Delineating pericyte contribution to hereditary cerebral small vessel disease using Organ-on-Chip technologies

Abstract

Cerebral small vessel disease (cSVD) is a group of disorders which affect the structure and function of small blood vessels in the brain and can result in strokes, dementia and cognitive decline. Despite being one of the most common age-related diseases, the underlying mechanisms of cSVD are poorly understood. As brain vessel formation requires interactions of multiple cell types in 3D, studies of cSVD commonly use animal models. However, challenges with visualising and accessing the blood vessels in the brain limit the experimental findings. To provide an alternative approach to studying cSVD, this project investigates the use of patient-derived induced pluripotent stem cells (iPSCs) and Organ-on-Chip technologies to study an inherited form of cSVD caused by the *COL4A1* mutation. In particular, the project focuses on the contribution of pericytes as they are important regulators of the extracellular matrix (ECM) which surrounds the blood vessels and perturbations in the ECM have been suggested as a convergent mechanism in cSVD. By characterising the patient-derived *COL4A1* mutant pericytes and investigating their response to inflammation, the role of *COL4A1* mutant pericytes in promoting an inflammatory phenotype and ECM dysfunction was highlighted. To extend these observations into a 3D *in vitro* model of the neurovascular unit (NVU), this project optimised the organ-on-chip platform where pericytes are cultured with astrocytes and endothelial cells to study vessel formation.

Overall, this contributes to the development of human *in vitro* models to investigate the disease mechanisms of cSVD. Importantly, it not only provides a more ethical approach compared to animal models and reduces harm to animals, but also offers a more physiologically relevant tool for investigating the causes of diseases and treatment options.

Introduction

Cerebral small vessel disease (cSVD) refers to a group of neurological disorders that affect the structure and function of the small vessels of the brain – including arteries, arterioles, capillaries and veins[1]. This disease is a major cause of stroke (causing about 25% of ischaemic strokes[1,2]), vascular dementia[3] and cognitive decline. A recent systematic review has also suggested an association between the neuroimaging features of cSVD with Alzheimer's disease (AD)[4]. Despite being one of the most common age-related conditions, affecting about 80% of 65-year-olds and almost all 90-year-olds, the underlying pathogenesis of cSVD remains poorly understood.

While most cases of cSVD are sporadic and multi-factorial, making the study of disease mechanisms very complex, inherited monogenic forms of cSVD (which arise from a mutation in a single gene) can allow for an insight into underlying mechanisms in a simplified context[5,6]. These monogenic forms include; cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), which arises due to a mutation in the *NOTCH3* gene[7], cerebral autosomal-recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), relating to *HTRA1* mutations[8], and type IV collagen (COL4) A1/A2 disease, relating to *COL4A1/2* mutations[9].

This study focuses on *COL4A1* mutations. Heterozygous mutations in COL4A1 were initially identified to cause porencephaly and it was Gould et al[10] who identified heterozygous mutations in humans to be associated with familial cSVD, causing a spectrum of cerebrovascular phenotypes. *COL4A* encodes for collagen type IV alpha - an extracellular matrix (ECM) protein which forms heterotrimers that constitute the main component of the basement membrane (BM) in the vasculature. These macromolecular structures not only provide structural integrity but also participate in cell-cell and cell-matrix communication of the neurovascular unit (NVU) by binding growth factors and interacting with integrins. As a result of aberrant heterotrimer formation, *COL4A1* mutations have been implicated to cause ECM dysfunction and blood-brain barrier (BBB) disruption – neurological pathologies proposed to be common across all forms of cSVD. Therefore, studying monogenic cSVD may be able to offer insights into the shared disease mechanisms of cSVD.

Among the various cell types in the neurovascular unit (NVU), this project focuses on the contribution of pericytes to the development of cSVD. Pericytes are mural cells embedded in the basement membrane of blood microvessels[11]. Positioned between endothelial cells, glial cells (eg: astrocytes) and neurons in the neurovascular unit (NVU), pericytes play a crucial role in integrating signals from neighbouring cells and regulating blood-brain barrier development, maturation and homeostatic functions[12,13]. Interest in pericyte function in cSVD pathogenesis is increasing, especially following studies on CADASIL where the importance of pericytes has been emphasised[14,15].

To investigate the role of *COL4A1* mutant pericytes in cSVD, this project utilises induced pluripotent stem cell (iPSC) and Organ-on-Chip technologies. While current research into cSVD and stroke is largely dominated by animal models[16,17] there are obvious species-specific differences[18] and technical difficulties with visualising and accessing small blood vessels *in vivo*. This limits the ability to translate mouse studies to humans, and the study of the basic biochemical and cellular mechanisms of cSVD, which has prompted research using human *in vitro* systems. The advantages that Organ-on-Chip microfluidic systems present over current *in vitro* conventional cell culture systems of the NVU or BBB include the ability to precisely control flow and the chemical environment and direct cell types through a more physiologically relevant microphysical 3D architecture[19]. Combined with the advantages of being able to use patient-derived cells by using iPSC technologies, this presents us with a more physiologically relevant human *in vitro* model.

