Determining the role of Protocadherin 1 in angiogenesis

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1. Note regarding resubmissions

This proposal to work on PCDH1 is novel, but it does relate to **BHF PhD Studentship Application, Case Reference 30325** "Investigating the role of Protocadherin 7 in endothelial motility and angiogenesis" submitted in 2013. Though this application was not funded, the committee invited a further application with more preliminary data. Subsequently a survey of the δ-1 protocadherins, of which PCDH7 is a member, led us to discover that PCDH1 was the most highly expressed of this family of protocadherins in endothelial cells. This finding is consistent with high expression of PCDH1 in the developing mouse vasculature reported by another group. Importantly knockdown of PCDH1 gives a profound and robust phenotype in terms of its inhibiting endothelial motility, sprouting and tubulogenesis. No endothelial function has been reported for PCDH1 and this studentship aims to build on these preliminary findings investigating its intracellular mechanism of action and its function *in vivo*.

2. Abstract

Determining the molecular regulation of angiogenesis will enable strategies to better treat atherosclerosis and vascular disease. We have identified a novel function for Protocadherin 1 (PCDH1) in mediating endothelial migration, sprouting and tube formation. PCDH1 is a member of the δ1-subfamily of protocadherins, belonging to the cadherin superfamily of adhesion proteins. No function in angiogenesis for this subgroup of cadherins has been reported and **this studentship aims to characterise this novel role for PCDH1.**

Specifically it aims to:

i) Determine the role of PCDH1 in endothelial migration, polarisation, tip and stalk cell specification and lumen formation and test whether it modulates the actin cytoskeleton and focal adhesions.

ii) Delineate intracellular binding partners and elucidate their role in regulating endothelial tubulogenesis and motility.

iii) Investigate the biological function of recombinant soluble PCDH1 extracellular domain.

iv) Use a knockout mouse to determine how PCDH1 affects vessel formation *in vivo*.

2. Background to the project and pilot data

Understanding the molecular regulation of angiogenesis, the formation of new blood vessels from pre-existing vessels, is critical in combating coronary and peripheral artery disease. On the one hand angiogenesis contributes to the pathogenesis of atherosclerosis by promoting plaque destabilisation and rupture (1), and it is plaque rupture and the ensuing thrombus formation and vessel occlusion that causes myocardial infarction and stroke. Conversely, inducing therapeutic angiogenesis to re-vascularise ischaemic tissue resulting from coronary and peripheral artery disease has long been a goal of angiogenesis research.

Angiogenesis is mediated by endothelial cells which line all blood vessels. In response to pro-angiogenic factors, endothelial cells must first degrade the extracellular matrix, then migrate and proliferate, form a lumen and finally stabilise the new vessel by the recruitment of pericytes (2). Vascular endothelial growth factors (VEGF) are key endothelial regulators and well characterised potent pro-angiogenic factors. However, their use and the use of other pro-angiogenic factors such as fibroblast growth factor (FGF)-2 in many clinical trials to treat both coronary and peripheral artery disease have proved disappointing (3). This highlights the urgent need to more fully understand the molecular basis of angiogenesis and devise ways to promote this process *in vivo*. We have identified Protocadherin (PCDH)1 as a novel modulator of endothelial tube formation, and this studentship proposal aims to delineate the molecular function and *in vivo* role of this little studied protein in angiogenesis. The exploratory work outlined in the proposal is ideal for a studentship; the methodology described is routinely used by the laboratory and collaborators, giving a student training in a wide portfolio of techniques which include molecular biology, biochemistry, confocal microscopy, cell based assays and mouse models of angiogenesis.

