

## Regulation of VEGF-activated signalling by the plasma membrane calcium ATPase 4 in endothelial cells

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**REGULATION OF VEGF-ACTIVATED SIGNALLING BY THE PLASMA  
MEMBRANE CALCIUM ATPASE 4 IN ENDOTHELIAL CELLS**

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A thesis submitted in partial fulfilment of the requirements of the

University of Wolverhampton for the degree of

Master of Philosophy

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## **DECLARATION**

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# **CONTENTS**

LIST OF FIGURES .....	iii
LIST OF TABLES .....	v
LIST OF ABBREVIATIONS .....	v
ABSTRACT .....	1
1. INTRODUCTION .....	4
BACKGROUND .....	5
1.1 PHYSIOLOGICAL ANGIOGENESIS .....	5
1.1.1.a Angiogenesis in embryonic development .....	5
1.1.1.b Angiogenesis in wound healing.....	7
1.1.1.c Angiogenesis in the female reproductive system.....	8
1.2 PATHOLOGICAL ANGIOGENESIS.....	9
1.2.1. PATHOLOGY ASSOCIATED WITH EXCESSIVE ANGIOGENESIS .....	9
1.2.1.a Tumor angiogenesis .....	9
1.2.1.b Diabetic retinopathy .....	11
1.2.2. PATHOLOGY ASSOCIATED WITH INSUFFICIENT ANGIOGENESIS .....	12
1.2.2.a Ischemic heart disease .....	12
1.2.2.b Peripheral arterial disease.....	14
1.2.2.c CLINICAL DEVELOPMENT IN PRO/ANTI-ANGIOGENIC THERAPIES.....	15
1.3 CELLULAR EVENTS IN ANGIOGENESIS.....	18
1.4 REGULATORS OF ANGIOGENESIS.....	21
1.5 VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY .....	24
1.5.1 VEGF-A.....	25
1.6 VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS (VEGFR) .....	27
1.6.1 VEGFR-1 .....	28
1.6.2 VEGFR-2 .....	28
1.6.3 VEGFR-3 .....	29
1.7 VEGFR2-ACTIVATED SIGNALLING IN ANGIOGENESIS.....	30
1.7.1 EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) PATHWAY .....	30
1.7.2 P38 MITOGEN ACTIVATED PROTEIN KINASE .....	31
1.7.3 PHOSPHATIDYLINOSITOL-3 KINASE (PI3K) PATHWAY.....	32
1.7.4 PROTEIN KINASE C.....	35

1.7.5 CALCINEURIN/NFAT PATHWAY.....	36
1.8 NOTCH SIGNALLING PATHWAY .....	37
1.8.1 MECHANISM OF NOTCH SIGNALLING.....	38
1.8.2 NOTCH SIGNALLING AND ENDOTHELIAL CELL SPECIFICATION.....	41
1.9 PLASMA MEMBRANE CALCIUM ATPase pump (PMCA) .....	42
1.9.1 STRUCTURE OF PMCA.....	42
1.9.2 PMCA BINDING DOMAINS .....	43
1.9.3 PMCA AS A REGULATOR OF INTRACELLULAR SIGNALLING PATHWAY VIA INTERACTION WITH PARTNER PROTEINS.....	44
1.9.4 CA <sup>2+</sup> TRANSPORTATION VIA PMCA PUMP.....	46
1.9.5 <i>PMCA</i> ISOFORMS AND THEIR TISSUE DISTRIBUTION.....	46
1.9.5.a <i>PMCA</i> 1.....	47
1.9.5.b <i>PMCA</i> 2.....	48
1.9.5.c <i>PMCA</i> 3.....	48
1.9.5.d <i>PMCA</i> 4.....	49
1.9.6 ROLE OF <i>PMCA</i> 4 AS AN INHIBITOR OF VEGF-INDUCED ANGIOGENESIS .....	51
2. AIMS AND HYPOTHESIS.....	53
3. MATERIALS AND METHODS .....	57
3.1 CELLS AND CELL CULTURE .....	58
3.2 CELL CULTURE IN TISSUE CULTURE FLASKS .....	58
3.3 COUNTING AND PLATING CELLS.....	59
3.4 TRANSFECTION OF PRIMARY ENDOTHELIAL CELLS WITH SMALL INTERFERING RNA (siRNA).....	60
3.5 CELL STIMULATION .....	61
3.6 TOTAL RNA ISOLATION .....	62
3.7 REVERSE TRANSCRIPTION.....	63
3.8 QUANTITATIVE REAL TIME PCR .....	64
3.9 PROTEIN DETECTION BY WESTERN BLOT.....	65
3.10 PCR-BASED SCREENING OF SMALL GENE ARRAYS .....	68
3.11 STATISTICAL ANALYSIS .....	69
4. RESULTS .....	71
4.1 SILENCING THE EXPRESSION OF <i>PMCA</i> 4 IN HUMAN ENDOTHELIAL CELLS BY TRANSFECTING siRNAs TARGETING <i>PMCA</i> 4.....	72

4.1.1 KNOCK-DOWN OF <i>PMCA4</i> AT RNA LEVEL.....	72
4.1.2 PROTEIN LEVEL DETECTION BY WESTERN BLOT .....	73
4.2 ANALYSES OF CHANGES IN GENE EXPRESSION CAUSED BY <i>PMCA4</i> SILENCING IN ENDOTHELIAL CELLS.....	75
4.2.1 SCREENING OF A GENE ARRAY RELATED TO NOTCH SIGNALLING PATHWAY .....	75
4.2.2 SCREENING OF GENE ARRAY RELATED TO EXTRA CELLULAR MATRIX AND CELL ADHESION MOLECULES.....	78
4.3 VALIDATION OF DIFFERENTIAL GENE EXPRESSION OF <i>PMCA4</i> -TARGET GENES AFTER <i>PMCA4</i> SILENCING IN ENDOTHELIAL CELLS ISOLATED FROM LARGE VESSELS (HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS, HUVEC) OR THE MICROVASCULATURE (HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS, HDMEC).....	81
4.3.1 VALIDATION OF GENES IMPLICATED IN NOTCH SIGNALLING PATHWAY USING TAQMAN qPCR TECHNIQUE.....	81
4.3.2 RNA EXPRESION OF GENES RELATED TO ECM-CAM SIGNALLING PATHWAY IN HUVEC.....	84
4.3.3 RNA EXPRESSION OF GENES RELATED TO ECM-CAM IN HDMECs.....	87
4.4. QUANTIFICATION OF <i>PMCA4</i> RNA EXPRESSION IN AGING HUVEC.....	91
5. DISCUSSION.....	92
6. CONCLUSION .....	101
REFERENCE.....	103

## LIST OF FIGURES

Figure 1.1.i Diagrammatic representation of the two types of angiogenesis...7	7
Figure 1.3.i. Diagrammatic representation of various stages involved in formation of sprouting angiogenesis in response to angiogenic stimulus.....20	20
Figure 1.5.i Exon assembly of vascular endothelial growth factor A (VEGF-A).....26	26
Figure 1.6.i Diagrammatic representation of different isoforms of VEGF receptors and their ligands.....27	27
Figure 1.8.i Cellular events involved in Notch Signalling.....40	40
Figure 1.9.i Diagrammatic illustration of P-type Plasma membrane Calcium transport ATPase pump.....43	43
Figure 2.1 Diagrammatic representation of various pathways involving VEGF/VEGFR signalling.....55	55

Figure 3.4.i. Presence of vacuoles within the cells implies good transfection uptake .....	61
Figure 4.1.1.i. siRNA-mediated <i>PMCA4</i> silencing in endothelial cells leads to a strong reduction in <i>PMCA4</i> expression at the RNA level.....	73
Figure 4.1.2.i. Western blot analysis of <i>PMCA4</i> after siRNA-mediated silencing of <i>PMCA4</i> expression in human endothelial cells.....	74
Figure 4.2.1.i. Volcano plot depicting changes in the expression of genes related to the Notch signalling pathway after <i>PMCA4</i> silencing in endothelial cells.....	76
Figure 4.2.2.i. <i>PMCA4</i> gene knockdown in HUVEC shows changes in the expression of genes related to ECM-CAM.....	79
Figure 4.3.1.i. Validation of <i>DLL1</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC.....	82
Figure 4.3.1.ii. Validation of <i>Hey1</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC.....	82
Figure 4.3.1.iii. Validation of <i>DLL4</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC. ....	83
Figure 4.3.1.iv. Validation of <i>c-Fos</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC. ....	83
Figure 4.3.2.i. Validation of <i>ADAMTS-1</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC .....	84
Figure 4.3.2.ii. Validation of <i>SELE</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC. ....	85
Figure 4.3.2.iii. Validation of <i>SELP</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC.....	85
Figure 4.3.2.iv. Validation of <i>VCAM1</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC.....	86
Figure 4.3.2.v. Validation of <i>SELL</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HUVEC.....	86
Figure 4.3.3.i. siRNA-mediated <i>PMCA4</i> silencing leads to a strong reduction in <i>PMCA4</i> expression at the RNA level in HDMECs.....	88
Figure 4.3.3.ii. Validation of <i>ADAMTS-1</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HDMEC .....	88
Figure 4.3.3.iii. Validation of <i>SELE</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HDMEC.....	89
Figure 4.3.3.iv. Validation of <i>SELL</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HDMEC.....	89

Figure 4.3.3.v. Validation of <i>SELP</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HDMEC.....	90
Figure 4.3.3.vi. Validation of <i>VCAM1</i> RNA expression during <i>PMCA4</i> gene silencing in VEGF-stimulated HDMEC.....	90
Figure 4.4.1.i. Bar chart representing increase in levels of <i>PMCA4</i> in aging HUVECs.....	91
Figure 5.1. Graphical representation of CaN/NFAT and NFκB signalling pathway which leads to increase of ADAMTS-1, SELE and VCAM-1 regulated by VEGF/VEGFR signalling.....	98

## **LIST OF TABLES**

Table 1.4.a. Stimulators of angiogenesis.....	21
Table 1.4.b. Inhibitors of angiogenesis.....	23
Table 3.4.a. Composition of the complexes used in transfection of HUVECs using siRNA-NT or siRNA- <i>PMCA4</i> .....	60
Table 3.7.a. Composition of the Master mix used for reverse transcribing RNA to cDNA.....	63
Table 3.8.a. Components of master mix used for TaqMan PCR assays.....	64
Table 3.8.b. Various TaqMan primers and their gene reference code used to run the Applied BioSystem 7500 Fast real-time PCR.....	65
Table 3.9.a. Composition of Resolving Gel (6% and 10%) and Stacking gel..	66
Table 4.2.1.a. List of 84 genes screened in RT2 profiler PCR array depicting changes in genes related to the Notch signalling pathway after <i>PMCA4</i> silencing in endothelial cells.....	77
Table 4.2.2.a. List of 84 genes screened in RT2 profiler PCR array depicting changes in genes related to ECM-CAM after <i>PMCA4</i> silencing in endothelial cells.....	80

## **LIST OF ABBREVIATIONS**

**µg-** microgram

**ADAM-** A Disintegrin and Metalloprotease

**ADP -** Adenosine Diphosphate

**AMD-** Age-Related Macular Degeneration

**ANOVA-** Analysis of Variance

**APS- Ammonium Per Sulphate**  
**ATA- Aurin Tricarboxylic Acid**  
**ATP- Adenosine Triphosphate**  
**ATPase- Adenosine Triphosphatase**  
**CAM- Calmodulin**  
**CAM-BD- Calmodulin binding domain**  
**cAMP- Cyclic Adenosine Monophosphate**  
**CASK- Calcium/Calmodulin Dependent Serine Protein Kinase**  
**CDK- Cytokine dependent Kinase**  
**cDNA- Complementary Deoxyribonucleic Acid**  
**CLI- Critical Limb Ischaemia**  
**Cn A- Calcineurin A**  
**Cn B- Calcineurin B**  
**CO<sub>2</sub>- carbondioxide**  
**Col 1A1- Collagen 1A1**  
**Cox- Cyclooxygenase**  
**CsA- Cyclosporin A**  
**CSL- CBF-1/RBPJ- $\kappa$ , Su(H), Lag-1**  
**Ct- Cycle Threshold**  
**Ctgf- Connective tissue growth factor**  
**DLL- Delta like ligand**  
**DME- Diabetic macular edema**  
**DNA- Deoxyribonucleic acid**  
**DSL- Delta/Serrate/Lag2**  
**EC- Endothelial Cell**  
**ECGM- Endothelial Cell Growth Medium**  
**ECM- Extracellular Matrix Components**  
**EGF- Epidermal Growth Factor**  
**EGFR - Epidermal Growth Factor Receptor**  
**eNOS- Endothelial Nitric Oxide Synthase**

**Erk- Extracellular Signal Regulated Kinase**  
**EXP- Experiment**  
**FBS- Fetal Bovine Serum**  
**FGF- Fibroblast Growth factor**  
**Flk-1- Foetal Liver Kinase-1**  
**HAT- Histone Acetyltransferases**  
**HDACs- Histone Deacetylases**  
**HDMECs- Human Dermal Microvascular Endothelial Cells**  
**Hes- Hairy/Enhancer of Split**  
**HGF- Hepatocyte Growth Factor**  
**HIF- Hypoxia Inducible Factor**  
**HPRT1- Hypoxanthine Phosphoribosyl transferase 1**  
**HUVECs- Human Umbilical Vein Endothelial Cells**  
**ICAM- Intracellular Adhesion Molecule**  
**Ig- Immunoglobulin**  
**IHD- Ischemic heart disease**  
**Jag- Jagged**  
**kDa- Kilo Dalton**  
**KO- Knock-out**  
**MAGUK- Membrane Associated Guanylate Kinase**  
**MAML- Mastermind like**  
**MAPK- MAP kinase**  
**MAPK- Mitogen Activated Protein Kinase**  
**MAPKK- MAP Kinase Kinase**  
**MAPKKK- MAP Kinase Kinase Kinase**  
**mg- milligram**  
**mL- Millilitre**  
**MMPs- Matrix Metalloproteinases**  
**mRNA- Messenger Ribonucleic Acid**  
**MW- Molecular Weight**

**Na<sup>2+</sup>/K<sup>+</sup> ATPase- Sodium Potassium ATPase**

**NFAT- Nuclear Factor of Activated T-Cells**

**NECD- Notch Extra Cellular Domain**

**ng- nanogram**

**NICD- Notch Intra Cellular Domain**

**nNOS- Neural Nitric Oxide Synthase**

**NO- Nitric Oxide**

**ns- Non-significant**

**PAD- Peripheral Arterial Disease**

**PAGE- Polyacrylamide gel electrophoresis**

**PBS- Phosphate Buffered Saline**

**PCR- Polymerase Chain Reaction**

**PDGF-B- Platelet Derived Growth Factor-B**

**PDGFR – Platelet Derived Growth Factor Receptor**

**PDR- Proliferative Diabetic Retinopathy**

**PDZ- Post Synaptic Density, Drosophila Disc Large Tumour  
Suppressor and Zona Occludens-1**

**PE/Ang II- Phenylephrine/ Angiotensin**

**PGI<sub>2</sub>- Prostaglandin**

**pH- Potential of Hydrogen**

**PI3K- Phosphatidylinositol-3 Kinase**

**PISP- PMCA Interacting Single-PDZ Domain**

**PKA- Protein Kinase A**

**PKC- Protein Kinase C**

**PIGF- Placental Growth Factor**

**PMCA- Plasma membrane calcium ATPase**

**POFUT 1- Protein O-fucosyl transferase**

**PVDF- Polyvinylidene Difluoride**

**qPCR- Quantitative Polymerase Chain Reaction**

**RASSF1- Ras-Associated Factor 1**

**RCAN1- Regulator of Calcineurin 1**

**RPM- Rotations per minute**

**RT PCR- Real time Polymerase Chain Reaction**

**SCs- Stalk Cells**

**SD- Standard Deviation**

**SDS- Sodium Dodecyl Sulphate**

**SE- Standard Error of Mean**

**SELE- Selectin E**

**SELL- Selectin L**

**SELP- Selectin P**

**SERCA - Sarcoplasmic Endoplasmic Reticulum Calcium ATPase**

**siRNA- Small Interfering RNA**

**svVEGF- Snake venom Vascular Endothelial Growth Factor**

**TAD- Transactivation Domain**

**TBS- Tris Buffered Saline**

**TC- Tip cell**

**TEMED- N,N,N',N'-Tetramethylethylenediamine**

**TGF $\beta$ -1- Transforming Growth Factor-B1**

**TIMP2- Tissue inhibitor of metalloproteinase-2**

**TM- transmembrane**

**TSP-1- Thrombospondin-1**

**VCAM-1- Vascular Cell Adhesion Molecule- 1**

**VEGF- Vascular Endothelial Growth Factor**

**VEGFR- Vascular Endothelial Growth Factor Receptor**

**VMSC- Vascular smooth muscle cells**

**WB- Western Blot**

**WT- Wildtype**

## **ABSTRACT**

**INTRODUCTION:** Angiogenesis, the formation of new blood vessels from pre-existing ones. It is a tightly regulated processes involving pro- and anti-angiogenic molecules. Deregulation of this process is associated with aberrant blood vessel formation (excessive or insufficient) in several human pathologies. Among the many pro-angiogenic factors promoting angiogenesis, the vascular endothelial growth factor (VEGF) has been characterised as a major regulator of both physiological and pathological angiogenesis. Therefore, the characterisation of the molecular mechanisms that regulate VEGF-induced angiogenesis is essential to develop therapeutic strategies that correct abnormal angiogenesis. In this sense, our group has previously reported a negative role for the Plasma Membrane Calcium ATPase 4 (PMCA4) protein in endothelial cells, acting *via* inhibition of the pro-angiogenic calcineurin/NFAT signalling pathway. However, we hypothesise that other intracellular pathways might be regulated by PMCA4 in endothelial cells during VEGF stimulation of angiogenesis.