Aims

The overall aim of this project is to investigate the contribution of pericytes to cSVD using iPSC and Organ-on-Chip technologies. In this project, *COL4A1* mutant pericytes generated from patient-derived iPSCs will first be characterised in normal and inflammatory conditions. Then, the Organ-on-Chip microfluidic platform for modelling the NVU will be optimised to allow for future studies to determine the influence of *COL4A1* mutant pericytes on vessel formation, vessel permeability and pericyte-vessel interactions.

Material and methods

Cell culture

Cells were obtained from our collaborators, the Granata Lab at the University of Cambridge. The four iPSC-derived cell types used were *COL4A1* mutant mural cells (pericytes), isogenic control mural cells, isogenic endothelial cells and isogenic astrocytes. Isogenic mural cells were generated by correcting the *COL4A1* mutation within the patient-derived iPSC cell line to minimise the effect of interindividual variations. Cells were cultured in DMEM + 10% FCS on plates coated with 1% gelatine. iPSC derived astrocytes were grown on matrigel coated plates in complete astrocyte media (ScienCell) and endothelial cells were grown on gelatine coated plates in EGM2 media (Lonza). All cells were grown in a humidified cell culture incubator at 37°C with 5% CO_2.

Immunofluorescence Staining

Mural cells were seeded on coverslips and incubated for 24 hours (for PDGFR-β, SMA-α, NG2, nestin, VCAM-1, ICAM-1 staining) or 1 week (for COL4A1/2 staining). After the respective incubation times, 10ng/ml TNF-α or 50ng/ml VEGF was used to treat cells and stimulate inflammatory conditions for another 24 hours. Cells were then fixed in 4% paraformaldehyde at room temperature for 10 minutes. This was followed by incubations in (1) permeabilisation buffer (PBST containing 0.1% Triton X-100) for 10 mins, (2) block buffer (PBST containing 10% donkey serum, 1% w/v BSA and 200μM glycine) for 1 hour and (3) respective primary antibodies (1:100 dilution polyclonal goat anti-human PDGFR-β antibody (R&D Systems), 1:200 dilution monoclonal mouse anti-human SMA-α antibody (Abcam), 1:200 dilution polyclonal rabbit anti-human NG2 antibody (Merck Millipore), 1:100 dilution monoclonal mouse anti-human Nestin antibody (Biolegend), 1:20 dilution monoclonal mouse anti-human VCAM-1 antibody (DSHB), 1:100 dilution monoclonal mouse anti-human ICAM-1 antibody (DSHB) and 1:100 dilution monoclonal mouse anti-human COL4A1/A2 antibody (DSHB)) for 1 hour. Subsequently, cells were washed 3x with PBST and incubated with 1:500 diluted Alexa Fluor 488-labelled donkey anti-mouse antibodies for 1 hour. Nuclei were stained with 1:2000 dilution of Hoechst stain for 3 minutes, then washed 2x with PBST. Coverslips were then lifted and transferred onto a microscope slide with mounting gel and left to dry overnight. Microscope images were visualised on an Axioskon2 fluorescence microscope and analysed using Image J.

Antibody Arrays

Human Inflammation Antibody Array A (Abcam, ab197449) and a Human MMP Antibody array (ab197453) were used to analyse the inflammatory cytokines and MMPs present in the supernatant of mural cells. For both isogenic and mutant mural cells, the supernatant was taken from cells grown with and without 24-hour treatments with 10ng/ml TNF-α. The array was carried out according to the protocol provided in the user's manual.

Platform design and fabrication

The microfluidic platform design and fabrication were based on the organ-on-a-chip platform by Phan et al (2017)[20]. To summarise the design shown in Figure 1 briefly, each microfluidic

device consists of 4 tissue chambers, with one gel loading inlet and outlet. There is a pressure regulator unit integrated into each device which consists of pressure-releasing burst valves and diversion channels to facilitate proper loading. The tissue chambers are connected to microfluidic perfusion channels which have an asymmetrical design with one medium inlet (M1) and outlet (M2). In-line fluidic resisters generate a hydrostatic pressure gradient across the tissue chambers. This will produce a laminar flow along the microfluidic channels, as well as an interstitial flow across the tissue chambers.