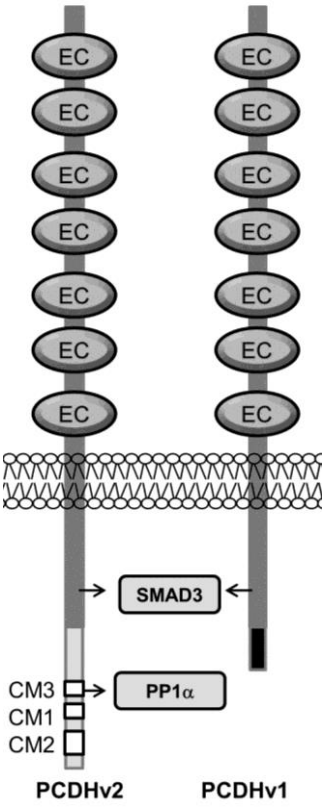


Figure 1. A cartoon of the splice variantsvof PCDH1. The extracellular domain contains 7 extracellular calcium-binding cadherin (EC) domains. The intracellular domains have distinct C-termini generated by alternative splicing represented by different shading. The longer isoform has three regions CM1, CM2 and CM3 and is known to interact with PP1α. Both variant 1 and 2 interact with SMAD3.

Protocadherin1

PCDH1 belongs to the cadherin superfamily which has over a hundred members, and within this it belongs to the δ-1 subfamily of protocadherins (4). Unlike classical cadherins such as vascular endothelial (VE)-cadherin with its critical role in mediating endothelial adhesion and signalling (5), the functions of δ-protocadherins are poorly defined. Complex expression patterns within the central nervous system suggest a role in functional regionalisation and differentiation within the brain (6, 7), and PCDH1

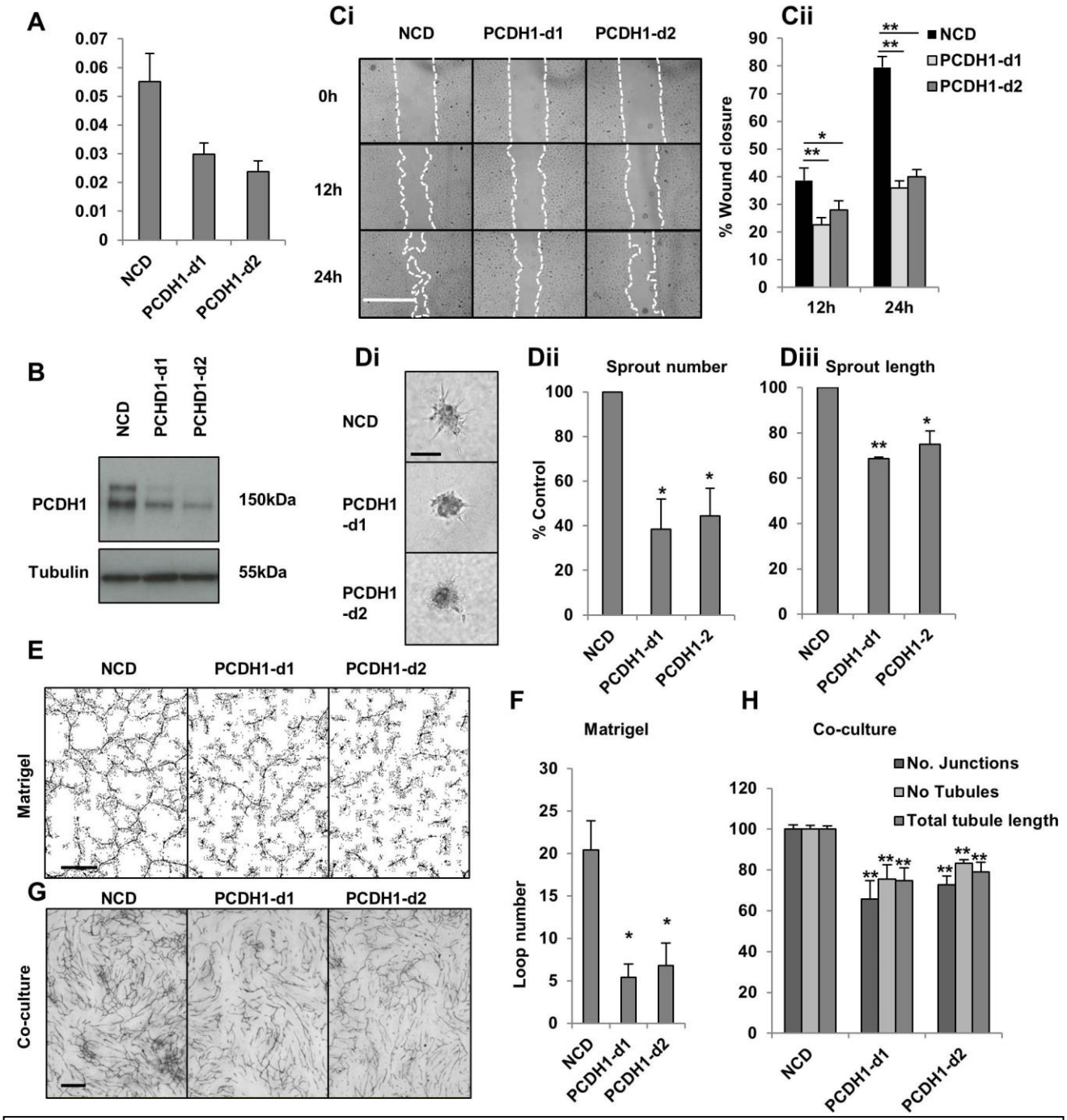
mediates cellular organisation in the prenotocord in early embryonic development in *Xenopus* (8). It is of note that members of neural guidance protein families such as the Roundabouts, Ephrins and Semaphorins play critical roles in angiogenesis (9). Unlike many protocadherins, PCDH1 expression has been reported outside neural tissue. Its expression was found to be high in the vessels of developing mice from embryonic day 10-15 (10) and in endothelial cells isolated from adult mouse lung capillaries (11). Epithelial expression has also been reported both in the mouse embryo (10) and human lung and nasal mucosa (12); with genetic links between PCDH1 and asthma suggested by a number of studies (13).