**METHODS:** To identify PMCA4 regulated pro-angiogenic signalling pathways, we have screened gene arrays related to Notch signalling or extracellular matrix-Cell Adhesion Molecule (ECM-CAM) pathway using RNA isolated from PMCA4-silenced (or control) HUVEC. Changes in gene expression after PMCA4 knockdown have been further validated by TaqMan-based qPCR in HUVEC or HDMEC. RNA levels of *PMCA4* in aging HUVEC were analysed by TaqMan qPCR using RNA isolated from HUVEC cultured from different passages (from 3 to 15 passages).

**RESULTS:** siRNA-mediated *PMCA4* knockdown led to increased expression of Notch ligand *DLL1* and Notch target gene *Hey1* in VEGF-stimulated

HUVEC. Expression of the transcription factor *c-Fos* was also elevated after PMCA4 knockdown in HUVEC stimulated with VEGF for 1h. Analysis of a gene array containing genes encoding extracellular matrix and cell adhesion molecules revealed that *PMCA4* silencing alters the basal expression of *P-Selectin* and *L-Selectin* in HUVEC. The expression of other genes in the array like, *ADAMTS-1*, *E-Selectin*, and *VCAM-1*, was affected by lack of PMCA4, but only when cells were stimulated with VEGF. Examination of changes in the expression of these genes in *PMCA4*-silenced HUVEC or HDMEC showed differences indicating that PMCA4 might differentially regulate these genes in different sub-types of endothelial cells. In conclusion, our results suggest that PMCA4 negatively regulates Notch signaling pathway, and it is required for proper synthesis of ECM-CAM molecules.

A first step to investigate the expression of PMCA4 in endothelial cells during aging has shown that *PMCA4* mRNA levels increase along cell culture passage in HUVEC. However, this initial result requires further verification of changes in PMCA4 protein levels and/or in other cellular types to conclude that *PMCA4* expression increases with aging.

## **1. INTRODUCTION**

## **BACKGROUND**

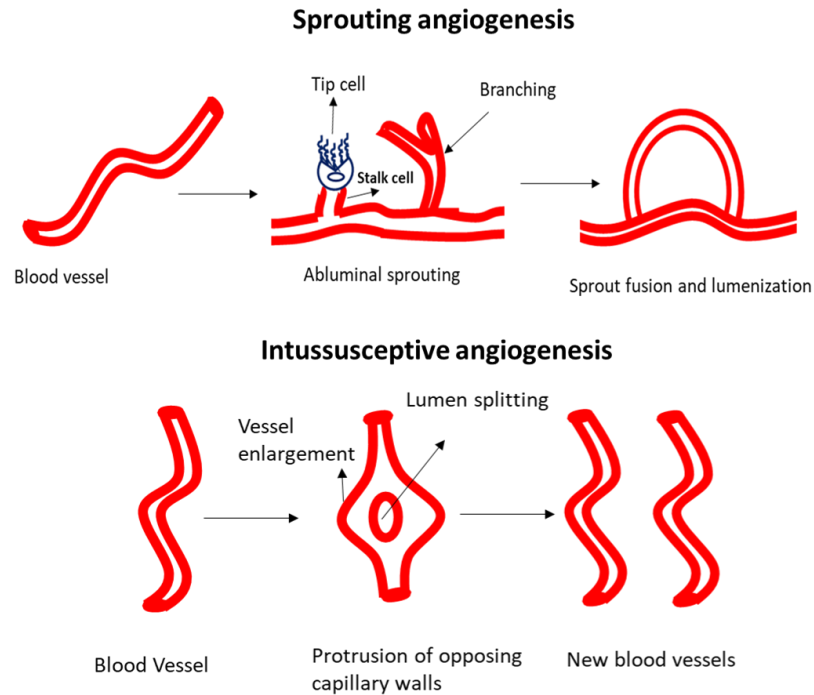
During embryonic development, a simple vascular system originates by de novo synthesis from mesoderm-derived endothelial precursor cells (angioblasts) that give rise to a first network of blood vessels called primary capillary plexus. This process is known as vasculogenesis (Herbert and Stainier, 2011; Semenza, 2007). Remodelling of this primitive plexus into a complex functional circulatory system takes place by angiogenesis, that is defined as the formation of new blood vessels from pre-existing vasculature (Herbert and Stainier, 2011; Semenza, 2007). In physiological conditions, angiogenesis is tightly controlled by a balance in the synthesis of pro-angiogenic and anti-angiogenic growth factors (Iruela-Arispe and Dvorak, 1997). However, imbalance between these factors leads to abnormal blood vessel formation, sometimes excessive, others insufficient, which has been associated with severe human pathologies (Carmeliet, 2003).

### **1.1 PHYSIOLOGICAL ANGIOGENESIS**

#### **1.1.1.a Angiogenesis in embryonic development**

Angiogenesis is the basic unit of physiological remodelling processes required for development, growth, and repair in the developing embryo (Coultas *et al.*, 2005). Human embryogenesis is a complex, multi-dimensional and heterogeneous process. Various mediators of angiogenesis such as vascular endothelial growth factor (VEGF) (Apte *et al.*, 2019), acidic/basic fibroblast growth factor (aFGF/bFGF) (Fu *et al.*, 1991) and platelet derived growth factor (PDGF) (Betsholtz, 2003) have been implicated in embryonic angiogenesis. Among them, VEGF and its receptors stand out as critical regulators of this process. Targeted deletion of *vegf* gene in mouse causes embryonic lethality

(Carmeliet *et al.*, 1996), even in heterozygous embryos (Hiratsuka *et al.*, 2005). Haploinsufficiency suggest that an appropriate dosage of VEGF is essential for proper vascular development, highlighting the importance of this factor. As it will be described in detail later, VEGF is a homodimeric glycoprotein which induces endothelial cell (EC) proliferation, migration, differentiation, and permeabilization in the growing embryo in response to hypoxic micro-environment (Sciorio and Smith, 2019). Hypoxia is a strong driving factor of angiogenesis (Sciorio and Smith, 2019). Oxygen metabolism plays an important role in the developing embryo. Oxygen levels in the developing embryo are extremely low (<3%-Hypoxia), which is critical in stimulating the angioblasts to divide and form the primary vascular plexus that later will be remodelled to form functional blood vessels in the embryo (Sciorio and Smith, 2019; Sherer and Abulafia, 2001). The endothelial basement membrane is degraded in response to VEGF, and new blood vessels are formed towards the region of hypoxia by sprouting or intussusceptive angiogenesis. Sprouting angiogenesis is initiated by basement membrane degradation, mural cell detachment, EC tip/stalk cell determination, EC proliferation, migration and finally establishment of vessel homeostasis (Duran *et al.*, 2017) (Figure 1.1.i). It is controlled by activation of conserved signalling pathways. Among them, NOTCH signalling pathway is particularly important in regulating the tip/stalk cell response mediated by VEGF gradient (Ferrara *et al.*, 2003). During the absence of VEGF gradient, intussusceptive angiogenesis occurs by circumferential enlargement of vessels followed by luminal wall splitting and tissue insertion (Uccelli *et al.*, 2019) (Figure 1.1.i).



**Figure 1.1.i Diagrammatic representation of the two types of angiogenesis.** In sprouting angiogenesis, the new blood vessels are formed from pre-existing vasculature with the help of tip cells and trailing stalk cells which fuses together to form nascent blood vessel. In intussusceptive angiogenesis, lumen is split into two to form new blood vessels.

### 1.1.1.b Angiogenesis in wound healing

In response to tissue injury, the ECs migrate forming capillary vessels and granulation tissue to supply oxygen and nutrition to the affected area (Li *et al.*, 2003). Wound healing is a three-phase process which involves inflammation, proliferation, and extra cellular matrix remodelling. A number of cells participate in wound healing like fibroblasts, smooth muscle cells, endothelial cells, immune cells, etc (Li *et al.*, 2003; Shah *et al.*, 2012). Most potent regulators of tissue remodelling in this dynamic process are VEGF (Bao *et al.*, 2009), fibroblast growth factors, and angiopoietins (Li *et al.*, 2003; Tonnesen *et al.*, 2000) which are secreted by wide range of cell types and tissues. While VEGF induces vascular permeability or hyperpermeability leading to collagen

deposition (Bao *et al.*, 2009; Takahashi and Shibuya, 2005). Ang-1 promotes tight non-leaky vasculature *in vivo* (Li *et al.*, 2003). FGF promotes fibrin deposition at the site of tissue ablation leading to clot formation which aids in the migration of ECs through the clot (Grazul-Bilska *et al.*, 2003). There are numerous signalling cascade mechanisms and interactions occurring between the ECs and the extra cellular matrix (ECM) components which provides a provisional scaffold required during the process of healing (Li *et al.*, 2003). At the first instance the wound develops a matrix immediately within few hours after injury which is followed by inflammation induced by cytokines. The inflammatory phase involves neutrophil and monocyte recruitment. During the proliferative phase, capillary sprouting and neovascularization occurs where the extra cellular matrix components stabilize the newly formed blood vessels (Shah *et al.*, 2012).

#### **1.1.1.c Angiogenesis in the female reproductive system**

Angiogenesis is important in female reproductive system during decidualization which is accompanied by vascularization of stromal bed during early pregnancy (Okada *et al.*, 2018; Redmer and Reynolds, 1996). Female reproductive organs serve as an excellent model to study the periodic growth, differentiation, and regression of blood vessels (Reynolds *et al.*, 1992). Pro-angiogenic and anti-angiogenic regulators are produced by uterine, placenta, corpus luteum, and ovarian tissues which helps in extra-cellular matrix remodelling (Reynolds *et al.*, 1992). Endometrial vascularization is aided by several key angiogenic regulators like VEGF (Reynolds *et al.*, 1992), relaxin (Parry and Vodstrcil, 2007), fibroblast growth factor (FGF) (Estienne and Price, 2018), etc. Altered expression of VEGF or FGF has shown to result in

several pathologies in the female reproductive system like endometriosis (Danastas *et al.*, 2018), ovarian carcinoma, failed implantation, etc (Reynolds *et al.*, 2002). Angiogenesis is required for regeneration of the endometrial layer after shredding in menstrual cycle. The menstrual life cycle consists of three phases: the menstrual phase, proliferative phase, and the secretory phase (Punyadeera *et al.*, 2006). VEGF is highly expressed in the menstrual and early proliferative phase, but levels decline in the late proliferative phase (Punyadeera *et al.*, 2006). Changes in VEGF expression during the different phases of the menstrual cycle underlie the relevance of angiogenesis in this process (Punyadeera *et al.*, 2006).

## **1.2 PATHOLOGICAL ANGIOGENESIS**

Abnormal formation of blood vessels due to excessive or insufficient angiogenesis is associated to several human pathologies (Ribatti D., 2005). Furthermore, VEGF is known to mediate vascular permeability leading to a leaky vascular environment and infiltration of inflammatory cells that is also present in many vascular pathologies (Ribatti D., 2005).

### **1.2.1. PATHOLOGY ASSOCIATED WITH EXCESSIVE ANGIOGENESIS**

#### **1.2.1.a Tumor angiogenesis**

Tumor cell populations are heterogeneous in nature consisting of stromal cells, extracellular matrix components and infiltrated vasculature (Pradeep *et al.*, 2005). These cells require access to the vasculature for oxygen and nutrients due to active proliferation. They acquire oxygen either by preying on the nearby non-malignant cells (termed as 'co-option') (Kuczynski *et al.*, 2019) or by sprouting angiogenesis initiated by hypoxia that leads to the formation

of new blood vessels from pre-existing ones (Lugano *et al.*, 2020). VEGF-A is highly expressed in almost all tumor types, induced by a number of factors like low pH, hypoxia, inflammatory cytokines, hormones or due to inactivation of tumor suppressor genes (Kerbel, 2008). It has been described that VEGF receptors 1 and 2 play a major regulatory role in EC barrier function. Blockade of these receptors leads to less tumor extravasation *in vivo* (Lee *et al.*, 2010). Thus, targeting VEGF and/or its receptor has a valuable potential as an anti-tumor therapeutic tool. However, anti-VEGF/VEGF-R approaches have only shown limited benefits in patients so far. Trials using Bevacizumab, an anti-VEGF human monoclonal antibody that blocks binding of VEGF to its receptor, only showed improved survival of 4-5 months in patients with advanced colorectal cancer. After an initial brief period of regression, a high percentage of patients develop resistance to the anti-VEGF therapy which may be associated with the expression of other pro-angiogenic factors such as bFGF (Bergers and Hanahan, 2008; Mitsuhashi *et al.*, 2015). Moreover, another problem of anti-VEGF therapy is that the drugs not only act on tumor cells but also disrupt the physiological availability of VEGF required for normal functions. Blockage of the physiological effects of VEGF on the cardiovascular system resulted in severe side effects in patients. Established evidence has proven that VEGF is arterio-protective and maintains vascular integrity as it stimulates synthesis of endothelial NO and PGI<sub>2</sub> (Laitinen *et al.*, 1997). Tumor patients receiving Bevacizumab in combination with standard chemotherapy show increased blood pressure and cardiotoxicity when compared with those receiving standard care alone (Laitinen *et al.*, 1997). Anti-VEGF drugs also have thromboembolic activity which is likely due to depletion of VEGF leading

to low production of NO and PGI<sub>2</sub> levels endogenously (Laitinen *et al.*, 1997; Wheeler-Jones *et al.*, 1997). Therefore, a full understanding of the molecular mechanisms implicated in the response of endothelial cells during tumoral angiogenesis is required to design proper anti-angiogenic therapies to treat tumor progression.

#### **1.2.1.b Diabetic retinopathy**

Diabetic retinopathy is a leading cause of ocular disease leading to progressive vision loss in patients over 65 years. Investigations into the biological processes underlying the pathogenesis of this complication have shown that chronic exposure of diabetic eye to hyperglycaemia triggers a number of biochemical events that resulted in retinal endothelial dysfunction (Fong *et al.*, 2004). Progression to the most severe, vision-threatening forms of retinopathy is characterised by an increase in vascular permeability and retinal neovascularisation, leading to diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR), respectively (Fong *et al.*, 2004). Evidence from several groups demonstrated that levels of VEGF are elevated in the vitreous and aqueous fluids of diabetic patients diagnosed with PDR or DME (Aiello *et al.*, 1994; Adamis *et al.*, 1994). It is now well established that VEGF plays a critical role in the formation of new, highly permeable blood vessels that represent the hallmark of diabetic retinopathy (Cheung *et al.*, 2010). As described more in detail later in section 1.5.1, alternative splicing of the *VEGF* gene generates several VEGF isoforms. VEGF<sub>121</sub> and VEGF<sub>165</sub> are the major isoforms expressed in healthy eyes (Kim *et al.*, 1999). Both VEGF<sub>121</sub> and VEGF<sub>165</sub> induce vascular permeability and endothelial cell proliferation (Gengrinovitch *et al.*, 1995). Experiments in animal models have

shown that VEGF165 is approximately twice as abundant as VEGF121 during physiological retinal vascularisation, however ischemia-induced pathological retinal neovascularisation is associated to a 25-fold increase in the expression of VEGF165 (with respect to VEGF121 expression), suggesting that VEGF165 is the main isoform implicated in the progression of pathological ocular neovascularisation (Ishida *et al.*, 2003). Anti-VEGF therapies using monoclonal antibodies Bevacizumab (Minnella *et al.*, 2008) and Ranibizumab, or the aptamer Pegaptanib (Klettner and Roider, 2008) (an anti-VEGF agent that specifically inhibits the isoform VEGF165) showed benefits to treat aggressive disease but disease progression was observed after two years (Klettner and Roider, 2008). Although anti-angiogenic therapies show enormous potential, there is a clear need to improve current treatments for the treatment of diabetic retinopathies.

## **1.2.2. PATHOLOGY ASSOCIATED WITH INSUFFICIENT ANGIOGENESIS**

### **1.2.2.a Ischemic heart disease**

Ischemic heart disease (IHD) is caused by insufficient or no supply of oxygen to the heart due to narrowing of cardiac blood vessels due to clots or plaque deposition (atherosclerosis) which may cause the death of cardiomyocytes, fibroblast and endothelial cells, and lead to cardiac hypertrophy and heart failure. Although several pharmacological interventions like coronary bypass, grafting, or angioplasty exist, the chances of recurrent heart failure are higher in patients with ischemic heart disease. These patients may be benefited from therapeutic strategies involving angiogenic activator molecules delivered by tailored methods to supply blood to the myocardium at risk (Koransky *et al.*,

2002). VEGF is reported to have anti-thrombolytic properties. It prevents endogenous and exogenous coagulation within a blood vessel. It increases the level of nitric oxide (NO) and prostacyclin (PGI<sub>2</sub>) within the blood vessel (Laitinen *et al.*, 1997; Wheeler-Jones *et al.*, 1997). NO is a potent inhibitor of platelet aggregation (Suchyta *et al.*, 2014). VEGF also promotes the expression of serine proteases, which helps in production of plasmin leading to thrombolysis (Hui-Liang Zhang *et al.*, 2017; Ambra *et al.*, 2019). VEGFA/VEGF-R plays an important role in the cardiac response during early events of myocardial infarction (MI) (Zhao *et al.*, 2010; Ucuzian *et al.*, 2010). Studies in rat myocardium showed VEGF mRNA and protein levels increase in the infarct border zone at day 1 post-MI and decrease thereafter. In the infarcted region of the heart VEGF levels gradually reduces after day 1 up to 4 weeks. VEGF-R density in the border zone increase during day 1 and returned to normalcy at day 3 whereas in the infarcted heart, it decreased during day 1 and returned to normal afterwards. VEGF-A and VEGF-R levels are reduced in newly generated micro vessels in the infarcted heart post MI. However, VEGFR levels returned to normalcy after day 1 whereas VEGF levels remain low suggesting that VEGF levels are critical in stimulation of angiogenesis during early stage and not implicated at later stages of the cardiac response to MI (Zhao *et al.*, 2010). These studies have highlighted the involvement of VEGF and its receptor in the post-MI cardiac response. Thus, therapeutic approaches to deliver VEGF to the ischaemic heart have attracted huge attention as potential strategies to increase cardiac reperfusion after MI events. Protein therapy to deliver pro-angiogenic cytokines like FGF-2 or VEGF-A via intra-venous or intracoronary introduction are in Phase I/II trials

(Chu and Wang, 2012). Pre-clinical trials in stress induced ischemic animal models using gene therapy (plasmids or adenovirus) showed significant improvement in perfusion and heart function (Chu and Wang, 2012). Although, these methods show vascular perfusion, their efficacy remains low.