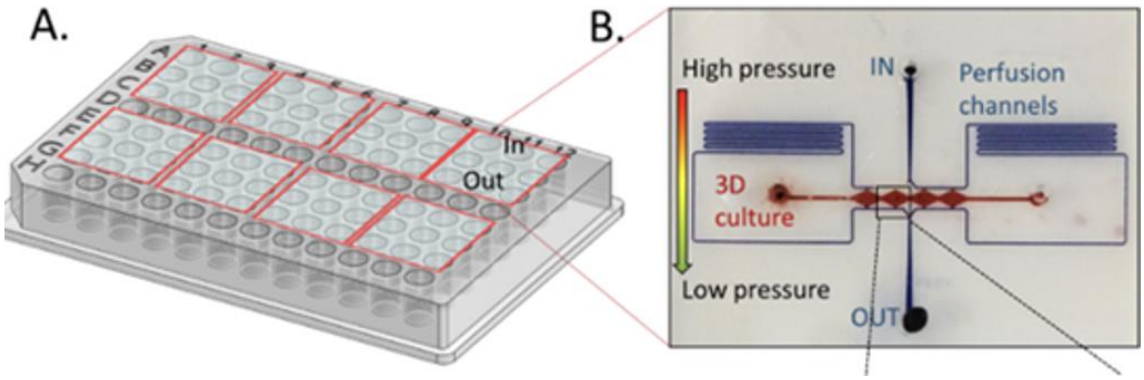

Figure 1 Platform Design

A) 96 well plate layout housing multiple devices with input reservoirs providing continuous perfusion without pumps. B) Image of a single microfluidic device with perfusion channels filled with blue dye and a 3D culture channel with red-dyed hydrogel.

The platform consists of an imprinted polydimethylsiloxane (PDMS) layer sandwiched between a 96-well plate and a glass coverslip. Customised polyurethane master moulds are fabricated with the device design and used to mould the PDMS device layer. These are then cut and punched with holes for the inlets and outlets. The PDMS layer is attached to the 96 well plate by a chemical glueing method (24) which consists of immersing the plates with 3-mercaptopropyl trimethoxysilane (Sigma-Aldrich) diluted in 98% methanol, rinsing and drying them. This is followed by treating both the well plate and PDMS later with oxygen plasma, aligning and bonding them together. Finally, a glass coverslip is bonded to the PDMS layer by treating with oxygen plasma. The fully assembled platform is placed in a 60°C oven overnight. Before use, the platform should be sterilised using UV light for a minimum of 30 minutes on each side, and de-gassed. An additional step of pre-treating the platform with poly-d-lysine overnight,

Loading of tissue chambers

Mural cells, astrocytes and endothelial cells were lifted using accutase, centrifuged, and then resuspended in DMEM + 10% FCS. Stock solutions were prepared with the following required cell densities: 7 million/ml for endothelial cells and 3.5 million/ml for astrocytes and mural cells. The cellular stock solution was then resuspended in filtered fibrinogen solution to obtain a final fibrinogen concentration of 5mg/ml. For device loading, the cell-matrix mixture is

quickly mixed in a 1:1 ratio with 5 units/ml thrombin and loaded into the gel loading inlet. After allowing the gel to polymerise for about 15 minutes, the microfluidic media channels are coated with fibronectin diluted 1:10 with endothelial cell media for another 15 minutes. Then, endothelial growth media is added asymmetrically into the medium wells to generate a hydrostatic pressure gradient. Every 24 hours, the medium should be replaced and the direction of flow switched to ensure a steady medium flow and cell stimulation from both directions. After 3 days, the endothelial growth media is switched to endothelial media to allow stabilisation of vessels. Microscope images were taken using an Axioskon2 fluorescence microscope from day 5 onwards.

Results and Findings

Characterisation of iPSC-derived COL4A1 mutant pericytes

To begin characterising the iPSC-derived *COL4A1* mutant and isogenic pericytes, immunofluorescence staining for four pericyte markers - platelet-derived growth factor receptor (PDGFR)-β, smooth muscle actin-α (SMA-α), neural/glial antigen-2 (NG2) and nestin were carried out[21]. Both mutant and isogenic pericytes expressed all of these markers. This validates the differentiation protocol of pericytes from iPSCs and their subsequent use in our assays.

Then, to compare the effect of the *COL4A* mutation on the expression of COL4A in mutant and isogenic pericytes, immunofluorescence staining for COL4A was performed. Cells were cultured for 1 week before staining to allow cells to reach confluency and develop a stable extracellular matrix. The collagen in mutant cells were found to be accumulated in aggregates within the cell, while the collagen in isogenic cells were more organised and evenly distributed extracellularly. (Figures 2A and B) This suggests that in mutant cells, collagen is not formed properly and aggregates in the cell instead of being secreted into the extracellular matrix. On quantification of COL4A expression, no significant differences were observed between isogenic and mutant cells. Thus, to determine whether there were differences between total COL4A and secreted COL4A incorporated into the ECM, cells were stained in the absence of permeabilisation to only stain extracellular COL4A. In the non-permeabilised cells, significant reductions in COL4A were found. (Figure 2C) Altogether, this suggests the intracellular accumulation of mutant COL4A protein.