PCDH1 is an integral membrane protein comprising 7 extracellular cadherin domains and an intracellular domain of either 186 or 363 amino acids due to alternative splicing giving rise to two different C-terminal sequences (Figure 1). Unlike classical cadherins, PCDH1 shows only weak homophilic interactions due to structural variation in its cadherin domains and it does not interact with intracellular proteins which are associated with adherens junctions or desmosomes (14, 15). We have found both PCDH1v1 and PCDH1v2 to be expressed in human umbilical vein endothelial cells (HUVECs), with v1 being about five-fold more abundant at the mRNA level. The cytoplasmic domain of both PCDH1v1 and v2 interacts with the transcription factor SMAD3 (16), while PCDH1v2 contains three cytoplasmic regions (CM1-3) which are conserved amongst δ1cadherins; CM3 contains a RRVTF motif which is known to interact with and inhibit protein phosphatase 1(PP1)-α (4, 17). SMAD3 is phosphorylated and activated by TGFβ signalling via its receptors ALK5 and TβR-II (18). Knockdown of PCDH1 in epithelial cells was found to enhance SMAD3 driven reporter activity, while its overexpression reduced it, and it was postulated that PCDH1 can sequester SMAD3 reducing its activation (16).

The importance of TGFβ in endothelial biology is underscored by the vascular defects causing embryonic lethality observed in mice lacking TGFβ and its receptors, as well as the association between genetic mutation of TGFβ signalling proteins and vascular malformations observed in Hereditary Haemorrhagic Telangiectasia (19). Signalling via SMAD3 has been associated with endothelial cell quiescence (18), and more recently has been associated with promoting the stalk cell phenotype in sprouting angiogenesis (20).

We have identified PCDH1 as being the most highly expressed δ1cadherin in endothelial cells and have investigated its role in endothelial tubulogenesis. In order to establish PCDH1 knockdown, two PCDH1- specific siRNA duplexes targeting sequences coding the extracellular region common to both splice variants of PCDH1 were used to reduce PCDH1 RNA (Figure 2A) and consequently reduce protein levels (Figure 2B) in human umbilical cord endothelial cells (HUVECs). These cells were then used in a variety of assays. PCDH1 knockdown reduced cell migration in a scratch wound assay (Figure 2C) and impaired sprouting of HUVEC from spheroids embedded in collagen, both in terms of their number and their length (Figure 2D). Additionally, two models of tubulogenesis were performed to test the impact of PCDH1 knock down in this process. Knockdown of PCDH1 impaired network formation when cells were plated on the solubilised basement membrane preparation Matrigel (Figure 2E). This short-term assay is considered to model the differentiation phase of angiogenesis (21) and PCDH1 knockdown significantly reduced the connectivity of the network, reducing the number of loops formed (Figure 2F). In the longer term co-coculture model of angiogenesis, endothelial cells are cultured for six days with confluent fibroblasts, during which time they proliferate and form tubules with lumens (22-24). PCDH1 knockdown a day prior to plating on the fibroblasts resulted in a reduction in total tubule length, tubule number and junction number (Figure 2G&H).