### **1.2.2.b Peripheral arterial disease**

Peripheral Arterial Disease (PAD) is an atherosclerotic disease mainly caused by plaque deposition lining the vascular bed. It is characterised by occlusive blood flow to the lower extremities due to narrowing of arteries (Olson and Treat-Jacobson, 2004). People with diabetes, hypertension, hyperlipidaemia are at increased risk of this disease (Khattab *et al.*, 2005). The risk of PAD increases with age, affecting both men and woman older than 60 years (Falconer *et al.*, 2008). It can be either symptomatic or asymptomatic both requiring treatment against cardiovascular complications and relief of symptoms like intermittent claudication leading to Critical limb ischemia (CLI) and amputation (Olson and Treat-Jacobson, 2004; Collins *et al.*, 2005). Symptomatic PAD is characterised by intermittent claudication 'limping' causing severe cramping of leg muscles due to insufficient flow of blood supply to meet with metabolic demand (Collins *et al.*, 2005). Over 20-30% of people with PAD have diabetes mellitus and are at 10 to 20-fold risk to develop CLI (Thiruvoipati *et al.*, 2015; Hochberg *et al.*, 2001). VEGF has been identified as a critical inducer of collateral blood vessels in the occluded area of peripheral arteries. VEGF-165 delivery in animals induced by gene therapy or protein therapy showed increase reperfusion in models of experimentally induced hindlimb ischemia (Chu and Wang, 2012). However, over-expression of VEGF-165 in lower limb ischemic mouse models induces abnormal leaky

vasculature and tissue inflammation (Hochberg *et al.*, 2001; Chu and Wang, 2012). Phase I/II clinical trials of gene therapy *via* delivery of adenovirus carrying *vegf* or *fgf* showed promising safety but not satisfactory efficacy (Chu and Wang, 2012). Intra-muscular gene therapy of VEGF-165 is given to patients with severe chronic lower limb ischemia who failed to meet with conventional therapy. Promising therapeutic effects were observed in patients with long term dose dependent treatment of plasmids carrying VEGF-165 gene although clinical benefit was not achieved (Anghel *et al.*, 2007). All these studies indicate that despite extensive research in this area, the promising results obtained in preclinical settings have not been translated into clinical benefits for patients. Hochberg *et al.*, 2001 have shown that diabetic patients with CLI present higher levels of VEGF than diabetic patients without CLI, suggesting that production of VEGF is not a limiting factor in diabetic patients with CLI and might be the reason why VEGF-based therapeutic approaches have not yielded any clinical benefits. Therapeutic approaches that deliver alternative pro-angiogenic factors are currently being investigated in this area.

#### **1.2.2.c CLINICAL DEVELOPMENT IN PRO/ANTI-ANGIOGENIC THERAPIES**

As we look at further sections of this thesis, VEGF is an important pro-angiogenic factor studied widely with regards to its angiogenic stimulating property. A number of clinical studies involving anti-VEGF molecules to target life threatening diseases exist but in all cases the patients suffer disadvantages. For example, tumor progression after a stable progression free survival may be due to the involvement of other proangiogenic factors or alternative survival pathways. Therefore, combination therapies with chemotherapeutic drugs or radiotherapy or even gene therapy delivering more

than two genes to target pathologies are gaining attention (Spooner *et al.*, 2019, Barc *et al.*, 2020). A clinical study involving the evaluation of safety and efficacy profile of anti-VEGF drugs Aflibercept, Bevacizumab, Ranimazimumab shows comparable effects in patients with Cystoid macular Edema (CME) secondary to Branch Retinal Vein Occlusion (BRVO) (Spooner *et al.*, 2019). Treat and Extend (T&E) randomized trials are increasingly popular in ophthalmological pathologies like neovascular age related macular degeneration (AMD) or patients with diabetic macular edema (DME) (Amoaku *et al.*, 2018). LUCAS (Lucentis Compared to Avastin Study) is a Norwegian multicentre, randomized clinical trial with (n=371) subjects divided into two groups. The subjects were randomized to receive either Ranimazimumab 0.5mg (n=187) or Avastin 1.25mg (n=184) on a monthly basis. The two years study revealed that the two drugs had equivalent effect on safety and efficacy between the two groups (Berg *et al.*, 2016, Khanna *et al.*, 2019). TREND (TReat and ExtEND) study, which is a US based multicentre, randomized trial involving n=650 subjects was studied in patients with wet age-related macular degeneration. N=327 received 0.5mg Ranimazimumab (monthly) or n=323 subjects received 0.5mg Ranimazimumab based on T&E protocol. The outcome of the study showed that subjects from T&E cohort were clinically benefitting (Berg *et al.*, 2016).

While the above-mentioned studies aimed to inhibit angiogenesis, there has also been major advancements in involving VEGF as a therapeutic target to improve angiogenesis to treat life threatening pathologies like the Ischemic diseases or Peripheral Arterial Diseases (PAD). Although there are many clinical interventions like miRNA therapy, cell therapy, guidance molecule

therapy available to treat conditions related to inadequate blood supply, the use of growth factors like VEGF, HGF has gained attention due to its ease of delivery and targeting.

Protein therapy trial using recombinant VEGFA-165 has shown clinical benefits in patients with cardiovascular disease. Phase I clinical trial in patients with angina showed safety and tolerability and improved myocardial perfusion (Henry *et al.*, 2001). Pharmacokinetic analysis showed improved uptake of rhVEGF by binding to endothelial receptors and heparin proteoglycans. Phase II VIVA trial (VEGF in ischemia for vascular angiogenesis) was done in n=178 patients randomized to receive Intracoronary infusion of placebo or low dose rhVEGF or high dose rhVEGF followed by 4 hours intravenous infusion and assessed at 60 and 120 days. Results showed no significant improvement in anginal score after 60 days in placebo or low dose treated groups. However, after 120 days, at high dose of rhVEGF, there was significant improvement in anginal score with good safety and tolerability to the therapy. Although this treatment regimen is good, it suffers limitation of short half-life of VEGF (Henry *et al.*, 2003).

Gene therapy for sustained delivery of cytokines using plasmid vectors or adenovectors has shown excellent therapeutic efficiency. In a phase I clinical trial using cDNA plasmids encoding VEGFA-165, phVEGF<sub>165</sub> by Intramyocardial injection via thoracotomy guided by catheter provided feasibility in a double blinded pilot study. Following this, several clinical trials followed this treatment route like the EUROINJECT-ONE and NORTHERN clinical trials (Kastrup *et al.*, 2005; Stewart *et al.*, 2009). Both trials showed no significance between VEGF treated versus placebo treated control groups.

TALISMAN, TAMARIS trials using plasmid vectors with FGF-1 showed negative results (Nikol *et al.*, 2008). Mostly trials using plasmid vectors showed to be negative due to low transfection efficiency of the plasmids (Zachary & Morgan, 2011).

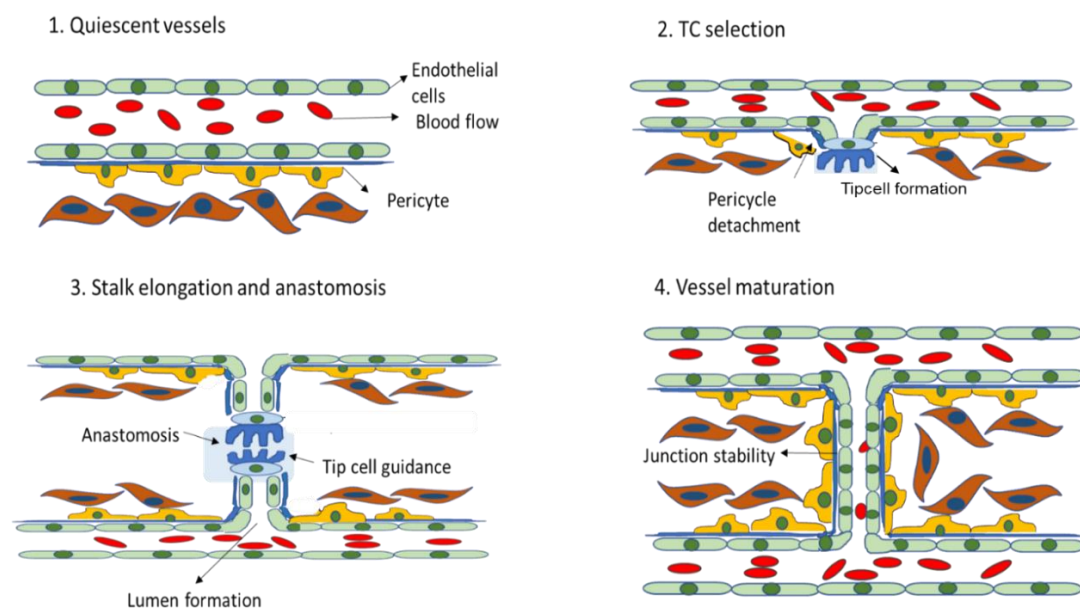
The adenoviral vectors are so far preferred for treating coronary vascular disease. In a phase I clinical trial involving n=21 subjects with Coronary Artery Disease treated with intramyocardial AdVEGF-121 via thoracotomy showed to be safe and well tolerated. REVASC trial using AdVEGF-121 intramyocardial injection via mini thoracotomy showed significant improvement in anginal symptoms in treatment group compared to control group (Stewart *et al.*, 2006). In another clinical study, patients with Critical limb Ischemia complicated by Diabetes mellitus were given an intramuscular injection of pIRES/VEGF-165/HGF which is a double VEGF/HGF gene therapy delivering the bicistronic plasmid vector. One group received the intramuscular injection while the second group was a control group and did not receive the plasmid. Based on the observation, the treatment regimen was safe and effective but is only reliable when applied at the early stage of disease progression (Barc *et al.*, 2020).

### **1.3 CELLULAR EVENTS IN ANGIOGENESIS**

ECs tightly regulate the integrity of the blood vessel wall. ECs in sprouting blood vessels have a heterogenous structure (continuous, fenestrated, or discontinuous) (Aird, 2007), phenotype (arterial, venular, capillary or lymphatic) (Phng and Gerhardt, 2009) and function (permeability, proliferation, migration) depending on its microenvironment (Aird, 2007; Phng and

Gerhardt, 2009). Sprouting angiogenesis is a complex multi-stage process that can be basically divided into 4 steps (Figure 1.3.i). The first stage is a quiescent stage where the monolayer of ECs bears tight junctions with an intact basement membrane, essential for barrier functions, and with pericytes and smooth muscle cells (Figure 1.3.i). Sprouting angiogenesis begins with activation of ECs along the pro-angiogenic gradient in response to hypoxia/injury leading to vascular dilatation which allows invasion of proteins that disrupt the endothelial basement membrane (Carmeliet and Jain, 2011). During this stage, extra cellular matrix is degraded, by proteases like those of the matrix metalloprotein family (MMPs) or suppressors of protease inhibitors like tissue inhibitor of metalloproteinase-2 (TIMP-2) (Jain, 2003) and endothelial cells lose their cell-cell junctions. Mural cells (vascular smooth muscle cells (VSMC), pericytes and fibroblasts) detach from the vessel wall. At stage 2, some ECs differentiate into "Tip cells" (TC) located at the growing end of the sprout (Figure 1.3.i). TC selection is regulated by the VEGF gradient (Jain, 2003). Tip cells guide the angiogenic sprout in the direction of the angiogenic stimulus. Every angiogenic sprout contains a single tip cell (Phng and Gerhardt, 2009; Jakobsson *et al.*, 2009). The TC is followed by "Stalk cells" (SC) which provides support to the growing sprout and forms lumen. Tip versus stalk cell selection is tightly co-ordinated by the Delta like ligand 4 (DLL4/NOTCH signalling axis (Hellstrom *et al.*, 2007). Stalk cells stop proliferating when the tip cell of a growing sprout anastomose with a tip cell originated from another sprout, which then fuse to form a nascent blood vessel (stage 3, Figure 1.3.i). The mechanism of anastomosis is thought to be spatially regulated by VEGF signalling and forms stable connections with

growing sprout by regulation of membrane localized Flt1 (mFlt1) receptor (Nesmith *et al.*, 2017). The newly formed blood vessel undergoes a series of molecular interactions between and within the layers of vascular beds (EC-EC; EC-Mural cells; EC-ECM) to form a stable network (stage 4, Figure 1.3.i) (Jain, 2003). Basement membrane reinforces, and the monolayer of endothelial cells is established and develops tight junctions. The process of angiogenesis continues until the angiogenic stimulus ceases and the mature new blood vessels attain a quiescent state (Jain, 2003).



**Figure 1.3.i. Diagrammatic representation of various stages involved in formation of sprouting angiogenesis in response to angiogenic stimuli.** 1) Quiescent Vessels: Monolayer of inactive EC with tight junctions, mural cells and basement membrane remain intact on the vessel wall; 2) Tip Cell selection: Angiogenic stimulus causes the EC to lose their cell-cell tight junctions, ECM and basement membrane degradation, pericytes and SMC detachment. Some ECs are selected as “Tip Cells”; 3) Stalk Elongation and Anastomosis: Tip cells are trailed by stalk cells that proliferate and establishes lumen, tip cells from two sprouting vessels anastomose; 4) Vessel Maturation: nascent blood vessels are stabilized by recruitment of pericytes and SMC. ECM, basement membrane and EC cell tight junctions are re-established. (adapted from Carmeliet and Jain, 2011; Herbert and Stainier, 2011).

## 1.4 REGULATORS OF ANGIOGENESIS

As we have seen in the previous section the process of angiogenesis is initiated in response to proangiogenic stimuli. Research in the last decades have identified several pro- and also anti-angiogenic factors that modulate the angiogenic process by binding to specific receptors in the membrane of ECs leading to activation of specific intracellular signalling pathways (Table 1.4.a and table 1.4.b) (Milkiewicz *et al.*, 2006).

**Table 1.4.a. STIMULATORS OF ANGIOGENESIS.** The table below shows selected molecules involved in the process of stimulating angiogenesis regulated by various molecular mechanisms.

MOLECULES	FUNCTION
<p><b>Vascular endothelial growth factor (VEGF)</b></p>	<p>VEGFA-VEGFR1/VEGFR2:EC proliferation migration, ECM degradation, tube formation, increase in vessel permeability (Rust <i>et al.</i>, 2019, Gaengel <i>et al.</i>, 2009)</p>
<p><b>Hepatocyte growth factor (HGF)</b></p>	<p><u>HGF-cmet (Epithelial/EC):</u></p> <ul style="list-style-type: none"> <li>• Activation of TF Ets-1 release HGFs to maintain a positive feedback loop on EC.</li> <li>• Regulates cell motility.</li> <li>• Negatively regulates TSP-1 (Suppressor of Angiogenesis) (Milkiewicz <i>et al.</i>, 2006).</li> <li>• EC and SMC proliferation and migration (Rust <i>et al.</i>, 2019)</li> </ul>

<p><b>Epidermal growth factor (EGF)</b></p>	<ul style="list-style-type: none"> <li>• EGF-EGFR (HER1) or HER2-4: Induces VEGF production via HIF 1<math>\alpha</math> protein synthesis (Milkiewicz <i>et al.</i>, 2006).</li> <li>• Increase epithelialization and shortened healing time for skin graft, diabetic foot ulcers (Bodnar, 2013).</li> <li>• Overexpressed in solid tumors involving in metastasis (Bodnar, 2013)</li> </ul>
<p><b>Fibroblast growth factor (FGF)</b></p>	<ul style="list-style-type: none"> <li>• FGF2/FGFR1 – Migration and differentiation of EC</li> <li>• FGF2/FGFR2 – Only migration (Milkiewicz <i>et al.</i>, 2006)</li> <li>• EC proliferation, migration, ECM remodeling (Gaengel <i>et al.</i>, 2009)</li> </ul>
<p><b>Prostaglandins (PG)</b></p>	<ul style="list-style-type: none"> <li>• PG/COX2 – EC survival &amp; migration (Milkiewicz <i>et al.</i>, 2006)</li> <li>• Synergism between PG/FGF2 signalling pathways, promotes EC proliferation (Finetti <i>et al.</i>, 2009)</li> </ul>
<p><b>Hypoxia inducible factor-1 (HIF1<math>\alpha</math>)</b></p>	<ul style="list-style-type: none"> <li>• HIF1<math>\alpha</math>/HIF1<math>\beta</math> dimerization acting as an inducer of HRE (hypoxic Response Elements)</li> <li>• Increases transcription of &gt;60 genes important in hypoxic adaption via PI3K signalling pathway (Milkiewicz <i>et al.</i>, 2006).</li> </ul>

**Table 1.4.b. INHIBITORS OF ANGIOGENESIS.** The table below shows selected regulatory molecules involved in the process of inhibiting angiogenesis controlled by various molecular mechanisms.