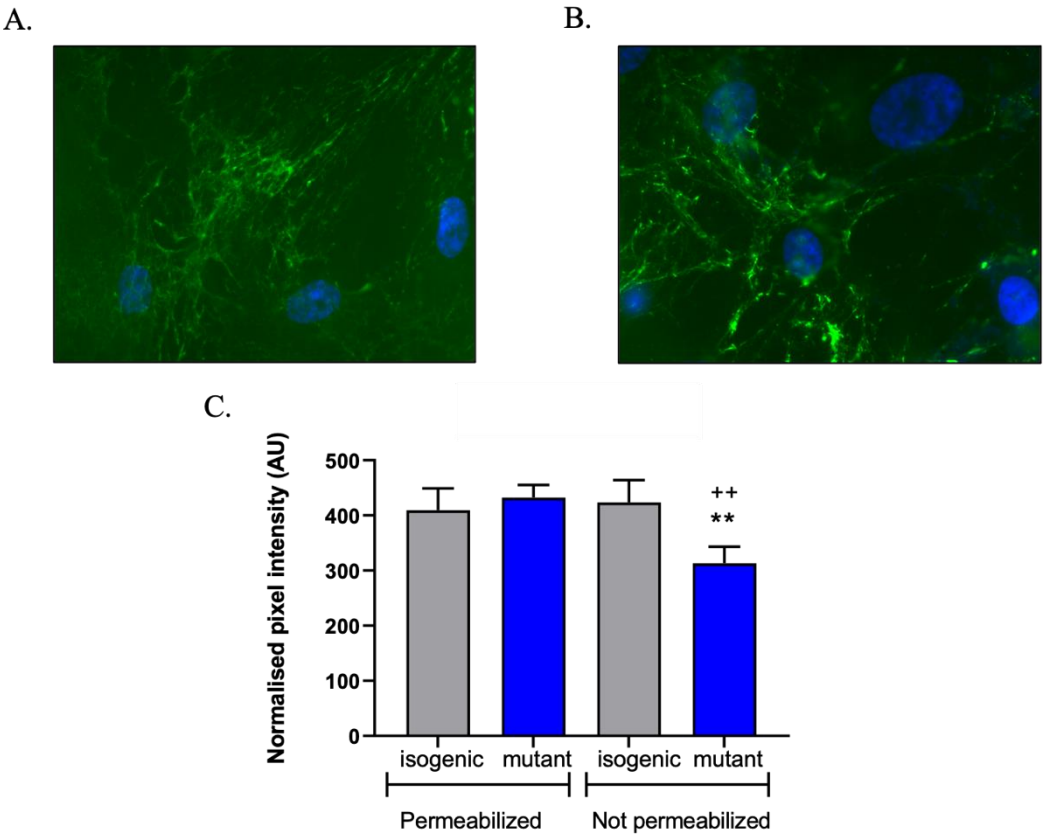

Figure 2 Immunostaining for COL4A Expression

A) COL4A expression in isogenic pericytes B) COL4A expression in *COL4A1* mutant pericytes. Cells were cultured for 1 week before staining. Images were taken at 100x. C) Quantification of COL4A staining in the presence and absence of cell permeabilisation. *denotes significance to control group of same cell type, + denotes significance to isogenic cells of the same treatment type

Investigating pericyte response to inflammation

Beyond their structural functions in vessel maturation and stabilisation, pericytes have also been implicated in immune regulation[22]. In response to pro-inflammatory stimuli, it has been reported that pericytes secrete a variety of chemokines and cytokines and by doing so, recruit immune cells to the inflammation site, induce cytokine secretion by other cells and promote inflammation-associated angiogenesis[23,24]. The expression of matrix metalloproteinases (MMPs) has also been associated with inflammatory conditions. MMP remodel the extracellular matrix and can cause increased vessel permeability and reduced stability.[25] To investigate the response of pericytes to inflammation, an inflammatory cytokine array and MMP array were conducted.

Using the inflammatory cytokine array, levels of 10 cytokines (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1, IFN-γ and TNF-α) were assessed in the supernatant of pericyte cells cultures. No significant changes in IL-1B, IL-4, IL-10, IL13 or IFN-y were observed across all

treatments and cell types. However, IL-1a and IL-6 were found to be elevated upon TNF treatment, with IL-6 levels in both control and TNF treated mutant cells being significantly higher than in isogenic cells. Furthermore, IL-8 and MCP-1 were found to be higher in control-treated mutant cells when compared to isogenic. However, this was not further elevated by TNF treatment. (Figure 3)