Having established a robust phenotype resulting from PCDH1 knockdown, the programme of work aims to determine more precisely the molecular function of PCHD1 in tubulogenesis.



PCDH1 knockdown impairs endothelial migration, sprouting and tube formation. HUVEC were transfected with a negative control siRNA duplex (NCD) or siRNA duplexes specific to PCDH1 (PCDH1-d1 and PCDH1-d2). **A** RNA was prepared two days after transfection and reverse transcribed, knockdown was assessed by performing probe-based quantitative PCR using both PCDH1 and actin specific primers, expression of PCDH1 relative to Actin was calculated for each sample. B Cellular lysate was prepared two days after transfection and blotted for PCDH1 and tubulin as a loading control. **C** Two days after transfection a scratch was made and wound closure imaged at 12 and 24 hours; the percentage wound closure measured using ImageJ; scale bar: 1 mm. **D** Endothelial sprouting was assessed by preparing spheroids in a hanging drop culture one day post transfection and embedding the spheroids in collagen two days after transfection. Endothelial sprouts were imaged 6 hours after embedding and the mean sprout number and length determined. Scale bar: 100 μm. **E** Two days after transfection endothelial cells were plated on Matrigel and imaged after 16 hours. Scale bar: 800 μm. **F** Network formation was assessed using the ImageJ-based Angiogenesis analyser and mean loop number determined. **G** A co-culture assay was performed by plating endothelial cells on to confluent fibroblasts one day post transfection. After a further 6 days cells were fixed and the endothelial cells histologically stained to reveal the endothelial tubules. Scale bar: 1 mm. **H** The networks were assessed using AngioSys 2.0 to determine the number of junctions and tubules and the total tubule length. For all experiments n=3, the error bars represent the standard error of the mean. Data from each duplex were compared with NCD, * p=0.01-0.05 and ** p<0.01 by Student's t test.

4. Original hypotheses

PCDH1 regulates angiogenesis by modulating endothelial motility and tubulogenesis

Aims:

i) Determine the role of PCDH1 in endothelial migration, tip and stalk cell specification and lumen formation; test whether it modulates the actin cytoskeleton and focal adhesions.

ii) Delineate intracellular binding partners and elucidate their role in regulating endothelial tubulogenesis and motility.

iii) Investigate the biological function of recombinant soluble PCDH1 extracellular domain.

iv) Use a knockout mouse to determine how PCDH1 affects vessel formation in vivo.

5. Experimental details and design of proposed investigation

i) Determine the role of PCDH1 in endothelial migration, tip and stalk cell specification and lumen formation; test whether it modulates the actin cytoskeleton and focal adhesions

In this section we aim to build on the preliminary data and determine more precisely in which aspects of tube formation PCDH1 is involved and how it modulates the actin cytoskeleton and focal adhesions. We have shown that siRNA mediated knockdown of PCDH1 affects endothelial motility as well as sprout and tubule formation. The effect of PCDH1 knockdown on cell motility will be explored in more detail by time lapse microscopy. In the preliminary data motility was assessed in a scratch wound assay, we will also investigate chemotaxis to pro-angiogenic factors such as VEGF using a Dunn chamber which allows the speed and trajectories of individual cells to be measured. PCDH1 knockdown results in impaired endothelial sprouting, we aim to determine whether PCDH1 is involved in the specification of stalk and tip cells. This will be achieved by mixing cell-tracker red-labelled siRNA control transfected cells with carboxyfluorescein succinimidyl ester-labelled PCDH1-siRNA transfected cells in spheroids and enumerating the source of tip and stalk cells within the sprouts.

