20. n.s. denotes not significant.

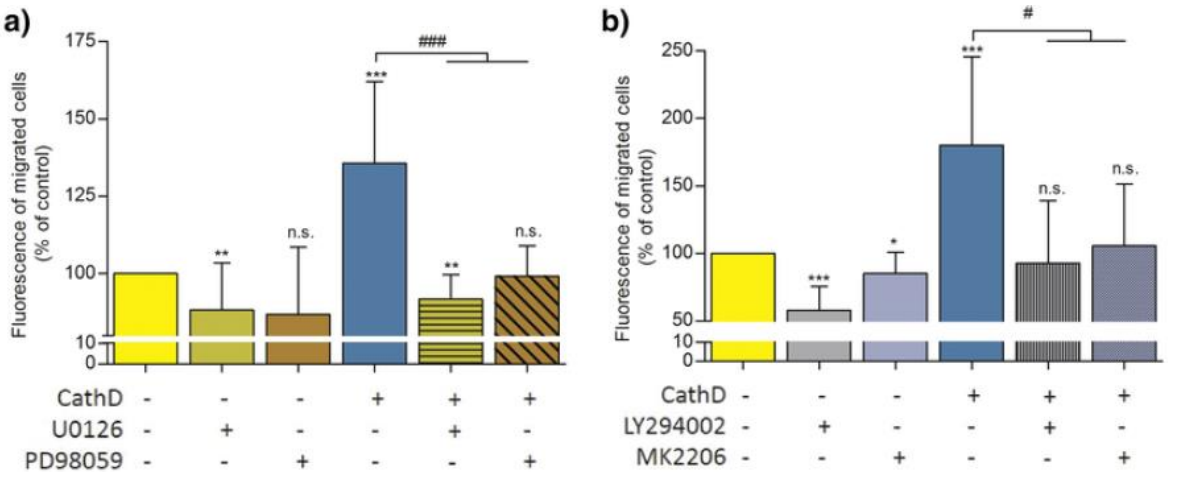

Fig. 6. **CathD induces HOMEC migration via activation of the a) ERK1/2 and b) AKT pathways.** Pre-treated (with corresponding kinase inhibitor) HOMECs were seeded in the upper transwell chamber and treated with or without CathD (50ng/ml) in the absence or presence of a) U0126 (10 µmol/l) and PD98059 (25 µmol/l) or b) PI3K and AKT inhibitors LY294002 (25 µmol/l) and MK2206 (5 µmol/l), respectively in media containing 0.5% FCS. The lower well contained correspondent treatments. After 6 hours, migrated cells were stained with calcein AM and fluorescence was quantified using a FLUOstar plate reader at Ex/Em: 485/520. Results are mean ± SD and shown as percentage of the control, n.s., *p<0.05, **p<0.01, ***p<0.001 vs control (100%), #p<0.05, ###p<0.001 vs CathD (normalised to control (100%)), n=6-12. n.s. denotes not significant.

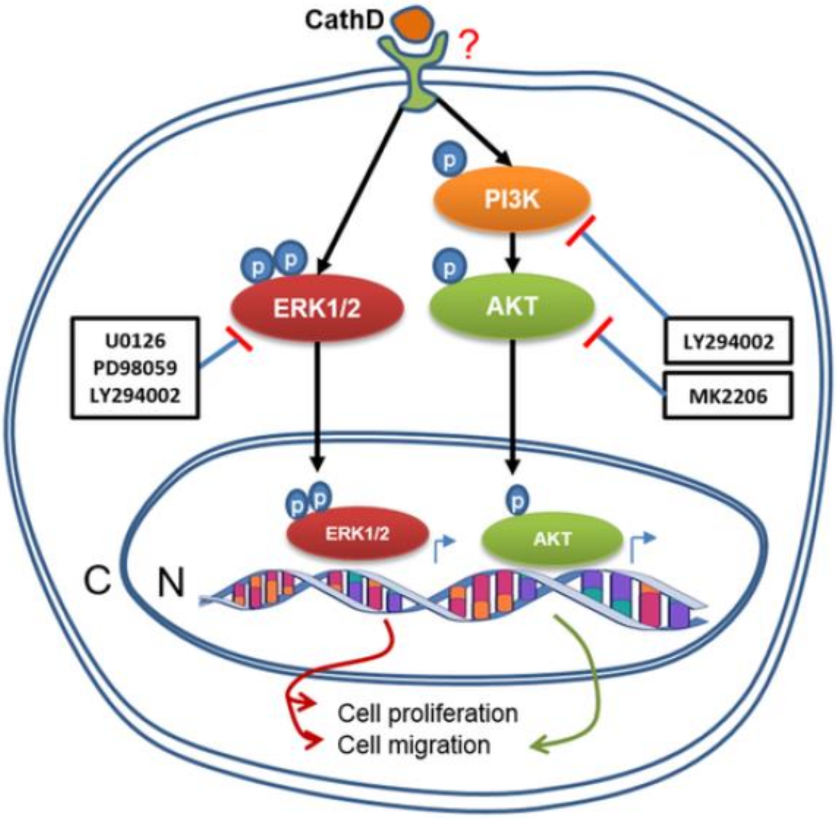

Fig. 7. **A summary of CathD -induced activation of the ERK1/2 and AKT pathways, and cellular functions in HOMECs.** CathD non-proteolytically activates a cell surface receptor, possibly a receptor tyrosine kinase, which leads to an increase in phosphorylation of ERK1/2 and AKT. The MEK/ERK1/2 inhibitors U0126 and PD98059 significantly reduce these cellular functions by inhibiting ERK1/2 phosphorylation. Both PI3K inhibitor LY294002 and AKT inhibitor MK2206 inhibit phosphorylation of AKT at Ser473 (S473) in CathD-treated HOMECs. However, only LY294002, but not MK2206, inhibits CathL-induced HOMEC proliferation, suggesting a cross-reaction of PI3K inhibitor LY294002 with the ERK1/2 pathway. Interestingly, both PI3K and AKT kinases are activated in CathD-induced HOMEC migration, which was reduced in the presence of both LY294002 and MK2206.