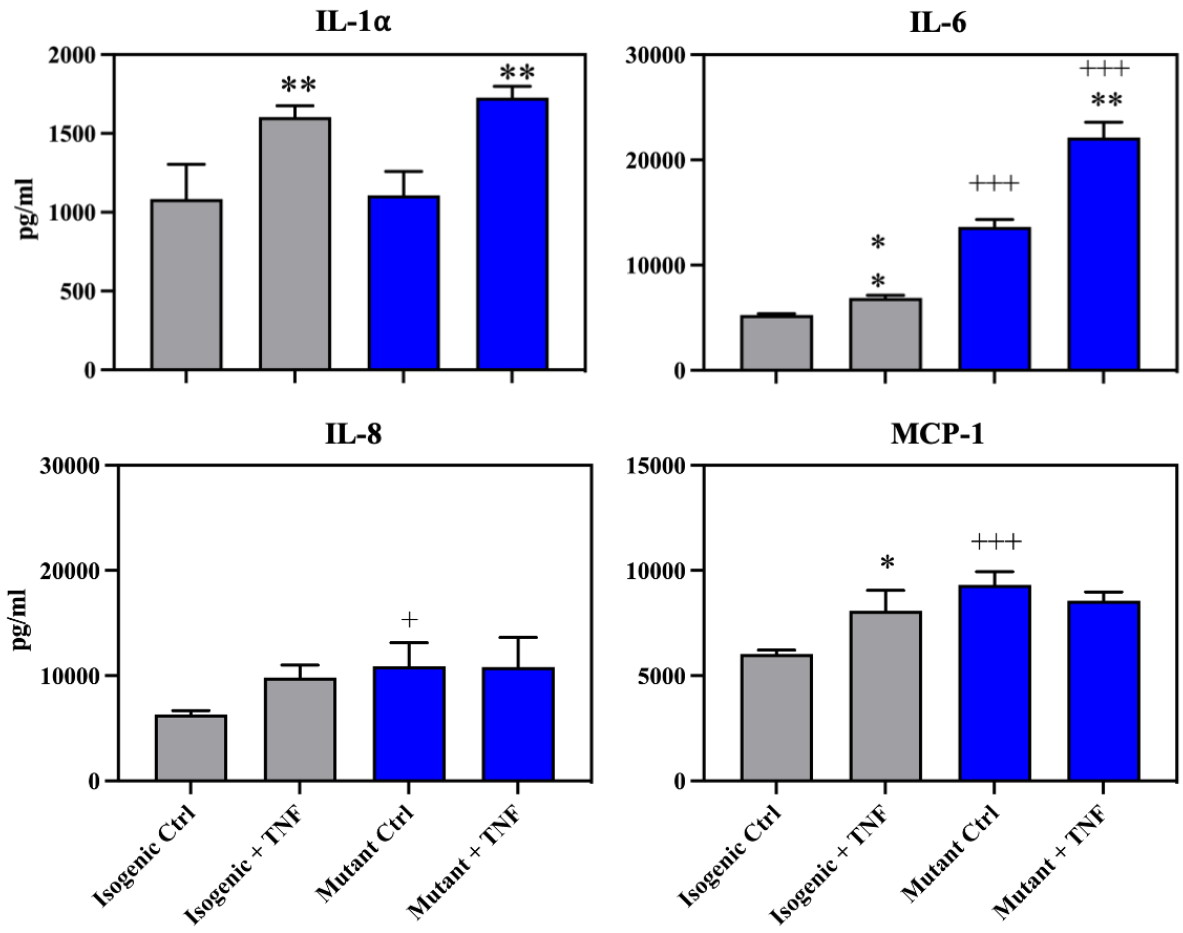

Figure 3 Inflammatory Cytokine Array

Significant results were observed for IL-1α, IL-6, IL-8 and MCP-1
*denotes significance to control group of same cell type, + denotes significance to isogenic cells of the same treatment type

Using antibody array quantification of MMPs released into the cell culture supernatant, MMP3, MMP8, MMP10, MMP13 and the MMP regulators TIMP2 and TIMP4 were found to be below detectible levels of the kit. However, MMP1, MMP9 and TIMP1 were all induced by TNF treatment and were further elevated in mutant cells both under control and TNF stimulated conditions. Additionally, MMP2 was found to be increased in TNF stimulated conditions in mutant pericytes, but this difference was not significant compared to isogenic cells. (Figure 4)