Cell motility is orchestrated by the rearrangement of the actin cytoskeleton combined with assembly and disassembly of the focal adhesions which connect cells with their substratum. The effect of PCDH1 knockdown will be assessed using immunofluorescent staining of filamentous actin (phalloidin), focal adhesions (vinculin) and microtubules (tubulin). Initially fixed cells will be examined, and depending on the results, we will then examine the cytoskeletal dynamics or focal adhesion turnover in live cells. We routinely use lentiviral transduction to express proteins of interest in HUVEC, and have previously used expression of RFP-paxillin (a focal adhesion protein) in combination with total internal fluorescence (TIRF) to monitor focal adhesion assembly and disassembly (25). We will use LifeACT-GFP/RFP to monitor changes in the actin cytoskeleton by confocal microscopy (26), and will examine this in the co-culture assay. In this assay endothelial cells are co-cultured on a monolayer of fibroblasts which induces their formation of tubules and this is considered one of the best in vitro models of the angiogenic process (27). In particular, we will use this assay to compare filopodia numbers in control and PCDH1 siRNA transfected endothelial cell expressing LifeACT-GFP, where we have shown that this strategy very effectively visualises the tip cell filopodia (Figure 3). The effect of PCDH1 knockdown on lumen formation will also be assessed in this assay.

ii) Delineate intracellular binding partners and elucidate their role in regulating endothelial tubulogenesis and motility

In this section the goal is to determine the intracellular binding partners of PCDH1 in endothelial cells. In addition to investigating the established binding partners identified in other cell types, we will undertake strategies to identify novel interactors. The identification of novel endothelial specific interactors may enable the development of strategies to specifically modulate vascular PCDH1. The relatively long intracellular domains of 186 or 363 amino acids and

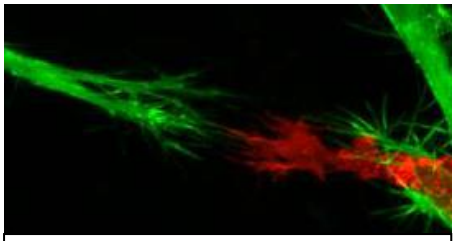


Figure 3. LifeACT-GFP and LifeACT-RFP labelled endothelial tip cells in a co-culture assay

the observation that truncated forms of a related δ1cadherin in *Xenopus* can act as a dominant negative proteins (28),suggests that the intracellular region of PCDH1 plays a critical role in its function.

Binding partners of the two alternatively spliced PCDH1 intracellular domains (PCDH1icd) will be characterised. These will be expressed as fusions with GST in bacteria and used in pulldown experiments with endothelial cell extract. We will use western blotting to establish interaction with reported binding partners SMAD3 and PP1-α and mass spectrometry in collaboration with Dr Debbie Cunningham to identify any additional binding proteins. Co-immunoprecipitation experiments will be performed to confirm interaction between PCDH1icd and candidate proteins. Confocal microscopy will be used to determine if candidates co-localise with PCDH1.

The regions and residues required for the PCHD1icd to bind its various partner proteins will be mapped using truncation mutants and point mutants. This will enable the generation of mutant forms of PCDH1 to determine the functional relevance of the interactions with the various binding partners. These will be lentivirally transduced into HUVEC to determine whether they can rescue migration and tubulogenesis defects caused by PCDH1 knockdown. Silent mutations will be engineered into PCDH1 constructs so that they will not be targeted by the transiently transfected siRNA duplexes. We will also test whether truncated forms of PCDH1 lacking the cytoplasmic domain can act as dominant negative molecules, as was found in studies of a *Xenopus* δ1cadherin (28), presumably due to it interacting with ligand but failing to transduce any signals. We will also determine if mutation of the RRVTF motif found in CM3 and known to interact with PP1-α (17) has any impact on the endothelial function of PCDH1.