MOLECULES	FUNCTION
<b>Angiostatin</b>	<ul style="list-style-type: none"> <li>• Inhibits EC proliferation &amp; neovascularisation; increases apoptosis.</li> <li>• Antagonistically binds to cmet, blocking HGF/cmet signalling pathway (Milkiewicz <i>et al.</i>, 2006)</li> <li>• Angiostatin/IL-12- Anti angiogenic activity as an immune modulator (Albini <i>et al.</i>, 2009)</li> </ul>
<b>Endostatin</b>	<ul style="list-style-type: none"> <li>• Inhibits VEGF induced migration binding to VEGFR2 directly.</li> <li>• Promotes EC apoptosis.</li> <li>• Inhibits catalytic activity of MMP2 &amp; MT1-MMP decreased EC invasion (Milkiewicz <i>et al.</i>, 2006)</li> <li>• Endostatin/TSP-1- Leads to increase in TSP-1 expression, anti angiogenic (Faye <i>et al.</i>, 2009)</li> <li>• Endostatin/VEGFR-3- Inhibits lymphangiogenesis (Han <i>et al.</i>, 2021)</li> </ul>
<b>Thrombospondin</b>	<p>TSP1 &amp; TSP2</p> <ul style="list-style-type: none"> <li>• Inhibitors of angiogenic sprouting by antagonistic activity of VEGF.</li> <li>• Activates Apoptosis (Milkiewicz <i>et al.</i>, 2006).</li> </ul>

	<ul style="list-style-type: none"> <li>• TSP4/Integrin <math>\alpha</math>2/HSF-1 promote EC proliferation in Gall bladder cancer via Akt pathway (Shi <i>et al.</i>, 2021)</li> </ul>
<b>TIMP (Tissue Inhibitor of Angiogenesis)</b>	<p>TIMP-2</p> <ul style="list-style-type: none"> <li>• Inhibits EC proliferation (PTP, Shp-1 signalling pathways).</li> </ul> <p>Inhibits MMPs &amp; selecting interact with MT1-MMP (Stetler-Stevenson and Seo, 2005).</p> <p>Induce cell cycle arrest at G1 phase- Anti apoptotic activity (Brew &amp; Nagase 2010).</p>

As it has been mentioned before in sections 1.1 and 1.2, vascular endothelial growth factor (VEGF) stands out as a key pro-angiogenic factor implicated in the formation of new blood vessels in both, physiological and pathological conditions. VEGF has been used extensively in this research project to activate pro-angiogenic gene expression in endothelial cells and thus, a more detailed description of the VEGF features is included in this introduction.

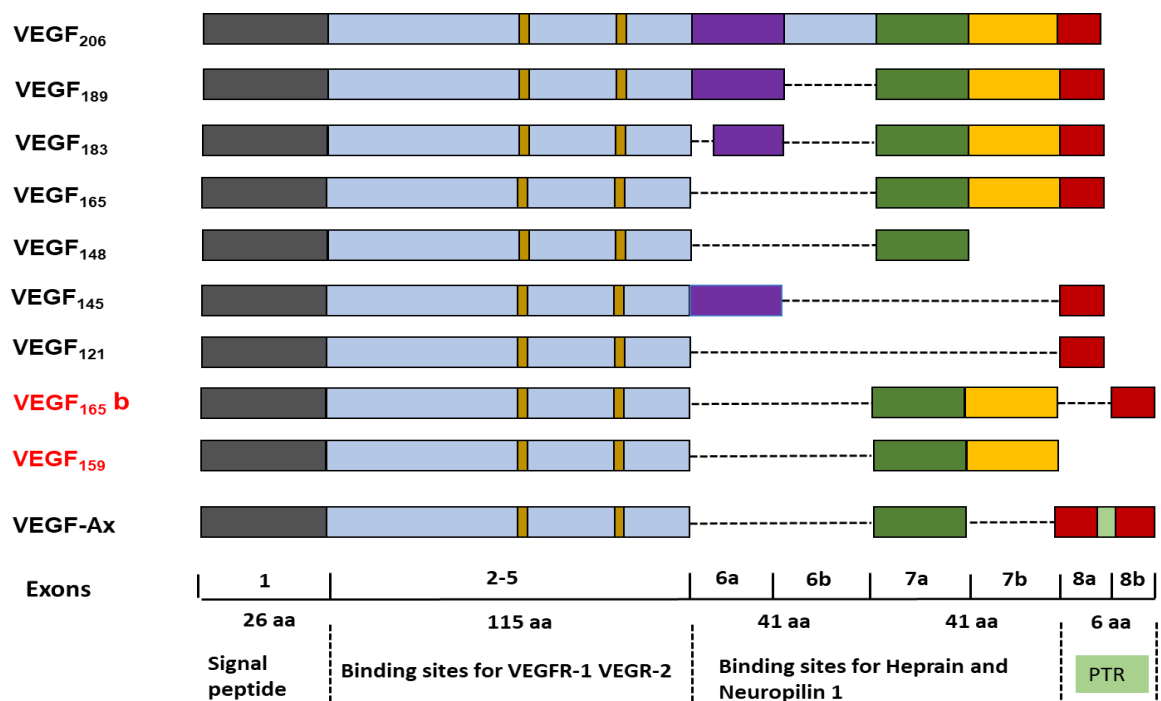
### **1.5 VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY**

The vascular endothelial growth factors belong to a protein family that comprises seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, svVEGF (snake venom VEGF) and PLGF (placental growth factor) (Takahashi and Shibuya, 2005). Among the several members of the VEGF family, VEGF-A has been identified as the major pro-angiogenic factor and is of particular interest for the study conducted in this thesis. For that reason, I focus on the description of the molecular and functional features of this member.

### 1.5.1 VEGF-A

VEGF-A is a dimeric glycoprotein that is produced by several cells and tissues. In humans, the *VEGF-A* gene spans around 14Kb with 8 exons and 7 introns. Alternate splicing of the *VEGF-A* gene into different specific combinations of functionally active exons generates distinct VEGF-A isoforms with different length (VEGF-A<sub>183</sub>, VEGF-A<sub>121</sub>, VEGF-A<sub>145</sub>, VEGF-A<sub>148</sub>, VEGF-A<sub>165</sub>, VEGF-A<sub>189</sub> and VEGF-A<sub>206</sub>) (Peach *et al.*, 2018). Some of the protein's variants synthesized by translation of these transcripts are produced as soluble proteins, whereas others can bind to the ECM that can act as a VEGF reservoir. Exons 1-5 (Figure 1.5.i) are included in all isoforms of VEGF-A. VEGF-A<sub>121</sub> lack exon 6 and 7 which encode the fragment of the VEGF protein responsible for binding to the ECM. Thus, this variant does not bind to ECM and is soluble (Grünewald *et al.*, 2010). VEGF-A<sub>145</sub>, VEGF-A<sub>183</sub>, VEGF-A<sub>189</sub> and VEGF-A<sub>206</sub> retain part or the whole of exon 6 and 7 (Figure 1.5.i). These isoforms bind to ECM with high affinity and are not freely diffusible. VEGF-A<sub>165</sub> and VEGF-A<sub>145</sub> are an intermediate between ECM-bound and freely soluble isoforms (Grünewald *et al.*, 2010; (Woolard *et al.*, 2009). Further, splicing of exon 8 can generate isoforms lacking exon 8a but containing exon 8b. These isoforms are called VEGF-A<sub>xxx</sub>b (where xxx represents amino acid length) and only differ from VEGF-A<sub>xxx</sub> in the last 6 amino acids of the C-terminus. VEGF-A<sub>xxx</sub> isoforms are associated with the physiological functions described for VEGF (pro-angiogenic properties, induction of vascular permeability and cell survival). However, VEGF-A<sub>xxx</sub>b isoforms have been reported to have anti-angiogenic properties (Peach *et al.*, 2018). An additional isoform of VEGF-A has been lately identified as VEGF-Ax. This isoform is formed due to

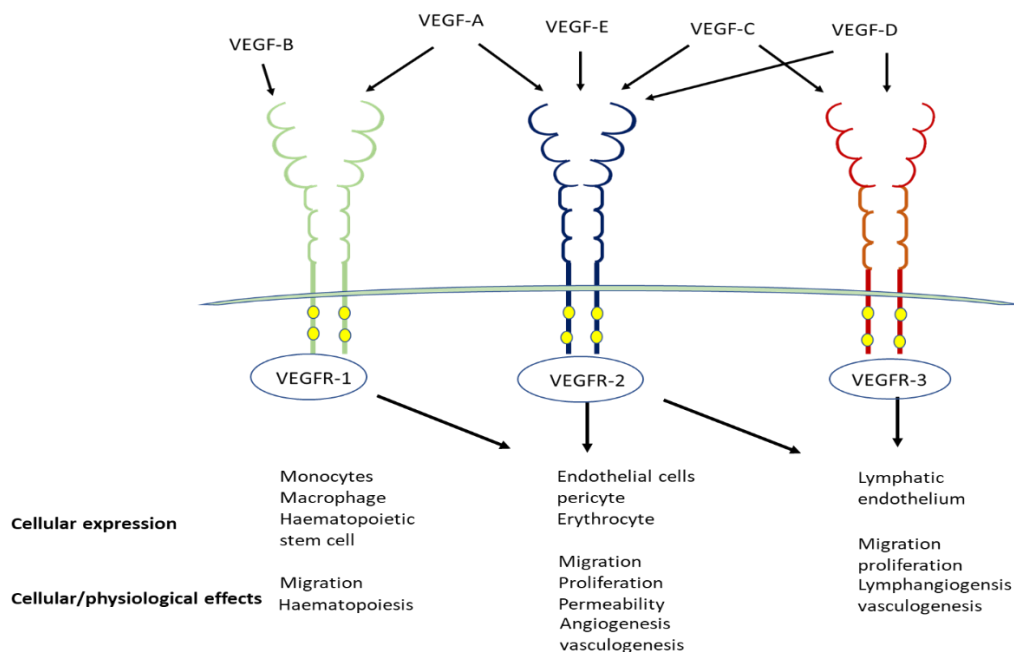
translation further to the canonical stop codon of VEGF-A isoforms located in exon 8a (Eswarappa *et al.*, 2014). Compared to VEGF-A<sub>165</sub>, RNA translation in VEGF-Ax extends further to exon 8a incorporating a new sequence of 16 amino acids and the 6 amino acids corresponding to exon 8b (Figure 1.5.i). The physiological role of VEGF-Ax has not been fully elucidated yet. Initially it was described as a VEGF-A variant with anti-angiogenic properties (Eswarappa *et al.*, 2014). However, a later work by Napoleone Ferrara's group has reported that VEGF-Ax exerts mitogenic, angiogenic and permeability enhancing functions in endothelial cells (Xin *et al.*, 2016). The reason for this discrepancy is not clear at present.



**Figure 1.5.i Exon assembly of vascular endothelial growth factor A (VEGF-A).** Exon variations in VEGF-A isoforms generated by alternative splicing along the mRNA are shown. After post-translational modification, the newly formed proteins are ready to bind to their specific receptors. (adapted from Felix S Grunewald *et al.*, 2010; Peach CJ *et al.*, 2018)

## 1.6 VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS (VEGFR)

VEGF-A isoforms bind to specific tyrosine kinase receptors (VEGFRs), which consist of an extracellular ligand binding domain, a transmembrane domain and an intracellular domain possessing inducible tyrosine kinase regions. Three different VEGFRs have been identified, VEGFR-1, VEGFR-2 and VEGFR-3, with variable ligand affinities and tyrosine kinase activity. VEGF-A binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk in mice) activating different downstream cellular responses in each case (Sakurai *et al.*, 2005). VEGF-C/VEGF-D bind to VEGFR-3 and mediate lymphangiogenesis (Figure 1.6.i).



**Figure 1.6.i Diagrammatic representation of different isoforms of VEGF receptors and their ligands.** The binding of ligands on to specific receptors leads to several cellular and physiological effects mediated by various downstream signaling pathways important in angiogenesis. (adapted from Holmes *et al.*, 2007)

### **1.6.1 VEGFR-1**

VEGFR-1 is a 180-185 kDa glycoprotein which has affinity to bind to VEGF-A or VEGF-B in its extracellular domain. Global knockout of VEGFR-1 in null *flt-1* mice causes embryonic lethality, with embryos showing overgrowth of EC and disorganized blood vessel formation in E8.5 (Sakurai *et al.*, 2005; Koch and Claesson-Welsh, 2012). These results suggest that VEGFR-1 plays a negative regulatory role in angiogenesis. In agreement with this idea, VEGFR-1 has been characterized as a decoy receptor and is not driven by VEGF gradient to promote angiogenesis. VEGFR-1 has high affinity to bind VEGF-A compared to VEGFR-2, but kinase activity is lower than its latter (Peach *et al.*, 2018). The VEGFR-1 (Flt-1) gene expresses 2 mRNAs: long (8 kb), and short form (2.5 to 3.0 kb). Short forms are expressed in the placenta and known as soluble Flt-1 (sFlt-1). They are very important to maintain barrier function of the placenta and to avoid abnormal vascular permeability (Shibuya, 2011).

### **1.6.2 VEGFR-2**

VEGFR-2 is a 151 kDa a membrane bound glycoprotein consisting of 7 extracellular immunoglobulin-like domains, a transmembrane domain, and an intracellular tyrosine kinase domain. VEGFR-2 is the key VEGF receptor of endothelial cells (Koch and Claesson-Welsh, 2012). All VEGF-A isoforms can bind VEGFR-2 (Peach *et al.*, 2018). But the activation of receptors varies in each case. Binding of VEGF-A/VEGFR-2 initiates a conformational twist or rotation of the transmembrane helix domain. Then the intracellular domain undergoes a conformational change of N and C lobes (Peach *et al.*, 2018). ATP binds to the N lobe and transactivates tyrosine phosphorylation of the C lobe. This initiates recruitment of adapter proteins which activate intracellular

signaling pathways. Phosphorylation of Y951 mediates regulation of cell adhesion, vascular permeability, and cell survival via the PI3K-AKT pathway (Peach *et al.*, 2018; Koch and Claesson-Welsh, 2012). P-Y1175 recruits PLC $\gamma$  and triggers Ca<sup>2+</sup> dependent signaling leading to EC proliferation and migration (Koch and Claesson-Welsh, 2012). VEGFR-2 can be quickly recycled to cell surface after being internalized to maintain persistent intracellular signaling, thereby regulating endosomal as well as plasma membrane signaling. VEGF/VEGFR signaling is a highly conserved mechanism which is linked with other pathways like Angiopoietin/Tie2 or DLL4/NOTCH signaling to mediate tight regulation of angiogenesis (Shibuya, 2011).

### **1.6.3 VEGFR-3**

VEGFR-3 (Flt-4) is expressed on all endothelial cells during embryonic development and becomes restricted to lymphatic endothelium in the adult (Tammela *et al.*, 2008). Upon binding to its ligands VEGF-C or VEGF-D, VEGFR-3 plays an important role in lymphangiogenesis but is not important for angiogenesis (Deng *et al.*, 2015; Zhang *et al.*, 2010). In this sense, transgenic mouse models harbouring mutant forms of VEGFR-3 (ligand binding domain/ tyrosine kinase domain) showed disrupted lymphatic growth but normal blood vessel development (Zhang *et al.*, 2010). Moreover, VEGF-C binding onto VEGFR-3 has been reported to regulate several downstream signalling pathways that are important in embryonic or post-natal lymphangiogenesis, further highlighting the major role of VEGFR-3 in lymphatic development (Krista *et al.*, 2017).

Since VEGFR-2 is one of the major receptors involved in angiogenesis, I will focus on signalling pathways mediated by VEGF-A/VEGFR-2.

## **1.7 VEGFR2-ACTIVATED SIGNALLING IN ANGIOGENESIS**

VEGFR-2 undergoes auto-phosphorylation upon binding to VEGF-A and leads to activation of downstream signalling pathways that mediate angiogenesis and cell survival (Abhinand *et al.*, 2016). Some of the most important signalling pathways activated by the VEGF-A/VEGFR-2 axis is: Extracellular Signal-Regulated Kinase (Erk) pathway, the p38 Mitogen-Activated Protein Kinase (MAPK) pathway, activation of phospholipase C, the phosphatidylinositol 3-kinase/Akt pathway, the calcineurin/Nuclear Factor of Activated T-cells (NFAT) pathway and the Notch signalling pathway.

### **1.7.1 EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) PATHWAY**

Activation of the Mitogen activated protein kinase (MAPK) signalling by VEGF-A plays a major role in EC proliferation, differentiation, and cell survival (Yoon and Seger, 2006). When VEGF binds to VEGFR-2, the intracellular kinase domain of the receptor get phosphorylated. Then, an adapter protein called GRB-2 (having SH2 binding domain) interacts with the phosphorylated tyrosine kinase domain of the intracellular domain of the VEGF receptor. GRB-2 recruits SOS, which is a guanine nucleotide exchange factor (GEF). SOS contains an allosteric binding pocket which binds Ras GTP and induces a conformational change that activates GEF activity in SOS (Jun *et al.*, 2013). SOS then removes GDP from membrane bound Ras which becomes activated and can initiate a cascade of phosphorylation events by phosphorylating MAP-KKK Raf, that in turn will phosphorylate/activate MAP-

KK MEK1/2, which finally will activate MAPK ERK1/2 (Thatcher, 2010). The Ras/Raf/MEK, ERK pathway regulates important events in endothelial cell angiogenesis by controlling the activation status of a number of transcription factors including c-myc, CREB, c-FOS, ATF2, etc which play pivotal roles in the onset/progression of angiogenesis (Armesilla *et al.*, 1999; Fearnley *et al.*, 2014; Srinivasan *et al.*, 2009). Underlying the important role of this pathway in pro-angiogenic processes, it has been reported that targeted deletion of the Ras pathway shows defective Erk 1/2 which is important in proliferation and migration in ECs in mouse embryo (Srinivasan *et al.*, 2009).