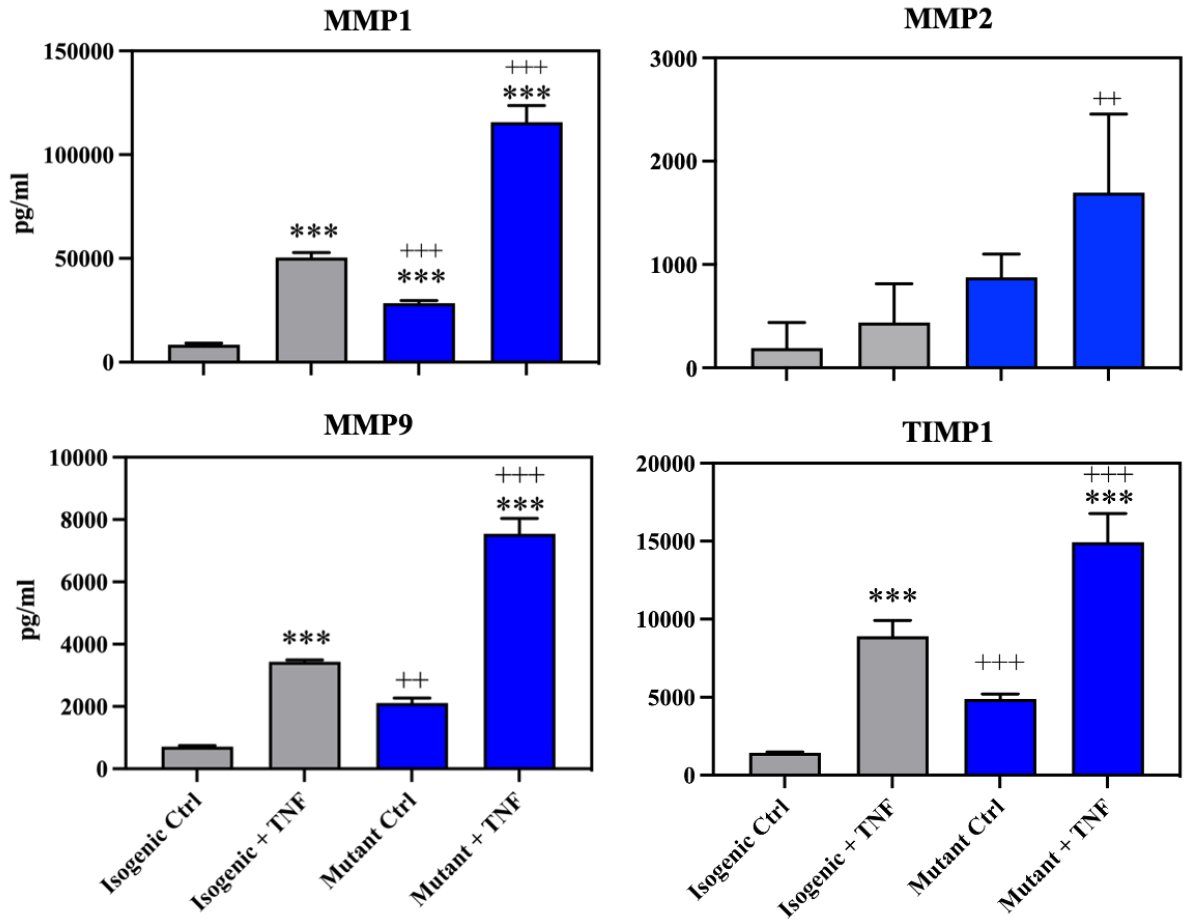

Figure 4 MMP Array

Significant results were observed for MMP1, MMP2, MMP9 and TIMP1
*denotes significance to control group of same cell type, + denotes significance to isogenic cells of the same treatment type

Pericytes have also been reported to overexpress adhesion molecules such as intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in response to inflammation to aid immune cell trafficking[24,26,27]. Thus, the expression of ICAM-1 and VCAM-1 was assessed using immunofluorescence staining in control and inflammatory conditions. An increase in ICAM-1 and VCAM-1 expression after treatment with TNF-α or VEGF for both mutant and isogenic pericytes was observed. Notably, the increase was greater in mutant compared to isogenic cells for most conditions. To quantify our observations, the images were analysed using Image J to calculate the average intensity per cell as a measure of expression level. Figure 5 reports the calculated percentage increase of ICAM-1 or VCAM-1 expression by mutant and isogenic pericytes in TNF-α or VEGF conditions compared to control conditions.

Overall, these results support the role of pericytes in the inflammatory response and prompt further investigations into how mutant pericytes respond to inflammation and contribute to ECM remodelling in cSVD.