Given the role of SMAD3 in angiogenesis and its modulation by PCDH1 in epithelial cells (16), a series of experiments will be undertaken to pursue this. The working model to be tested is that PCDH1 regulates TGFβ signalling by sequestering SMAD3; thus PCDH1 knockdown would enhance TGFβ induced activation of SMAD3 and lead to a more quiescent phenotype and impaired tubulogenesis. The co-applicant Dr Carol Murphy has considerable experience working with SMADs (29-33) and a wealth of reagents essential to this project enabling determination of whether:

i) PCDH1 knockdown impairs TGFβ-mediated SMAD3 phosphorylation.

ii) Modulating PCDH1 expression modulates TGFβ driven SMAD3 reporter expression.

iii) PCDH1icd binding of SMAD3 depends on phosphorylation of SMAD3; pulldowns will be performed with active and inactive mutants of SMAD3.

iv) PCDH1icd binds other SMAD proteins, in particular the related SMAD2 or SMAD1/5/8.

v) PCDH1 knockdown alters the nuclear trafficking of SMAD3. Immunofluorescent staining of SMAD2/3 will be performed on control and PCDH1 siRNA transfected HUVEC following TGFβ stimulation.

iii) Investigate the biological function of recombinant soluble PCDH1 extracellular domain

In this section we aim to determine if the recombinant extracellular domain of PCDH1 has any biological activity – and so potentially allow the development of a new therapeutic agent. Soluble extracellular domains of cell surface receptors involved in angiogenesis have been shown to disrupt signalling and interfere with tubulogenesis (34). A soluble extracellular domain could block interactions of PCDH1 with itself or other cell surface receptors. We will express the soluble extracellular domain of PCDH1 and determine its impact in modulating endothelial motility and tube formation in the various assays described previously. Should activity be observed, then smaller fragments of the extracellular domain will be tested. Should *in vitro* activity be demonstrated, the PCDH1-Fc protein will be tested *in vivo* sponge angiogenesis model, which is ideal for testing the angiogenic properties of soluble molecules (35). Given that protocadherins typically mediate homophilic interactions, we will use the BIAcore in collaboration with Prof Ben Willcox to determine its affinity, if any, for self-association.

iv) Use a knockout mouse to determine how PCDH1 affects vessel formation in vivo

Having determined a function for PCDH1 in endothelial tubulogenesis *in vitro,* the aim of this part of the project is to determine the role of PCDH1 in vivo by generating a knockout mouse. Embryonic stem cells with a targeted deletion of 6489bp encompassing exon 2 and part of exon 3 are available from the KOMP repository (project CSD69165). This region of the gene is common to all splice variants and encompasses the translational start site. The Transgenic Mouse Facility in the Birmingham University Technology Hub has extensive experience in creating chimeric mice and will use albino C57/BL6 blastocysts into which to inject these ES cells on a C57/BL6 background, our own group has made previously made knockout mice in this way (25, 36). After having generated the mice, progeny from the offspring of heterozygous matings of PCDH1 knockout mice will be scored for Mendelian inheritance, and if there is embryonic lethality, timed matings will be set up and the time of embryonic death established. The embryos and particularly their vasculature would be examined using platelet/endothelial cell adhesion molecule (PECAM)-1 staining, of particular interest will be E8-E10 when angiogenesis occurs (37). If there is no obvious developmental defect, then adult angiogenesis will be assessed using the rodent subcutaneous sponge angiogenesis assay and in hind limb ischaemia (in collaboration with Stuart Egginton). Additionally we will perform a thorough histological examination on the different organs and tissues of the knockout mice, and vessels will be visualised by staining PECAM-1 using immunohistochemistry. Because PCDH1 is expressed in other cell types, non-vascular phenotypes could be observed, however it is anticipated that unless they caused early embryonic lethality these would be unlikely to obscure any vascular phenotype. If there are technical difficulties with the generation of the knockout mice, such as a failure for germline transmission of the targeted DNA from the chimera, then we will develop mouse specific PCDH1 duplexes to be used in vivo in angiogenesis assays where the duplexes are imbedded into matrigel plugs which are implanted subcutaneously (38).