### **1.7.2 P38 MITOGEN ACTIVATED PROTEIN KINASE**

The p38 MAPK family consists of four isoforms; p38- $\alpha$ , - $\beta$ , - $\gamma$ , and - $\delta$  encoded by four different genes (Corre *et al.*, 2017). p38 $\beta$  is found in the brain, p38 $\gamma$  is expressed in skeletal muscles, and p38 $\delta$  is found in the pancreas, kidneys, testis, and small intestines. p38 $\alpha$ /MAPK14 is ubiquitously expressed and is simply referred to as p38 (38 kDa) (Corre *et al.*, 2017). p38 becomes phosphorylated by an upstream MAPKK called MEK3/6. They are heterogeneously expressed at different levels throughout all human tissues (Corre *et al.*, 2017). Targeting Y1214 residue within the VEGFR-2 tyrosine kinase domain can specifically inhibit p38 activation indicating that phosphorylation of VEGFR-2 at that amino acid plays a critical role in the subsequent molecular events that lead to VEGF-mediated activation of the p38 pathway in endothelial cells (Corre *et al.*, 2017). Several studies have showed the role of p38 in regulating hypoxia, angiogenesis and notably its importance in migration of endothelial cells via VEGFR-2 signalling (Corre *et al.*, 2017; Yoshizuka *et al.*, 2012). p38<sup>-/-</sup> knockout mouse models reveal that

p38 mediated signalling pathway is essential for HIF1 and VEGFA-165 expression. VEGFA-165 on binding to its receptor VEGFR2 results in a series of downstream phosphorylation mechanisms via VEGFR2-Nck/p38/MK2/HSP27 which leads to actin remodelling into stress fibres contributing to cell migration (Corre *et al.*, 2017; Yoshizuka *et al.*, 2012). p38 is thought to regulate the ECM degradation by regulating the proteolytic activity of metalloproteases (MMP9 and MMP2/uPA) which also implicates the p38 pathway as a regulator of angiogenesis (Corre *et al.*, 2017).

### **1.7.3 PHOSPHATIDYLINOSITOL-3 KINASE (PI3K) PATHWAY**

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that transduce intracellular signals implicated in the regulation of various cellular functions including cell proliferation, adhesion, migration, survival, and angiogenesis (Graupera *et al.*, 2008). This pathway plays an essential role in the formation of normal blood vessels during development. There are three classes (I, II and III) of PI3K proteins that have been identified. Class IA PI3K isoform has p110 catalytic subunits namely p110 $\alpha$ , p110 $\beta$ , p110 $\gamma$  or p110 $\delta$  (Graupera *et al.*, 2008; Soler *et al.*, 2013). p110 $\alpha$  and p110 $\beta$  are ubiquitously expressed and p110 $\gamma$  is expressed majorly in leucocytes. P110 $\alpha$  is highly expressed in ECs when stimulated by VEGF-A (Graupera *et al.*, 2008). Mouse embryos lacking p110 $\alpha$  catalytic subunit develop vascular defects, indicating that this subunit is essential for EC migration and angiogenesis (Graupera *et al.*, 2008; Karar and Maity, 2011). Inhibition of p110 $\alpha$  reduced the viability of tumor associated ECs serving as an excellent therapeutic target (Soler *et al.*, 2013). Transgenic mouse models lacking p110 $\alpha$  subunit showed that this subunit is required in a cell-autonomous manner to promote embryonic vascular patterning and

survival (Graupera *et al.*, 2008). Activation of PI3K/AKT pathway increases VEGF secretion in cancer cells and also modulates the expression of NO and angiopoietins (Soler *et al.*, 2013; Karar and Maity, 2011). Thus, PI3K pathway is important in both physiological and pathological blood vessel formation. Three members have been identified in class II- P13KC2 $\alpha$ , P13KC2 $\beta$ , and P13KC2 $\gamma$ , while Class III contains only one member- VpS34 (Fruman *et al.*, 1998). Although a possible role of P13KC2 $\alpha$  in blood vessel formation has been predicted in knockout mouse models, their downstream effector pathways have not been clearly studied (Yoshioka *et al.*, 2012). Mutagenic studies of class III VpS34 PI3Ks have not associated this kinase with human diseases but have shown that it is predominantly involved in membrane trafficking functions and thus this enzyme is of less relevance to angiogenesis (Jean and Kiger, 2014).

P110 $\alpha$  is an interesting target to treat occlusive vascular disease, like bypass graft stenosis or restenosis, which is characterised by migration and proliferation of Vascular Smooth Muscle cells (VSMC) induced by growth factors (Schnerer *et al.*, 2011). By blocking or inhibiting the activity of p110 $\alpha$  or p110 $\beta$  using siRNA or VSMC specific deletion, Scherner *et al.*, 2011 have demonstrated that p110 $\alpha$  but not p110 $\beta$  is crucial for growth factor-induced proliferation and migration of VSMC *in vitro* and *in vivo* (Schnerer *et al.*, 2011).

Exercise induced hypertrophy is thought to be cardioprotective due to increase in cardiac output whereas pathological hypertrophy leads to serious irreversible complications (Weeks *et al.*, 2012). It was identified that activation of phosphoinositide 3-kinase p110 $\alpha$  (PI3K(p110 $\alpha$ )) is important in regulating physiological hypertrophy. p110 $\alpha$  is a lipid kinase that catalyses the

phosphorylation of downstream signalling proteins. Study by Lin *et al*, 2010 showed a declined cardiac function in dominant negative PI3K(p110 $\alpha$ ) (dnPI3K) with myocardial infarction (MI) when compared to Ntg (non-transgenic) mice with MI. GRB14 is an adapter protein which is expressed more in the heart compared to other tissues, negatively regulates insulin receptor and FGFR-1 making it an interesting target (Lin *et al.*, 2010). Akt activation was reduced in GRB14 KO models and dnPI3K models in the heart making the animal more susceptible to myocardial infarction. Increased activity of PI3K is directly involved in maintaining fatty acid oxidation and Akt activation thereby protecting the heart against cardiac dysfunction (Lin *et al.*, 2010)

Weeks *et al.*, 2012 showed that dnPI3K mouse with attenuated PI3K levels showed faster disease progression and increased mortality rate in models of MI. Increase activity of PI3K in constitutively active phosphoinositide 3-kinase (caPI3K) models showed better cardiac function (Weeks *et al.*, 2012). HSP70 (Heat shock protein 70) had previously shown to protect the heart under stress. An interesting observation of increase in Akt activity was found in models of caPI3K when HSP70 was knocked out when compared with caPI3K under basal conditions. However, transgenic overexpression of HSP70 in dnPI3K mouse did not rescue the hypertrophic response. These results demonstrated that PI3K(p110 $\alpha$ ) protected the heart independently of HSP70 (Weeks *et al.*, 2012). Genes related to exercise-induced protection of the heart were mimicked in caPI3K models with enhanced PI3K(p110 $\alpha$ ) activity (Weeks *et al.*, 2012). rAAV6-caPI3K (recombinant adeno-associated viral 6-constitutively active phosphoinositide 3-kinase) vector specifically targeting

the cardiomyocytes showed restoration of cardiac function in TAC (Transverse aortic constriction) mouse models, 4 weeks post vector delivery demonstrating increased Akt activation when compared to control vector-TAC mouse (Weeks *et al.*, 2012).

PI3K(p110 $\alpha$ ) induces tumorigenic properties in other cell types (Weeks *et al.*, 2012). Silencing or blocking the activity of p110 $\alpha$  using selective inhibitors in several small cell lung cancer (SCLC) cell lines results in increased cell apoptosis and activation of Akt and phosphorylation of mTOR targets (Wojtalla *et al.*, 2013). Furthermore, angiogenesis experiments performed in the chick embryo chorioallantoic membrane (CAM) showed that p110 $\alpha$  inhibition impaired SCLC tumor formation and vascularization *in vivo* suggesting that pharmacological inhibition of p110 $\alpha$  stands as an excellent therapeutic target to treat tumor progression (Wojtalla *et al.*, 2013).

Evidence from literature shows that there are several signalling pathways that regulate tumorigenesis and survival of cardiomyocytes (Cheng & Force, 2010). SF-1126 and XL765 inhibitors are given to block the activity of PI3K(p110 $\alpha$ ) in cancer cells but as we saw in this section, p110 $\alpha$  is essential in restoring physiological heart growth and protection from pathological stress. Therefore, targeted delivery of anti-p110 $\alpha$  specifically to cancer cells without causing any effect in the heart, is gaining attention (Cheng & Force, 2010).

#### **1.7.4 PROTEIN KINASE C**

PKC signalling enhances angiogenic activity in endothelial cells induced by VEGF stimulation. It belongs to a family of serine/threonine kinases grouped into 3 classes: conventional PKC (cPKC) ( $\alpha$ ,  $\gamma$  and the alternatively spliced  $\beta$ I

and  $\beta$ II), novel PKC (nPKC) ( $\delta$ ,  $\epsilon$ ,  $\eta$  /L,  $\theta$ ), and atypical PKC (aPKC) ( $\eta$ ,  $\iota/\lambda$ ). Inhibition of PKC $\alpha$  in HUVECs inhibits vessel formation in *in vitro* studies and myocardial neovascularization in *in vivo* models in mice (Xu *et al.*, 2008). As we see an increase in evidence of VEGF in maintaining vascular homeostasis, this positive feedback loop is an important feature in regulating angiogenesis (Xu *et al.*, 2008). It has been demonstrated that PKC promotes angiogenesis via VEGF induction in the ECs via VEGF/VEGFR2 signalling. VEGF mRNA levels were upregulated when they were subjected to PKC activation by Phorbol-12-myristate-13-acetate (PMA) (Xu *et al.*, 2008). Interestingly the reverse effect was also observed with VEGF activating PKC $\alpha$  in HUVECs. Thus, an autocrine feedback loop is demonstrated where PKC enhances VEGF production which in turn acts like a positive feedback loop activating the PKCs in ECs promoting angiogenesis (Xu *et al.*, 2008).

#### **1.7.5 CALCINEURIN/NFAT PATHWAY**

Calcineurin (CaN) (80kDa) is a heterodimeric protein composed of two subunits; calcineurin A (a catalytic subunit) and calcineurin B (a regulatory calcium-binding subunit) of approximately 60 kDa and 20 kDa respectively. It is a calcium and calmodulin-dependent serine/threonine phosphatase. There are three isoforms of calcineurin A ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), encoded by three different genes (Klee *et al.*, 1998). The isoforms  $\alpha$  and  $\beta$  are distributed throughout the body, while  $\gamma$  is majorly distributed in the brain and testis (Kung *et al.*, 2001). The calcium dependent activity of calcineurin is regulated by calmodulin (CaM). The enzyme is activated upon calmodulin binding to calcineurin's active sites. This activation is dependent on the Ca<sup>2+</sup> concentrations within the cell. The CaN/CaM activity is directly proportional to the Ca<sup>2+</sup> concentration (Klee *et al.*,

1998). Increase in intracellular  $\text{Ca}^{2+}$  concentration results in dephosphorylation of the best characterized substrate of CaN the transcription factor nuclear factor of activated T-cells (NFAT) which is then translocated into the nucleus (Armesilla *et al.*, 1999). NFAT belongs to a family of transcription factors (NFATp (NFAT1), NFATc (NFAT2), NFAT3, and NFAT4) which is involved in the activation of genes important in angiogenesis or inflammatory immune responses. VEGF has been reported to induce the intracellular  $\text{Ca}^{2+}$  concentrations which then activate the CaN/NFAT pathway in endothelial cells (Armesilla *et al.*, 1999). Inhibition of CaN activity with Cyclosporin A (CsA), as an Immunosuppressive drug identified as a strong inhibitor of calcineurin, had been reported to inhibit VEGF-induced angiogenic process *in vitro* and *in vivo* (Hernandez *et al.*, 2001). These studies demonstrate the relevance of the CaN/NFAT pathway in the regulation of angiogenesis and highlight the potential of manipulating CaN activity to therapeutically modulate angiogenic processes.

## **1.8 NOTCH SIGNALLING PATHWAY**

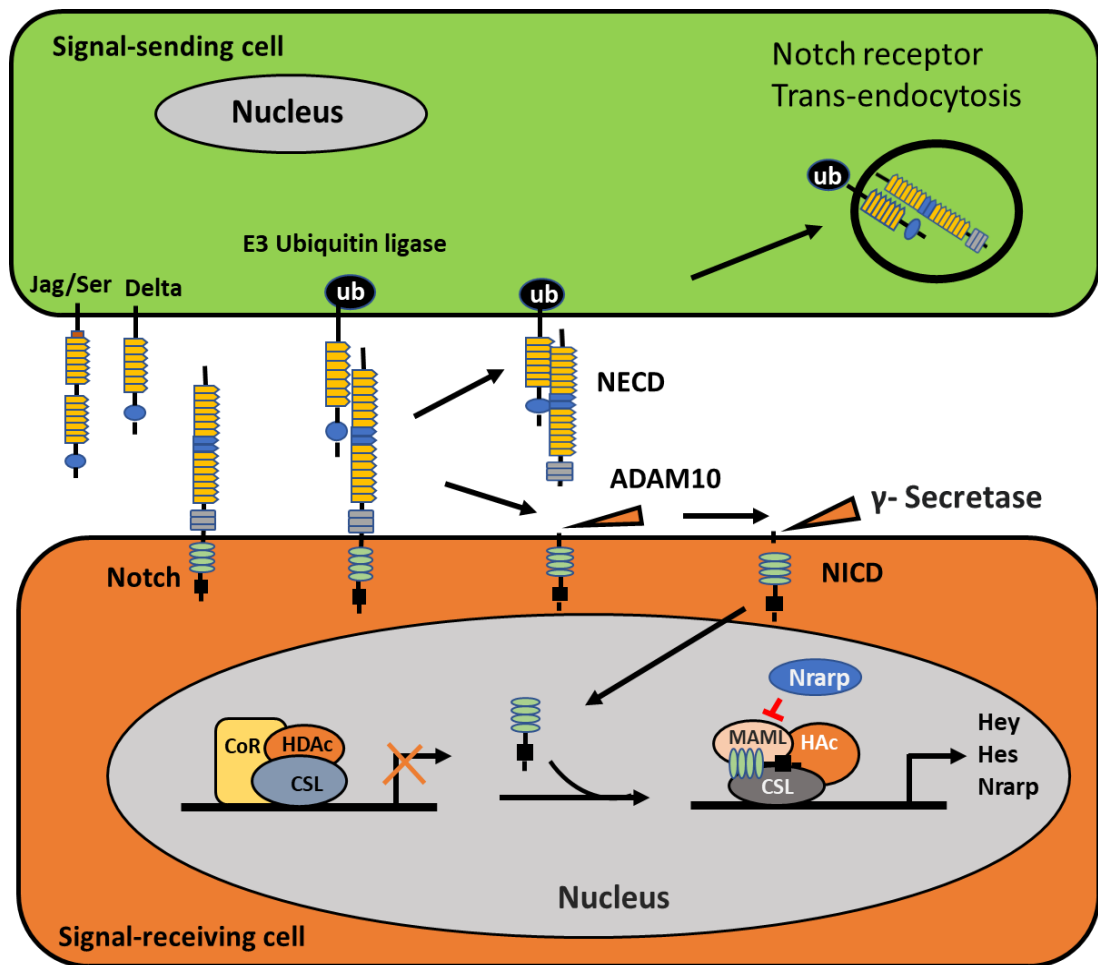
Notch signalling is a tightly regulated pathway which controls cell fate specification, growth, differentiation, and cell death in many organisms (Bray, 2006). Notch receptors are transmembrane proteins which undergo glycosylation within the endoplasmic reticulum involving the enzyme Protein O-fucosyl transferase (POFUT-1) (Roca *et al.*, 2007). The pre-protein is then processed in the trans-Golgi network by a protease called furin resulting in a molecule with a non-covalently linked extracellular domain (NECD) and a notch intracellular domain (NICD) subunit (Roca *et al.*, 2007). The NECD is made up of EGF-like repeats possessing specific sites for ligand binding.

NICD is made up of 6 tandem ankyrin repeats, glutamine rich domain and a PEST sequence in its C-terminal region (Roca *et al.*, 2007). (Figure 1.8.i). Four Notch receptors have been identified (Notch1–4) in mammals which can interact with five ligands, namely Delta-like ligand 1, Delta-like ligand 3, Delta-like ligand 4, Jagged1, and Jagged2 (Hofmann *et al.*, 2007). The ligands are collectively referred to as DSL (Delta/Serrate/Lag-2) and containing an extracellular N-terminal DSL motif that binds to the receptor, followed by a variable number of EGF-like repeats (Figure 1.8.i). In the cytoplasmic region the only feature of Notch ligands is the presence of a C-terminal PDZ binding domain (Roca *et al.*, 2007).