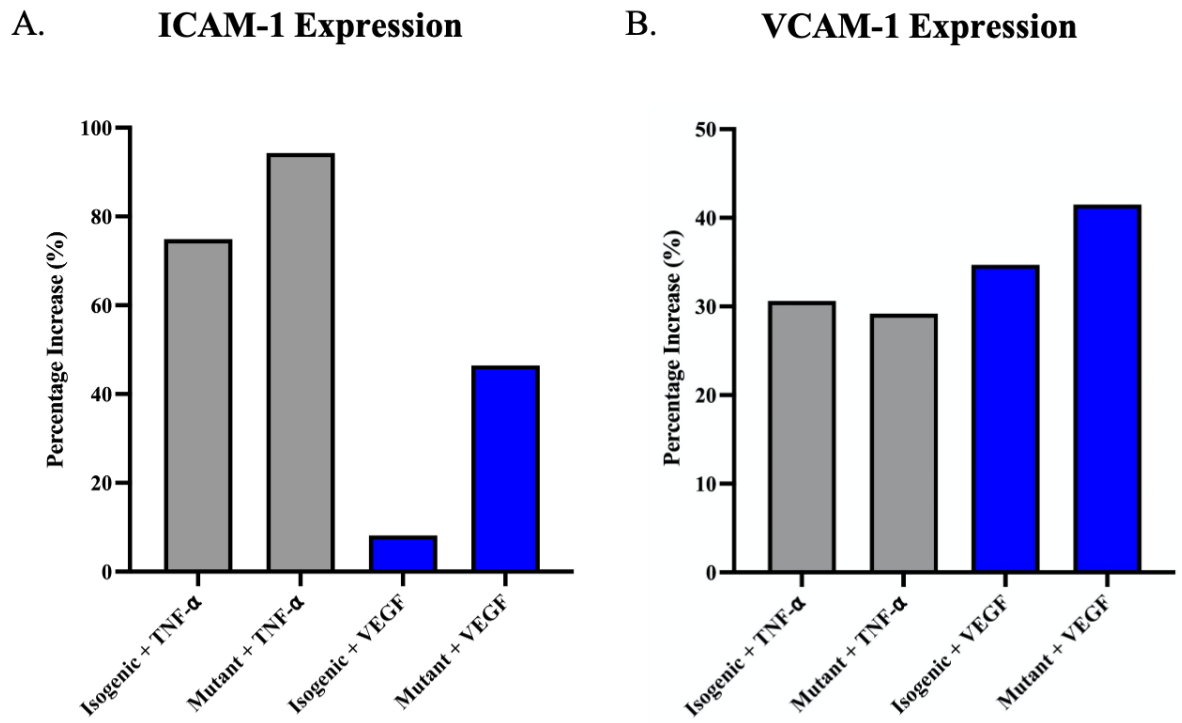

Figure 5 Analysis of ICAM-1 and VCAM-1 expression

A. Percentage increase in ICAM-1 expression in TNF-α and VEGF conditions B. Percentage increase in VCAM-1 expression in TNF-α and VEGF conditions

Optimising the Organ-on-Chip microfluidic platform for modelling the NVU

Having characterised the iPSC-derived pericytes, microfluidic devices were loaded with a combination of pericytes, astrocytes and endothelial cells in a mixture of fibrinogen and thrombin which quickly forms a gel when combined. Initial attempts were challenging as the loading protocol was complex. Few devices were successfully loaded as the devices were either loaded too slowly (resulting in the gel solidifying before the channels were loaded) or with much pressure (resulting in the channels bursting). Of the devices that were successfully loaded, no vessel formation (Figure 4). This could be attributed to the low cell density, the high prevalence of cell death, or the pulling away of the gel from the edges of the channels.

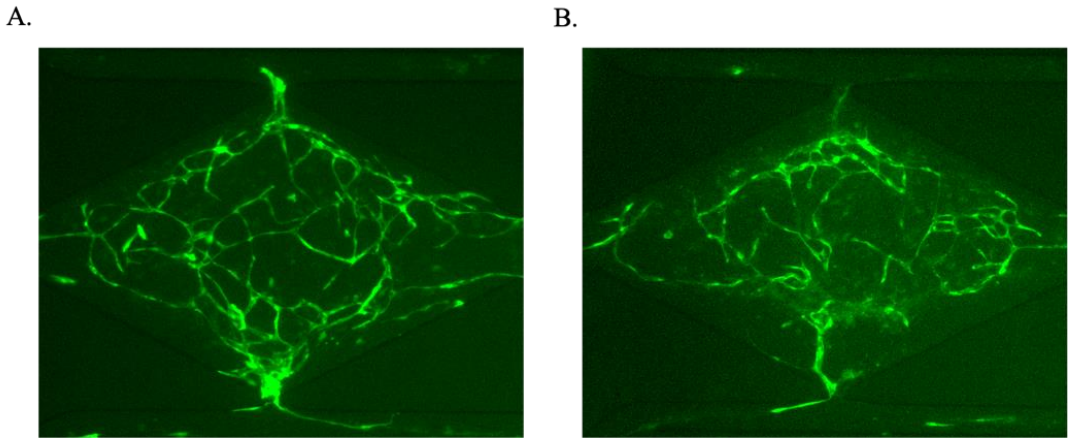

Figure 4 Initial attempts at loading microfluidic devices

A. Loading with isogenic pericytes B. Loading with *COL4A1* mutant pericytes. Images were taken 5 days after devices were loaded. Endothelial cells were visualised using green fluorescence to indicate vessel formation.

To overcome these issues, the protocol was adapted and streamlined. Improvements included increasing the final cell densities (to account for any inaccuracies in cell counting), pre-treating the gel channels with poly-d-lysine (to help the gel to adhere better) and chilling the trypsin and pipette tips (as lowering the temperature slows down the hardening of the gel). With practice, the success rate of loading the devices also improved. The time taken between the lifting of the cells to the loading of the devices was also shortened, reducing the possibility of cell death due to the cells being lifted for an extended period. Eventually, morphological improvements in vessel formation were observed (Figure 5) as the endothelial cells seemed to form lumen and a vascular network. However, in perfusion assays, where dextran dyes are added into the channels to assess vessel permeability, the vessels were not perfusable, indicating that they have not formed correctly and there are still issues to address.