6. Power calculations

Small scale experiments would always be analysed first to determine precisely the number of mice that would be required to give statistically significant results. Estimates of animal group sizes for the hind limb ischaemia model are made as follows: variables will be compared between two groups of mice, W/T and PCDH1-KO. Assuming n animals in each group and using a T-test with a pooled variance estimate of S, then: $t = D / S * N (2/n)$, where D is the difference in means between the two groups, e.g. typically detecting a 25% difference in regional capillary density. If the critical region is set to give a Type 1 error of 5% the size of change which can be detected with a similar error is given by: $D / S * N (2/n) = 6$. In data from previous experimental studies a value of S around 0.22 may be expected, thus according to the above calculation we would require n=8 animals per experimental group, in agreement with previous experience. Our experience with knockout mice for genes which when knocked down give a similar magnitude of effect in assays in vitro suggest that we would typically need three mice per group with two sponges per mouse for the sponge angiogenesis model.

7. Expected value of results

This project will give novel data regarding the role of PCDH1 in angiogenesis. To date, no role for PCDH1 in endothelial cell biology or angiogenesis has been described in the scientific literature, and so identification of a new protein involved in this process would mark a significant advance in the field. A more thorough understanding of the molecular mechanisms of angiogenesis will ultimately enable the promise of therapeutic angiogenesis to vascularise damaged heart tissue to be realised. Characterising the effect of the soluble extracellular domain of PCDH1 may yield exciting new strategies to modulate angiogenesis, which may have potential for therapeutic development. The generation of the PCDH1 knockout mouse would allow for the contribution of this gene to be assessed in additional models of cardiovascular disease. This project will advance our understanding of angiogenesis and may ultimately lead to the identification of new therapeutic targets or strategies for treating heart disease.

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10. Additional pages for appendices (e.g. charts, graphs, photos); must be kept to a minimum and should be submitted only if essential to the understanding of the application

None included

11. Statement detailing the scientific techniques in which the student will be trained

This project offers a student training in a diverse range of techniques ranging from molecular biology to *in vivo* mouse work.

Molecular biology and protein work
The student will learn how to make expression plasmids, perform site directed mutagenesis and produce recombinant protein in bacteria and mammalian systems. They will learn biochemical techniques and use mass spectrometry to test for protein-protein interactions. They will routinely use western blotting and learn how to do immunoprecipitation experiments.

Cell culture, *in vitro* angiogenesis assays and microscopy
The student will become skilled in the culture of human endothelial cells, perform siRNA transfection and use lentiviral systems to ectopically express proteins. They will learn a range of *in vitro* endothelial motility, chemotaxis and tube forming assays. They will be trained in performing immunofluorescence experiments and live cell imaging, and use standard wide field and confocal microscopy, as well TIRF microscopy, if necessary.

***In vivo* work**
The student will gain valuable experience of mouse knockout technology and studying the vasculature in embryonic and adult mice. The student will learn how to use *in vivo* angiogenesis models such as the rodent subcutaneous sponge assays and the hind limb ischaemia model.

Laboratory environment
The student will carry out their PhD in a stimulating laboratory environment in a space shared with Prof Roy Bicknell and with a number of post-doctoral researchers and PhD students with a diverse experience and an interest in angiogenesis and endothelial cell biology. As the student would be co-supervised by Dr Carol Murphy, the student would be exposed to the expertise of her group in cell signalling and endothelial biology. They will attend weekly laboratory meetings with both the Heath/Bicknell groups and with a group of laboratories from the School of Biosciences (which includes the Murphy group) with an interest in cell signalling and mass spectrometry. They would be expected to present their data approximately every 3 months to each of these groups.

The Laboratory is in the newly formed Institute for Cardiovascular Science whose multidisciplinary research brings together researchers in endothelial, platelet, leukocyte and myocardial cell biology. There is a weekly institutional seminar series which allows the student to be exposed to a range of both national and international cardiovascular research. Our laboratory is part of the strategic vascular inflammation, thrombosis and angiogenesis (VITA) grouping. Within VITA, there are monthly meetings where two students or post-doctoral scientists present their work to this group, and the student would be expected to attend these meeting and present their work at least twice during their studies.