### **1.8.1 MECHANISM OF NOTCH SIGNALLING**

Notch signalling takes place between two cells which possess ligand or receptor on different cells (*trans*) and inhibition occurs when the ligand and receptor are present on neighbouring cell (*cis*) (Kopan, 2012) (Figure 1.8.i). Ubiquitination of notch ligand occurs with the help of a protein ligase called the E3 ubiquitin. This protein interacts with the notch ligand as well as E2 ubiquitin-conjugated enzyme, aiding in transfer of ubiquitin onto the notch ligand. (Dutta *et al.*, 2021, Kopan, 2012). Upon binding of ligand to its receptor, the juxtamembrane negative control region (NRR) recruits ADAM10 which cleaves the receptor at the S2 site. The ubiquitin catalyzes lysosomal endocytosis of the ADAM10 cleaved notch ligand-notch ECD receptor complex. Further cleavage in site S3 occurs with the help of  $\gamma$ -secretase, and results in the release of the NICD (Kopan, 2012). Interestingly, two distinct types of cleavage at the S3 site have been identified in Notch1 after activation. The N-terminal S3-V cleavage with a valine end produces a stable NICD and

a strong transcription regulation (Tagami *et al.*, 2008). Whereas S3-S or S3-L with serine/leucine terminus generates unstable NICD resulting in a weaker intracellular signal (Tagami *et al.*, 2008). The NICD translocates into the nucleus and binds to a nuclear adaptor protein called CSL (CBF-1/RBPJ- $\kappa$ , Su(H), Lag-1) along with other proteins like histone deacetylases (HDACs) or Histone acetyltransferases (HATs) either causing suppression or activation of the chromatin conformation. Nuclear NICD also recruits other components of the transcription machinery such as mastermind like (MAML) and p300 (Kopan, 2012). Transcriptional activation involves phosphorylation of NICD within its PEST domain by kinases such as the cyclin-dependent kinase 8 (CDK8) kinase (Kopan, 2012). This process triggers transcription of notch target genes like Hairy/Enhancer of Split (Hes) and Hes related proteins (Hey), basic helix-loop-helix (bHLH) which further upregulate the expression of downstream signalling genes (Roca *et al.*, 2007).



**Figure 1.8.i Cellular events involved in Notch Signalling.** Angiogenic signal sending cell possess the notch ligand. Cell receiving the angiogenic signal possess notch receptor. Upon recruitment of ubiquitin by E3 ubiquitin ligase, the Notch ligand binds to the notch extracellular domain (NECD). The notch receptor is processed by a metalloprotease called ADAM10 which releases the Notch ligand-receptor ligated complex and is trans endocytosed into the ligand presenting cell.  $\gamma$  Secretase cleaves the notch intracellular domain (NICD) which is released into the cell. It is then translocated into the nucleus and transcribes genes which are important in cell proliferation and migration. Notch receptor domain showed in yellow are EGF-like repeats, some of them shown in blue are involved in ligand binding, Notch/LIN-12 domains (purple), single transmembrane domain, ankyrin repeat domain (blue) and PEST domain (black) in the C-terminal. Ligand domains shown are EGF-like repeats (yellow), transmembrane region, and an additional feature called von Williebrand factor (vWF) (red) is present in Jag/Ser ligand in its C-terminal. (Adapted from Roca C., & Adams R. H., 2007); Phng L.-K., & Gerhardt H., 2009)

## 1.8.2 NOTCH SIGNALLING AND ENDOTHELIAL CELL SPECIFICATION

Notch signalling controls multiple aspects of endothelial cell specification depending on their position and location in the vascular beds (Phng and Gerhardt, 2009). The signalling between DLL4/Notch in the endothelial cells direct towards arterial or venous phenotype. Targeted deletion of Notch-related genes in mouse or in zebrafish showed deformed arteries and veins in the mutant animals (Napp *et al.*, 2012; Siekmann and Lawson, 2007). ECs stimulated by VEGF-A compete for the tip cell position via DLL4/Notch signalling (Phng and Gerhardt, 2009). Heterozygous deletion of *dll4<sup>+/-</sup>* in a mouse model produced excessive filopodial extension in the presence of VEGF signalling (Suchting *et al.*, 2007). Decreased expression of VEGFR1 and increased expression of VEGFR2 was detected in *dll4<sup>+/-</sup>* mice suggesting that VEGF signalling in Notch-signalling is involved in the regulation of tip cell proliferation and migration (Suchting *et al.*, 2007). DLL4 has therefore been identified as a negative regulator of vessel sprouting and branching (Suchting *et al.*, 2007). The cell that produces more DLL4 becomes the tip cell (Phng and Gerhardt, 2009). In the presence of VEGF, endothelial tip cells promote lateral inhibition between the neighbouring cells and creates a non-uniform population. The stalk cells receive more notch signal and undergo transcriptional repression of VEGF receptors Kdr, Nrp-1, Flt4 while stimulating decoy receptor s(Flt-1) (Phng and Gerhardt, 2009). Regulation of VEGF-activated intracellular signaling in endothelial cells is regulated by a plethora of cellular proteins. Among these regulators, our laboratory has recently identified an important role for the Plasma Membrane Calcium ATPase 4 (PMCA4) protein as a negative regulator of the calcineurin/NFAT pathway in

endothelial cells. Given the relevance of this work for my project, the features of PMCA and its role in angiogenesis are described in detail in this introduction.

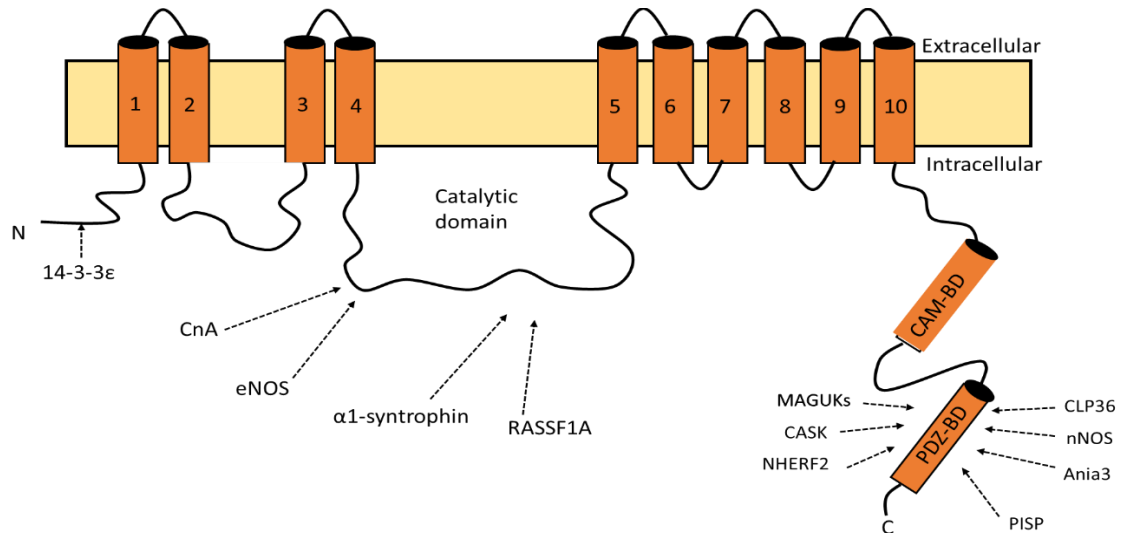
## **1.9 PLASMA MEMBRANE CALCIUM ATPase PUMP (PMCA)**

PMCA is a member of P-type ATPase family which is characterised by formation of aspartyl phosphate intermediate during its functional cycle. It has high affinity to bind  $\text{Ca}^{2+}$  ions but has lower extrusion capacity when compared to the sarcoendoplasmic reticulum (SERCA) pumps (Stafford *et al.*, 2017; Strehler and Zacharias, 2001). It releases one  $\text{Ca}^{2+}$  ion from intracellular cytosol to the extracellular space for every ATP hydrolysed. PMCA extrudes  $\text{Ca}^{2+}$  to avoid cytotoxic overload which may lead to apoptosis and cell death (Bruce., 2018). In mammals, there are 4 different isoforms of PMCA (PMCA1-4). All of them transport  $\text{Ca}^{2+}$  to the extracellular space but their affinity for calcium varies between the different isoforms.

### **1.9.1 STRUCTURE OF PMCA**

Like other types of P-type ATPase pumps, all isoforms of PMCA consist of hydrophobic transmembrane domains flanked by  $-\text{NH}_2$  and  $-\text{COOH}$  cytoplasmic tails. The putative structure of PMCA based on their sequence is illustrated in Figure 1.9.i. PMCA intracellular domain contains 80-90 amino acids. In addition to the cytoplasmic ends, PMCA has two large intracellular loops spanning transmembrane domains 2-3, known as “transducer domain”, and 4-5, which corresponds to the catalytic domain. The catalytic domain contains an autoinhibitory region regulated by interaction with a calmodulin binding site located in the  $-\text{COOH}$  terminal tail. Interaction between the C-

terminal domain and the catalytic loop of the pump renders an open or closed conformation in the presence or absence of  $\text{Ca}^{2+}$ -calmodulin complexes, and thus regulates the activity of the pump. RNA splicing in the N- and C-terminal regions gives rise to more than 20 splice variants (Stafford *et al.*, 2017).



**Figure 1.9.i Diagrammatic illustration of P-type Plasma membrane Calcium transport ATPase pump.** The  $\text{Ca}^{2+}$  transport pumps possess 10 transmembrane domains connected by 5 extracellular loops, 2 intracellular loops (loop connecting transmembrane domain 4 and 5 contains the catalytic domain with ATP binding site), and N- and C-terminal ends flanking either end of the pump inside the cell. Various interacting partners of the pump are shown. (Figure adapted from Stafford N *et al.*, 2017). CAM-BD, calmodulin binding domain; PDZ-BD, domain binding to protein PDZ-domains.

## 1.9.2 PMCA BINDING DOMAINS

**PDZ binding domains:** These binding domains are located at the end of the C-terminal tail. PDZ binding domain derives its name from the originally identified conserved sequence elements contained within postsynaptic density protein PSD95/SAP90, *Drosophila* tumor suppressor protein dlg-A, and tight junction protein ZO-1 (Fanning and Anderson, 1999). These domains possess amino acid residues which serves as a scaffold for protein interactions. In this way, PMCAs have been reported to interact with several

partner proteins, such as members of the membrane-associated guanylate kinase (MAGUK) family, neuronal nitric oxide synthase (nNOS), calcium/calmodulin-dependent serine protein kinase (CASK), Ania3, C-terminal LIM domain protein (CLP36), PMCA-interacting single-PDZ domain (PISP), etc (Stafford *et al.*, 2017; Strehler and Zacharias, 2001).

**Calmodulin binding site:** A calmodulin binding domain is located at the C-terminal intracellular domain above the PDZ binding site. The auto-inhibitory function of the pump is regulated by binding of the complex  $\text{Ca}^{2+}$ -calmodulin to this domain. The calmodulin binding domain binds to the second and the third intracellular domains of the pump generating a closed, inactive version. Increments in the concentration of cytoplasmic  $\text{Ca}^{2+}$  leads to the formation of active  $\text{Ca}^{2+}$ -calmodulin complexes that bind to the CAM-BD of the pump releasing the interaction between the intracellular domains and freeing the pump from auto-inhibition (Oceandy *et al.*, 2011).

### **1.9.3 PMCA AS A REGULATOR OF INTRACELLULAR SIGNALLING PATHWAY VIA INTERACTION WITH PARTNER PROTEINS**

PMCA is a  $\text{Ca}^{2+}$  dependent regulator of signal transduction pathways which functions by complex protein interactions. Some of the interacting protein families are common to all isoforms of PMCA whereas a few are specific. PMCA serves as a scaffold protein which anchors many interacting proteins at the plasma membrane (Stafford *et al.*, 2017). Recent studies show that, PMCA interacts and inhibits the activity of  $\text{Ca}^{2+}$ /calmodulin dependent proteins. Some of the identified functions of PMCA include organizing and regulation of  $\text{Ca}^{2+}$  efflux, modulation of NO signalling, or acting as a structural or scaffolding protein etc (Stafford *et al.*, 2017, Huang *et al.*, 1994). For

example, as described in the paper by Stafford *et al.*, 2017, PMCA4b-nNOS interaction has shown to play a pivotal role in excitable cells like neuronal cells, cardiac and smooth muscle cells. Neuronal NOS-I deficiency had shown reduced susceptibility to cerebral ischemia in mouse models (Huang *et al.*, 1994). Scuh *et al.*, 2001 demonstrated that PMCA4b is a negative regulator of nitric oxide synthase I (NOS-I). The interaction is initiated by binding of -COOH terminal of PMCA4b and PDZ domain of NOS-I. Cytoskeletal protein called  $\alpha$ 1-syntrophin forms a ternary complex with PMCA and nNOS and binds at the catalytic intracellular loop of PMCA and inhibits  $\text{Ca}^{2+}$ /calmodulin enzyme dependent synthesis of NO in the heart (Stafford *et al.*, 2017). Tumor suppressor Ras-associated factor 1 (RASSF1) was also found to interact with the second intracellular loop of PMCA which has same binding domain as that of  $\alpha$ 1-syntrophin. Thus, PMCA regulates the EGF dependent activation of ERK signalling pathway. Upon binding to RASSF1, the cell survival pathway is inhibited therefore PMCA has a role in cellular apoptosis (Armesilla *et al.*, 2004). Previous studies established that PMCA negatively regulates the endothelial nitric oxide synthase (eNOS) via its interaction with the catalytic domain of the pump (Holton *et al.*, 2010). Disruption of the PMCA-eNOS interaction showed a reduction of phosphorylation of Thr-495 of eNOS thus suggesting a negative regulatory activity. PMCA 2 and 4 had been reported to bind with eNOS at the same binding region of the catalytic domain as in the case of Calcineurin A (Holton *et al.*, 2010). Increase in intracellular  $\text{Ca}^{2+}$  levels lead to activation of PMCA which in turn functions by extruding calcium into the extracellular region. This creates a low calcium microdomain which restores PMCA interaction with eNOS and thus inhibiting NO synthesis.

Isoform  $\epsilon$  of the trafficking protein 14-3-3 interacts with NH<sub>2</sub>-tail region of PMCA4.

#### **1.9.4 CA<sup>2+</sup> TRANSPORTATION VIA PMCA PUMP**

Calcium transportation via the PMCA takes place in two conformational states namely E1 and E2. E1 state has high affinity for Ca<sup>2+</sup> binding whereas E2 phase has low affinity (Mangialavori *et al.*, 2013) In the E1 state, the pump exists in a conformation state exposing the high affinity sites for calcium binding within the cellular cytosol. Upon binding of calcium onto the intracellular catalytic domain, the protein undergoes phosphorylation by hydrolysis of ATP. ATP can bind to the protein even in the absence of Ca<sup>2+</sup> but cannot affect the reaction cycle. Ca<sup>2+</sup> is essential for conformational transition within the transmembrane helices (Mangialavori *et al.*, 2013). The pump then exists in E1-P unstable conformation state. Mg<sup>2+</sup> ion stabilizes this unstable state into E2-P stable intermediate. This configuration displays Ca<sup>2+</sup> outside the cell effecting the release into the extracellular region. Dephosphorylation of E2-P phase renders a stable inactive E1 configuration with high Ca<sup>2+</sup> binding affinity (Mangialavori *et al.*, 2013).

#### **1.9.5 PMCA ISOFORMS AND THEIR TISSUE DISTRIBUTION**

Alternative splicing at the amino (N) terminal of pre-mRNA of PMCA gives rise to x or z splice variant and splicing at carboxy (C) terminal gives rise to a, b, or d variants (Strehler and Zacharias, 2001). There are four known isoforms of PMCAs which are encoded by different genes *ATP2B1-4*. In human and mouse models, PMCAs are differentially distributed and have functional diversity in different cell types. PMCA1 and PMCA4 are ubiquitously expressed whereas PMCA2 and PMCA3 are restricted to specific cell types

(Strehler and Zacharias, 2001; Faddy *et al.*, 2008). Interestingly, the accumulation of specific isoforms within specialized type of cells was explained by Zacharias and Strehler in a study on neuronal maturation directed by transcriptional regulation of  $\text{Ca}^{2+}$  ions (Zacharias and Strehler, 1996). Thus, the regulation of PMCA expression is critical for cell survival which is directly proportional to  $\text{Ca}^{2+}$  activity (Brini and Carafoli, 2011).

#### **1.9.5.a PMCA1**

PMCA1 is a member of the P-type ATPase transport pump, which actively extrude  $\text{Ca}^{2+}$  ions into the extracellular space from cytosol. It is encoded by the gene *ATP2B1* in human and is important in maintaining  $\text{Ca}^{2+}$  homeostasis and blood pressure (Kobayashi *et al.*, 2012). *PMCA1* has been described as an important housekeeping gene. Embryonic lethality was observed in homozygous global knockout of *Atp2b1* (Okunade *et al.*, 2004). PMCA1 has an important role in maintaining blood pressure (BP) as verified in tissue-specific gene ablation studies in mice (Kobayashi *et al.*, 2012). Specific ablation of *Atp2b1* in vascular smooth muscle cell (VSMC) leads to higher systolic BP in KO animals. Intracellular calcium concentration in VSMC of KO animals was significantly increased both basal and phenylephrine-stimulated conditions highlighting the relevance of PMCA1 in the regulation of intracellular calcium levels (Kobayashi *et al.*, 2012). Interestingly, *PMCA1*<sup>+/-</sup> knockout mice did not show major alterations in BP at 6 months of age, however, increments in BP were observed in aging animals in association with arterial remodelling (Little *et al.*, 2017). The importance of PMCA1 as a regulator of blood pressure has been well established in these animal models,

however the molecular pathways implicated in this process remain unclear at present.