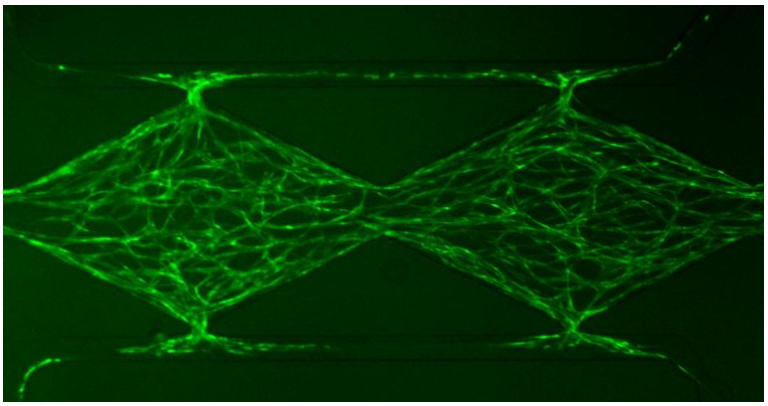

Figure 5 Later attempts at loading microfluidic devices

A more successful attempt at loading the microfluidic devices

Discussion

Given the lack of understanding of the pathogenic mechanisms of cSVD, this project sets out to investigate a hereditary monogenic form of the disease associated with mutations in the *COL4A1* gene to gain insights into common disease mechanisms. Specifically focusing on pericytes in the NVU, this project utilizes pericytes generated from patient-derived iPSCs for disease modelling.

As iPSC differentiation is complex and variable for each cell line, the pericytes were first characterized by their expression of PDGFR-β, SMA-α, NG2, and nestin. This is important for validating the cell line for use in subsequent experiments. The effects of the *COL4A1* mutation on the expression of COL4A were also investigated and it was observed that the mutant collagen in mutant pericytes were aggregated within the cell. This would support the hypothesis that *COL4A1* mutant cells would have a deficiency of COL4A in the ECM, giving us an indication of how *COL4A1* mutations lead to ECM dysfunction[28].

To investigate the potential roles of pericytes in the inflammatory response in cSVD, the secretion of cytokines, MMPs and the expression of adhesion molecules were analysed. Results from the antibody array kits suggest an inflammatory phenotype in our samples as the expression of certain cytokines (IL- IL-1α, IL-6, IL-8 and MCP-1) and MMPs and their regulator (MMP1, MMP2, MMP9 and TIMP1) varied in mutant pericytes in TNF stimulated conditions. This suggests that pericytes contribute to an inflammatory microenvironment and that high levels of ECM remodelling occur in the COL4A disease. IL-6 has been reported to be secreted by pericytes in previous studies[24], thus our study confirms this. Additionally, the upregulation of ICAM-1 and VCAM-1 is interesting as regions of low densities of COL4A and laminin 10 in the basement membrane have been associated with the upregulation of ICAM-1 and neutrophil extravasation.[29] Overall, these findings suggest the role of pericytes in COL4A disease progression and present them as therapeutic targets to reduce inflammation and stabilise vessels.

Then to study the contribution of pericytes in an *in vitro* model of the NVU, pericytes were cultured with astrocytes and endothelial cells in a microfluidic device to observe the effect of *COL4A1* mutant pericytes on vessel formation. Although initial technical challenges were faced, adjustments to the protocol and repeated practice resulted in improvements in the loading and subsequent vessel formation in the device channels. This helped to establish the model and also highlighted ways to modify the protocol to make it more accessible to a non-expert. However, while vessels formed within the device, perfusion of these vessels was unsuccessful. To improve the ability to perfuse these vessels, future work could also seed endothelial cells into the microfluidic perfusion channels to improve the connection of these channels with the 3D vessels that were formed in the fibrin gel. This work has helped reveal the problem of high levels of variability in vessel connection with the perfusion channels and has since led to Dr Holloway modifying the device design to provide wider connecting ports between the gel and perfusion channels and larger perfusion channels, to facilitate the connection of the 3D network with the microfluidic channels. Thus, this project has helped with the development of this model that will facilitate its wider use to explore cell interactions in cSVD.

Overall, this project investigates the combination of two technologies – iPSCs and Organ-on-Chip microfluidic disease modelling – for the study of monogenic forms of cSVD. With the adaptability of iPSCs to differentiate into any somatic cell type and the ability of Organ-on-Chip platforms to provide tools to study the interactions between various cell types of the NVU in a more physiologically relevant 3D environment, these technologies present novel tools to gain insights into the disease mechanisms of cSVD. Combined, these two technologies can provide experimental readouts of NVU functions that are currently only possible in animal models. Thus, it is important to establish these technologies to reduce the use of animal models and develop more physiologically relevant disease models.

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