The Graduate School
The student would belong to the Graduate School in the College of Medical and Dental Sciences. This would ensure that students have access to a broad range of training opportunities to best equip them to undertake their research successfully and to enhance their personal development. Training is specifically tailored to enhance generic skills appropriate for employment in the wider scientific community as well as in academia. The Graduate School of the College works closely with the University Graduate School to co-ordinate delivery of other aspects of training. These aspects include transferable skills in topics such as team-building, communication, writing, career development, publishing and thesis preparation. These opportunities are delivered through the University's Academic Practice and Organisational Development, the Student Guild and the Information Services Department.

12. Supporting letters from collaborators

School of Biomedical Sciences

Faculty of Biological Sciences

University of Leeds

Leeds

Victoria Heath DPhil
School of Immunity and Infection
Institute for Biomedical Research
University of Birmingham
Birmingham B15 2TT

27.04.16

Dear Dr Heath,

I have followed you work with great interest, and find the molecular insights you are providing to the field to be very helpful and interesting. My *in vivo* studies on angiogenesis induction and control offers a complementary approach that I am happy to share with you. To this end, I am happy to provide collaborative support to your proposed project entitled 'Determining the role of Protocadherin 1 in Angiogenesis', by way of surgical training and advice.

Yours sincerely,

Stuart Egginton BSc, PhD, DSc

Professor of Exercise Science

UNIVERSITY OF BIRMINGHAM

School of Biosciences,
Edgbaston,
Birmingham B15 2TT,
United Kingdom

Dr Debbie Cunningham

Lecturer in Molecular Cell Biology and Proteomics

Direct Line 0121 414 2652

E-mail d.cunningham@bham.ac.uk

28th April 2016

Dear Committee Members,

I am very happy to write this letter to support Dr. Vicky Heath in her application to the BHF for a studentship grant.

I have many years of experience in quantitative proteomics in the field of cell signaling and protein phosphorylation. In my group, we regularly use SILAC (stable isotope labelling by amino acids in cell culture) combined with high-resolution mass spectrometry to identify protein-protein interactions. I would be very happy to collaborate with Vicky and provide expertise in the use of SILAC-based mass spectrometry. As a collaborator I will assist in the design, methodology and data interpretation of quantitative SILAC experiments to profile the interactome of protocadherin 1 (PCDH1).

Both Vicky and I are attend regular shared lab meetings that have a common cell signaling theme and this creates a great collaborative environment in which to share data and ideas. If I can provide any further information then please do not hesitate to contact me,

Sincerely,

Debbie Cunningham

Professor of Molecular Immunology
Director, Cancer Immunology and Immunotherapy Centre
Telephone (+44) (0)121 414 9533
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UNIVERSITY OF BIRMINGHAM

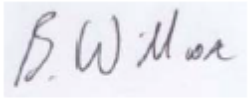
Institute of Immunology and Immunotherapy
University of Birmingham
Vincent Drive
Edgbaston
Birmingham B15 2TT
United Kingdom

Dear Sir or Madam,

I am writing to confirm I am delighted to collaborate with Dr Victoria Heath on her project to investigate the role of Protocadherin 1 in endothelial motility and angiogenesis. I have considerable experience in studying protein interactions and affinities and am happy to assist in using BIAcore-based methods to measure the affinity between extracellular domains of PCDH1. As Academic Director of the University of Birmingham Protein Expression Facility (BPEF), I can also confirm that the BPEF facility is in an excellent position to produce recombinant purified forms of PCDH1 for use in the various biological assays Dr Heath has planned. Clearly, investigation of homophilic interactions by PCDH1 using BIAcore technology is one significant aim. There is both a BIAcore 3000 and also a BIAcore T200 machine available for these studies and I would be happy to collaborate with Dr Heath to facilitate her work in this area.

In summary, I am fully committed to helping progress the project Dr Heath outlines, both by facilitating production of recombinant PCDH7 via the BPEF facility, and in providing collaborative BIAcore expertise.

Yours faithfully,



Benjamin E. Willcox