#### **1.9.5.b PMCA2**

PMCA2, is an important  $\text{Ca}^{2+}$  transporter protein critical for the clearance of calcium from excitable cells. A high level of PMCA2 is identified in neuronal cells, cochlear outer hair cells, and mammary cells (Empson *et al.*, 2010; Kozel *et al.*, 1998; VanHouten *et al.*, 2010). Aberrant expression of PMCA2 results in severe neuronal damage due to the lack of calcium homeostasis and is closely associated with aging and sensitivity to inflammation (Boczek *et al.*, 2019). Homozygous deletion of *Atp2b2* showed growth impairment when compared with heterozygous or wildtype mouse models with additional impairment in motor functions. (Kozel *et al.*, 1998). Heterozygous loss of *Atp2b2* in mouse shows progressive high frequency hearing impairment whereas homozygous *Atp2b2* null mutants are completely deaf (Smits *et al.*, 2019). In mammary epithelial cells, loss of PMCA2 due to weaning after lactation shows sensitisation towards apoptosis by increasing intracellular  $\text{Ca}^{2+}$  levels (VanHouten *et al.*, 2010). On the other hand, overexpression of PMCA2 causes breast cancer due to resistance towards apoptosis (VanHouten *et al.*, 2010). Overall, PMCA2 plays a role in calcium transportation involved in maintaining homeostatic balance, hearing, protecting the cells from calcium toxicity and apoptosis.

#### **1.9.5.c PMCA3**

In contrast to other isoforms of PMCA, PMCA3 has a more restricted tissue distribution to specific neurons like cerebellar parallel fiber-Purkinje neuron synapse (Boczek *et al.*, 2019). Loss of function of *ATP2B3* is involved in

sensory neuronal communication disorders but has not been extensively studied so far (Strehler, 2013; Strehler and Thayer, 2018).

#### **1.9.5.d PMCA4**

PMCA4 is ubiquitously expressed in all cell types. Among the two splice variants “a” and “b”, originated by alternative splicing in the C-terminal end, the sperm tail principally expresses “a” splice form whereas “b” splice variant is the major isoform expressed in cardiomyocytes, endothelial cells, pancreas, and epithelium (Padanyi *et al.*, 2016). Studies using genetic alteration of PMCA4 expression in animal models are starting to elucidate the involvement of PMCA4 in the pathophysiology of several diseases. The first analyses of *Atp2b4*<sup>-/-</sup> showed that mice lacking *Pmca4* were viable (Schuh *et al.*, 2004). *Pmca4* null animals showed infertility in male due to reduction in sperm motility (Schuh *et al.*, 2004). Further studies using animal models with altered expression of PMCA4 have revealed that deregulation in *PMCA4* expression is linked to cardiac pathologies. In a study by Wu *et al.*, 2009 it has been shown that the overexpression of *PMCA4b* splice variant antagonizes cardiac hypertrophy due to pressure overload by transverse aortic constriction or neuroendocrine agonist phenylephrine/Ang II (PE/Ang II) stimulation (Wu *et al.*, 2009). Prasad *et al.*, in their work showed that a double mutant mouse model generated by crossing transgenic mutant (Glu180Gly)  $\alpha$ -tropomyosin (Tm180) and a *PMCA4* KO had a protective role in cardiac hypertrophy by diminishing its calcineurin activity and showed reduced expression of L-type Ca<sup>2+</sup> ion channels. Tm180 mutation is a commonly observed cardiac hypertrophic mutation which causes increase in Ca<sup>2+</sup> sensitivity leading to impaired diastolic relaxation. Elevated levels of PMCA4, increase in pro-

fibrotic genes like collagen 1a1 (Col1a1) and connective tissue growth factor (Ctgf), increase in expression of calcineurin A (CnA) and Rcan1 are commonly observed in Tm180 related hypertrophy. It has been well established in previous studies that PMCA4 inhibits NO production. Loss of PMCA4 leads to increase in localized  $Ca^{2+}$  concentration and leads to activation of NOS. The increase in NO availability thus leads to the activation of cGMP-PKG signalling which is associated with prevention of pathological hypertrophy (Prasad *et al.*, 2014). Mohamed *et al.*, 2016 demonstrated that fibroblast specific KO of PMCA4 reduced cardiac hypertrophy by producing high levels of secreted frizzled related protein 2 (sFRP2) whereas cardiomyocyte directed KO did not show any protective response (Mohamed *et al.*, 2016). Systemic deletion of PMCA4 attenuated the hypertrophic response in the heart when the KO mouse was subjected to transverse aortic constriction (TAC) for 5 weeks inducing cardiac pressure overload (Mohamed *et al.*, 2016). Treatment with aurintricarboxylic acid (ATA) reversed cardiac hypertrophy (Mohamed *et al.*, 2016). As described previously, PMCA4 directly interacts with neuronal nitric oxide synthase nNOS and regulates NO-dependent cardiac signal transduction pathways in cardiomyocytes. This was studied in global depletion models of PMCA4 (PMCA4 KO) versus acute inhibition of PMCA4 using aurintricarboxylic acid (ATA) (Lewis *et al.*, 2018). Global ablation of PMCA4 does not alter BP, arterial structure, or arterial contractility but acute inhibition of PMCA4 using ATA showed nNOS dependent reduction in BP and arterial contractility (Lewis *et al.*, 2018).

### **1.9.6 ROLE OF PMCA4 AS AN INHIBITOR OF VEGF-INDUCED ANGIOGENESIS**

Regarding PMCA4 function in endothelial cells, previous work by our laboratory has shown that PMCA4 plays an important role as a negative regulator of VEGF-induced angiogenesis via inhibition of the calcineurin/NFAT pathway in endothelial cells (Baggot *et al.*, 2014). They demonstrated in their work that PMCA4 inhibits VEGF-driven angiogenesis via interaction with calcineurin in endothelial cells. The interaction between PMCA4 and calcineurin A, at a low calcium microdomain (due to calcium efflux activity) impaired the endothelial cell motility and blood vessel formation but not endothelial cell proliferation in response to VEGF stimulation (Baggot *et al.*, 2014). This interaction further revealed the downregulation of NFAT target genes *RCAN 1.4* and *Cox-2*. *In vivo* experiment conducted on *Pmca4<sup>+/+</sup>* or *Pmca4<sup>-/-</sup>* sham operated non-ischemic legs or femoral ligated ischemic legs showed enhanced perfusion of blood flow in *Pmca4<sup>-/-</sup>* model at day 5 and 14 after surgery when compared with WT animals. In the study, it was established that the interaction between PMCA4 and calcineurin downregulated the calcineurin/NFAT activity and thus served as an endogenous inhibitor of VEGF induced angiogenesis (Baggot *et al.*, 2014).

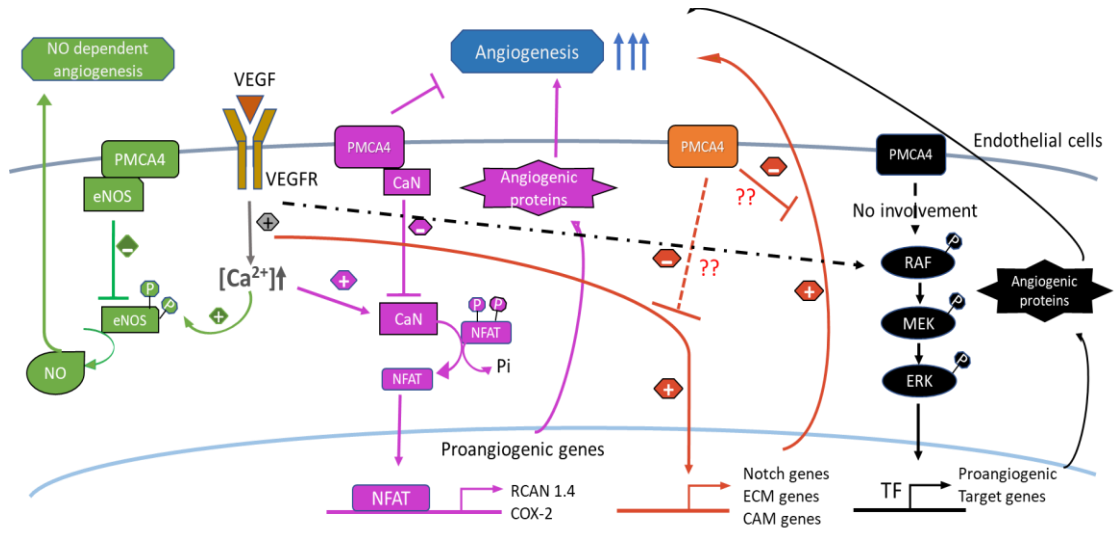
Following this, our group worked on characterising the effects in angiogenesis of a selective inhibitor of PMCA function-aurintricarboxylic acid (ATA) previously identified by Mohammed *et al.*, 2013. Kurusamy *et al.*, 2017 reported that the inhibition of PMCA4 activity by ATA lead to enhanced VEGF-mediated endothelial cell motility and blood vessel formation by releasing the calcineurin from its catalytic site. ATA attenuated VEGF-induced synthesis of

nitric oxide with an increase in interaction between PMCA4 and eNOS in endothelial cells (Kurusamy *et al.*, 2017). No side effects were observed at nanomolar levels of ATA, however, increasing micromolar concentration, resulted in embryonic toxicity in endothelial cell cultures and zebrafish *in vivo* models (Kurusamy *et al.*, 2017). Having focused on the functional relevance of the ion transport pump, the molecular mechanisms underlying PMCA4 regulation of VEGF-activated signalling pathways in endothelial cells were poorly understood which led my focus on this aspect.

## **2. AIMS AND HYPOTHESIS**

Therapeutic angiogenesis has gained attention since the discovery of pro-angiogenic factor VEGF (Zachary *et al.*, 2011). However, therapeutic strategies aimed to induce or inhibit blood vessel formation have failed to yield clinical benefits for patients suffering from diseases occurring with angiogenesis. Therefore, a full understanding of the molecular pathways that regulate angiogenesis is an essential pre-requisite to develop more efficient therapeutic interventions. It is well established that binding of VEGF-A to VEGFR2 leads to an increase in the concentration of cytoplasmic Ca<sup>2+</sup> in endothelial cells (Brock *et al.*, 1991) that activates intracellular signalling pathways such as calcineurin/NFAT, Erk, and others (Armesilla *et al.*, 1999; Yoon and Seger, 2006). Previous research in our group revealed endothelial PMCA4 as a negative regulator of VEGF driven angiogenesis *via* inhibition of the calcineurin/NFAT pathway (Baggot *et al.*, 2014; Kurusamy *et al.*, 2017). However, studies performed in non-endothelial cells have shown that PMCA4 regulates other transduction pathways, through its interaction with a plethora of cytoplasmic partner proteins (Stafford, N. *et al.*, 2017). Therefore, we hypothesise that PMCA4 might be regulating several intracellular signalling pathways in endothelial cells in its role as a negative modulator of VEGF-activated angiogenesis. In fact, VEGF-signalling is known to induce the expression of genes such as *DLL4*, extracellular matrix proteins, or cell adhesion molecules that play a critical role in the progression of angiogenesis (Fish *et al.*, 2017; Liu *et al.*, 2014). However, whether PMCA4 is involved in the regulation of these genes in endothelial cells activated with VEGF has not been studied so far. Thus, in this project, we would like to test the following hypothesis: “PMCA4 regulates VEGF-triggered intracellular signalling

pathways that are translated into the expression of critical angiogenic molecules such as components of the notch signalling pathway, extracellular matrix proteins (ECM) or cell adhesion molecules (CAM) in human endothelial cells” as illustrated in Fig 2.1.



**Figure 2.1. Diagrammatic representation of various pathways involving VEGF/VEGFR signalling.** Left to right: PMCA4 mediated NO-dependent angiogenic pathway regulated by VEGF/VEGFR given in green. CaN/NFAT pathway regulated by VEGF/VEGFR is shown in purple. Notch signalling and ECM-CAM signalling pathways activated by VEGF/VEGFR are shown in orange, gaps in knowledge about the involvement of PMCA4 in the regulation of these pathways is denoted by “?”. Extracellular Signal-Regulated Kinase (Erk) pathway regulated by VEGF/VEGFR is represented in black.

To this purpose, the following are the objectives established for this project:

- 1) To silence the expression of *PMCA4* in human endothelial cells by transfecting siRNAs targeting *PMCA4*.
- 2) To analyse the effect of *PMCA4* downregulation in endothelial cells on the VEGF-induced expression of genes related to Notch signalling and encoding extra-cellular matrix components or cell adhesion molecules.

- 3) To validate the effects observed in the expression of PMCA4-target genes identified above in endothelial cells isolated from large vessels (human umbilical vein endothelial cells, HUVEC) or the microvasculature (human dermal microvascular endothelial cells, HDMEC) using TaqMan qPCR.

It has been reported that aging leads to sensitization of cells to apoptosis, which disrupts the integrity of endothelial cell wall leading to atherosclerosis. Aging impairs NO synthesis and endothelial cell dysfunction (Hoffmann *et al.*, 2001). As mentioned in section 1.8.5.d, PMCA4 is a negative regulator of NO synthesis (Fig 2.1). Thus, we hypothesise that changes in the expression of PMCA4, might be involved in decreased NO synthesis during aging. As a first step to elucidate the role of PMCA4 in abnormal functioning of EC during aging, another objective of my work in a parallel project was:

- 4) To investigate whether aging of endothelial cells by high number of tissue culture passages alters the expression of *PMCA4*.

### **3. MATERIALS AND METHODS**

### **3.1 CELLS AND CELL CULTURE**

Early passage Human Umbilical Vein Endothelial Cells (HUVEC) containing approximately 500,000 pooled multiple donor viable cells, were purchased from Cellworks Ltd., and were cultured in 0.1% gelatin pre-coated plates. Endothelial Cell growth media (ECGM, PromoCell, UK) for cell culture was supplemented with 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich) and ECGM supplement mix (PromoCell) containing: 2% FBS, 0.4% endothelial cell growth supplement, 0.1 ng/mL epidermal growth factor (recombinant human), 1 ng/mL basic fibroblast growth factor (recombinant human), 90 µg/mL heparin, and 1 µg/mL hydrocortisone). ECGM containing supplements will be hereafter referred as ECGM complete. For transfection/gene expression experiments HUVEC were used at passages (P4-P8) under a controlled aseptic sterile environment. Longer passages were required in experiments assessing *PMCA4* expression during senescence.

Human Dermal Microvascular Cells (HDMEC) were purchased from PromoCell. Cells were grown in a 0.05% gelatin pre-coated plates in ECGM complete. Cell growth was comparatively slower than in HUVEC. Cells were used for the experiments at passages (P4-P8).

### **3.2 CELL CULTURE IN TISSUE CULTURE FLASKS**

Primary cells were cultured in a small, ventilated flask (T25) immediately after purchasing. The flask was first coated with 10 mL of 0.1% gelatin and incubated for 15 minutes or more. The cells were suspended in 15 mL of ECGM complete media and incubated in a 5% CO<sub>2</sub> incubator at 37°C. Once the flask was fully confluent, the cells were washed using 1X PBS and

detached using 2 mL of Trypsin-EDTA solution (Sigma-Aldrich) to digest the cell surface proteins attached to the gelatin-coated flask. Incubation with trypsin lasted not longer than two minutes as longer incubation might kill the cells. Once the cells were detached, trypsin was neutralised using 8 mL of ECGM complete. The cell suspension was then centrifuged at 200g for 5 minutes. The supernatant was discarded, and the cell pellet was suspended in 20 mL of ECGM complete and transferred to a medium flask (T75) that was incubated in a 5% CO<sub>2</sub> incubator at 37°C until cells reached confluency. The same procedure was repeated to expand cells to a big flask (T175) except that the final volume of culture medium was increased to 30 mL.

### **3.3 COUNTING AND PLATING CELLS**

Cells were washed with 1X PBS followed by detachment by adding 3 mL of trypsin-EDTA solution and incubation for approximately 2 minutes. The flask was gently tapped along the sides to completely detach the cells and the process was neutralised by adding 8 mL of ECGM complete. The cell suspension was centrifuged at 200g for 5 minutes. The cell pellet was suspended in 10 mL of ECGM complete after discarding the supernatant carefully. A small aliquot of the cell suspension was taken in an empty eppendorf and gently mixed-up pipetting and down. The cells were then counted using a haemocytometer. The cells are counted from two outer opposite squares and the average is calculated. The number of cells per mL of cell suspension is calculated according to the following formula:

$$\text{No. of cells per square} \times 10^4 = \text{No. of cells/mL of suspension.}$$

The calculated cell count was mixed with required quantity of ECGM complete and evenly distributed into the wells. The wells maybe pre-coated with or without gelatin prior to the experiment. The plates were incubated under controlled conditions of 37°C and 5% CO<sub>2</sub> for 24 hours.

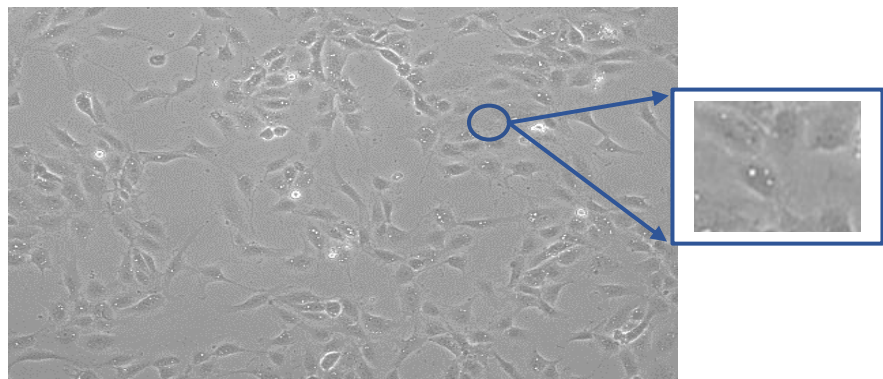
### 3.4 TRANSFECTION OF PRIMARY ENDOTHELIAL CELLS WITH SMALL INTERFERING RNA (siRNA)

siRNA-mediated knock down of PMCA4 (siRNA-PMCA4) was performed using a siRNA targeting specifically human PMCA4; “ON-TARGET-plus SMART pool human ATP2B4” (Thermo Scientific). “ON-TARGET-plus non-targeting pool control duplexes” (Thermo Scientific) (siRNA-NT) was used as a control. HUVECs were plated in 6 well gelatin-coated plates (3 ×10<sup>5</sup> cells/well) one day prior to transfection. siRNAs were transfected into the cells using Lipofectamine 2000 (Thermo Scientific) as following. The plates were washed two times with 1X PBS. 5mL of serum free OPTI-MEM was added to each well and cells were incubated for one hour at 37°C and 5% CO<sub>2</sub>. The complexes siRNA-Lipofectamine were prepared by diluting siRNA stocks (20 μM) in OPTI-MEM according to the indications showed in Table 3.4.a.

siRNA-NT			siRNA-PMCA4		
<b>Sol A</b>	<b>1 Well</b>	<b>7 Wells</b>	<b>Sol A</b>	<b>1 Well</b>	<b>7 Wells</b>
<b>siRNA-NT</b>	4.5μL	31.5μL	<b>siRNA-PMCA4</b>	4.5μL	31.5μL
<b>Opti-MEM</b>	245.5μL	1718.5μL	<b>Opti-MEM</b>	245.5μL	1718.5μL
<b>Sol B</b>	<b>1 Well</b>	<b>7 Wells</b>	<b>Sol B</b>	<b>1 Well</b>	<b>7 Wells</b>
<b>Lipofectamine</b>	5μL	35μL	<b>Lipofectamine</b>	5μL	35μL
<b>Opti-MEM</b>	245μL	1715μL	<b>Opti-MEM</b>	245μL	1715μL

**Table 3.4.a. Composition of the complexes used in transfection of HUVECs (Sol A and Sol B) using siRNA-NT or siRNA-PMCA4.**

The contents of solution A and B corresponding to each complex were mixed dropwise and incubated for 30 minutes. 500  $\mu$ L of the complex Lipofectamine-siRNA were added to each well dropwise while gently swirling. The plates were incubated for 6 hours and checked for transfection features. We have noticed that after the 6-hours incubation period small, bubble-like vacuoles are observed inside the cells (Figure 3.4.i). This is an empirical observation that indicates good transfection of the cells with the siRNA. After the 6-hour incubation period, the medium containing the transfection solution was aspirated and replaced with ECGM complete. The plates were finally incubated for 72 hours and then used as required.



**Figure 3.4.i. Presence of vacuoles within the cells implies good transfection uptake** Microscopy image of HUVEC after 6 hours of incubation in Opti-MEM containing Lipofectamine-siRNA complexes. Image was recorded using a Nikon DSFi1 digital camera coupled to a Nikon ECLIPSE TS100 microscope at 10x magnification. Bubble-like vacuoles can be observed inside the cells indicating good transfection of the siRNA into the cells.

### 3.5 CELL STIMULATION

Transfected cells were washed once with 1X PBS and incubating in ECGM supplemented only with 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich) (hereafter referred as serum-free ECGM). After serum starvation for 3 hours, Vascular Endothelial Growth Factor-A (VEGF) was added to the cells

at 25 ng/mL final concentration. Unstimulated cells were used as control. Each experimental point was performed in triplicate. The VEGF used in the experiments was prepared by dissolving 10 µg of lyophilized VEGF (Peprotech) in 400 µL of PBS 1X. 10 µL of this stock solution were added to 5 mL of culture medium to make a final concentration of VEGF 25 ng/mL.

### **3.6 TOTAL RNA ISOLATION**

RNA isolation was performed using a “Total RNA Purification Plus Kit” (Norgene) according to the manufacturer’s recommendations. Briefly, the media was discarded from the plates and cells were washed with 1X PBS. Cells were lysed in 300 µL of RNA lysis buffer. The plates were swirled for 1 minute to completely lyse the cells. The lysate was collected and stored at -80°C or immediately processed as following. The lysate was transferred into gDNA Columns provided in the kit and passed throughout the columns by centrifugation for 1 minute at 15,600 g. The flow-through was transferred to a fresh tube and 200 µL of 100% ethanol was added. The mixture was vortexed for 10 seconds and passed throughout RNA purification columns by centrifugation at 9,200 g for 1 minute. The flow-through was discarded and the columns were washed 3 times with 400 µL of washing buffer by centrifugation for 1 minute at 15,600 g. An extra final centrifugation was performed to totally eliminate any traces of washing buffer as it contains ethanol. After washing, the columns were put into fresh eppendorfs for eluting RNA. 40 µL of RNA elution buffer was added, followed by 1 minute of incubation at room temperature. The samples were then centrifuged for 2 minutes at 300 g, followed by centrifugation for 1 minute at 15,600 g. RNA was collected and

quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK) using elution buffer as blank.

### 3.7 REVERSE TRANSCRIPTION

500 ng of total RNA isolated as described in the previous section were diluted in nuclease-free-water to a final volume of 10  $\mu$ L. A cDNA master mix containing the reagents required for the retro-transcription was prepared as described in the Table 3.7.a.

COMPONENTS	VOLUME( $\mu$ L)/ REACTION
10x RT Buffer	2.0 $\mu$ L
25x dNTP mix	0.8 $\mu$ L
10x RT Random primers	2.0 $\mu$ L
Multi Scribe reverse transcriptase	1.0 $\mu$ L
RNase inhibitor	1.0 $\mu$ L
Nuclease free H <sub>2</sub> O	3.2 $\mu$ L
<b>Total Per Reaction</b>	<b>10<math>\mu</math>L</b>

**Table 3.7.a. Composition of the Master mix used for reverse transcribing RNA to cDNA**

10  $\mu$ L of cDNA master mix were added to the 10  $\mu$ L of RNA solution previously prepared. The mixture was then briefly centrifuged to eliminate any air bubbles. Retro transcription took place in a PTC-200 Peltier thermal cycler, according to the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C for until collection. After completion, the samples were diluted with 80  $\mu$ L of nuclease-free water and stored at -80°C until further use in real-time PCR experiments.

### 3.8 QUANTITATIVE REAL TIME PCR

A PCR reaction mix was prepared for each primer according to the instructions shown in Table 3.8.a.

REAGENTS	X1
TaqMan primer	1.0 $\mu$ L
TaqMan universal PCR master mix (2X)	10 $\mu$ L
Nuclease free water	3.4 $\mu$ L
<b>Total</b>	<b>14.4<math>\mu</math>L</b>

**Table 3.8.a. Components of master mix used for TaqMan PCR assays**

A fast optical 96-well reaction plate (Applied Biosystems) was loaded with 14.4  $\mu$ L of TaqMan reaction mix and 5.6  $\mu$ L of cDNA making it up to a final volume of 20  $\mu$ L/well. The plate was sealed with optical adhesive film, centrifuged for 1 minute at 100 g and loaded into an Applied BioSystem 7500 Fast real time PCR system. The machine was setup for amplification programme with holding stage at 95°C for 10 minutes specifically for enzyme activation, followed by 40 cycles of denaturization at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. After PCR amplification, the raw data was exported to an Excel document to calculate fold change. Ct value or the cycle threshold value is a relative measure of concentration of target in a PCR reaction.  $\Delta$ Ct value was calculated as the difference between Ct value in each sample to the Ct value of the housekeeping gene *Hprt-1*.  $\Delta\Delta$ Ct algorithm is a method to analyse relative changes in gene expression. It is calculated as the difference between  $\Delta$ Ct (treated sample) -  $\Delta$ Ct (untreated sample).

TaqMan primers used on this study were purchased from Applied Biosystems and are indicated in Table 3.8.b.

TAQMAN PRIMERS	ID ASSAY
Plasma Membrane Calcium ATPase Isoform 4 ( <i>PMCA4</i> )	<i>Hs00608066_m1</i>
Delta like ligand 1 ( <i>DLL1</i> )	<i>Hs00194509_m1</i>
Delta like ligand 4 ( <i>DLL4</i> )	<i>Hs00184092_m1</i>
c-FOS	<i>Hs04194186_s1</i>
Selectin L ( <i>SELL</i> )	<i>Hs00174151_m1</i>
Selectin P ( <i>SELP</i> )	<i>Hs00927900_m1</i>
Selectin E ( <i>SELE</i> )	<i>Hs00174057_m1</i>
ADAM Metallopeptidase with Thrombospondin Type 1 Motif 1 ( <i>ADAMTS1</i> )	<i>Hs00199608_m1</i>
Vascular Cell Adhesion Molecule 1 ( <i>VCAM1</i> )	<i>Hs01003372_m1</i>
Hypoxanthine Phosphoribosyl transferase 1 ( <i>HPRT1</i> )	<i>Hs99999909_m1</i>

**Table 3.8.b. Various TaqMan primers and their gene reference code used to run the Applied BioSystem 7500 Fast real-time PCR.**

### 3.9 PROTEIN DETECTION BY WESTERN BLOT

HUVEC were plated in 6-well tissue culture plates at a density of 250,000 cells/well. The cells were transfected with siNT or siPMCA4 as described in section 2.4. After 72 hours of incubation at 37°C and 5% CO<sub>2</sub>, cells were lysated in 100 µL of 2X NuPAGE® LDS protein sample buffer (Life Technology, UK) containing 0.05% β-mercaptoethanol. The protein lysate was separated according to molecular weight by Polyacrylamide gel electrophoresis (PAGE).

Different composition of the resolving and stacking gel is depicted in Table 3.9.a.

<b>RESOLVING GEL-6%</b>	
<b>Components</b>	<b>Composition</b>
Deionised water	7.35mL
Bis-Acrylamide	2.8mL
Resolving buffer	3.75mL
10% Ammonium Per Sulphate (APS)	100µL
TEMED	80µL

<b>RESOLVING GEL-10%</b>	
<b>Components</b>	<b>Composition</b>
Deionised water	5.45 mL
Bis-Acrylamide	4.7 mL
Resolving buffer	3.75 mL
10% Ammonium Per Sulphate (APS)	100µL
TEMED	80µL

<b>STACKING GEL</b>	
<b>Components</b>	<b>Composition</b>
Deionised water	6.1 mL
Bis-Acrylamide	1.4 mL
Stacking buffer	2.5 mL
10% Ammonium Per Sulphate (APS)	100µL
TEMED	80µL

**Table 3.9.a. Composition of Resolving Gel (6% and 10%) and Stacking gel**

The components of stacking gel remain always the same, however the composition of resolving gel varies across different proteins. PMCA4 has a molecular weight of 135 KDa hence a 6% resolving gel was prepared to separate the protein bands. Tubulin which was used as loading control has a molecular weight of 50 KDa hence requires a 10% resolving gel. The crude protein lysate was initially heated at 100°C for 10 minutes to cleave genomic DNA in the samples and facilitate loading into the gel. Just before loading, the samples were heated at 100°C for 2 minutes and cooled on ice. This is an essential step as it renders denaturation of the proteins. BLUeye protein ladder (GeneFlow, UK) or Protomarkers™ (National diagnostics) pre-stained protein ladder was loaded as markers of protein molecular weight along with the samples. PAGE took place in Tris-Glycine buffer Running buffer (0.025M Tris, 0.192M Glycine, 0.1% SDS) at 150 volts.

Once separated by PAGE, proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane using a wet-transfer blotting apparatus (Invitrogen). In brief, the PVDF membrane was hydrated in methanol for 30 seconds and immediately washed with deionized water to remove the methanol from the membrane. The membrane was placed on top of the resolving gel inside a “sandwich” formed by several layers of Whatman 3MM chromatography paper (Sigma-Aldrich, UK) and foam sponges. Transfer took place in “Transfer Buffer” (0.025M Tris, 0.192M Glycine, 20% methanol) for 90 minutes at 35 volts. The membrane was then incubated in a solution of 5% semi-skimmed milk in 1X TBS for 1 hour at 4°C to block unspecific binding of primary and secondary antibodies. After blockage, milk solution was discarded and the membrane washed twice with TBS-T (TBS, 0.05% Tween

20) followed by incubation with primary antibody JA3 mouse monoclonal anti-PMCA4 (Santa Cruz Biotechnology; 1:1000) or mouse monoclonal anti-tubulin antibody (Sigma; 1:2500) overnight at 4°C. The following morning, membranes were washed with TBS-T five times for five minutes and incubated in a 1:5000 dilution of peroxidase-conjugated sheep anti-mouse immunoglobulin antibody in TBS-T for 4 hours. Unbound secondary antibody was removed by washing the membrane five times for five minutes with TBS-T. Bound antibodies were detected by ECL using an EZ-Chemiluminescence Detection Kit (GeneFlow) following the recommendations of the manufacturer. Chemiluminescence signal was detected by exposing the membrane to Kodak BioMax MS autoradiographic film (Sigma-Aldrich, UK) in dark conditions for different periods of time and subsequent development.

### **3.10 PCR-BASED SCREENING OF SMALL GENE ARRAYS**

An RT<sup>2</sup> Profiler™ PCR Array Human Notch Signalling Pathway Plus Kit (Ref PAHS-059Y) was purchased from Qiagen. Total RNA was isolated from HUVEC transfected with siRNA-NT or siRNA-PMCA4 and stimulated with VEGF-A (25 ng/mL) for 1 hour, and subsequently retrotranscribed as described in previous sections. To screen RT<sup>2</sup> Profiler™ PCR Arrays, a reaction mixture was prepared by mixing 100 µL of cDNA with 1350 µL of RT<sup>2</sup> SYBR Green ROX master mix (Qiagen) and 1248 µL of RNase-free water. 25 µL of this reaction mixture were added into the wells of PAHS-059Y PCR array plate (Qiagen) containing primers to detect the expression of 84 genes related to the Notch signalling pathway, along with primers for several “housekeeping” genes used for normalization. PCR reaction was carried out using an Applied BioSystem 7500 Fast real-time PCR system under the following conditions:

10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The Ct values obtained in the real time PCR were exported to Excel to calculate the fold change. Screening of RT<sup>2</sup> Profiler™ PCR Array Human Extracellular Matrix & Cell Adhesion Molecules Kit (Ref PAHS-013Z) (Qiagen) containing primers to detect the expression levels of 84 genes encoding proteins related to extracellular matrix and cell adhesion molecules, along with primers to detect several “housekeeping” genes for normalization purposes, was carried as described above except that transfected HUVEC were stimulated with VEGF (25 ng/mL) for 3 hours. PCR-based screening of gene array plates was performed in three independent experiments. Ct values in obtained in PCR reactions performed from HUVEC containing (siNT) or lacking PMCA4 (siPMCA4) were analysed for statistically significant differences in gene expression using the RT<sup>2</sup> Profiler PCR Data Analysis facility <https://dataanalysis2.qiagen.com/pcr> according to the instructions of the manufacturer (Qiagen).

### **3.11 STATISTICAL ANALYSIS**

Results are shown as mean ± SE. Statistical differences between two groups were analysed by unpaired, two-tailed Student’s t-test. One-way ANOVA with *post hoc* Tukey’s comparison test was used to analyse differences between more than two groups. Samples with more than two groups and two independent variables were analysed for statistical significance by two-way ANOVA with post-hoc multiple comparison Bonferroni’s test. Statistical significance was set at  $P \leq 0.05$ . Experimental biological replicates, indicated by ‘n’, are defined as a sample of cells plated in an individual tissue culture well, and transfected independently of others. After transfection, biological

replicates were lysed independently, and RNA was isolated and converted into cDNA in an independent manner for each biological replicate. Levels of gene expression in each biological replicate were analysed by qPCR.

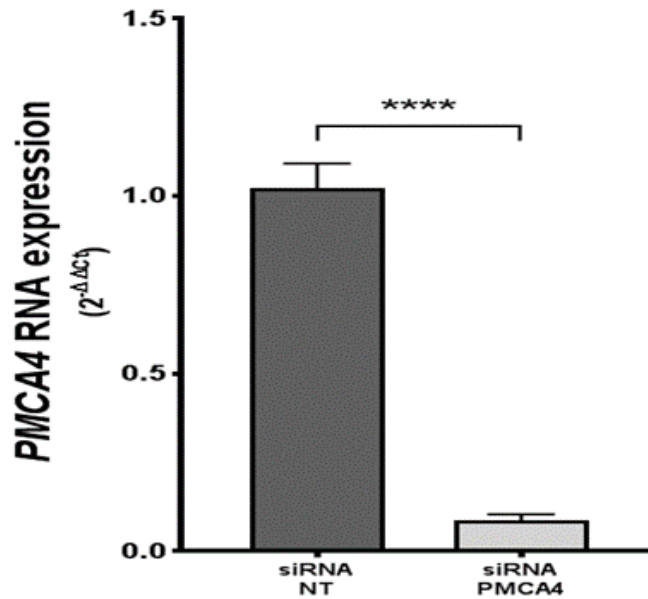
## **4. RESULTS**

## **4.1 SILENCING THE EXPRESSION OF PMCA4 IN HUMAN ENDOTHELIAL CELLS BY TRANSFECTING siRNAs TARGETING PMCA4.**

### **4.1.1 KNOCK-DOWN OF *PMCA4* AT RNA LEVEL**

As we have mentioned in section 2, we have previously reported that endothelial *PMCA4* inhibits VEGF-induced angiogenesis by downregulating the activity of the calcineurin/NFAT pathway (Baggot *et al.*, 2014; Kurusamy *et al.*, 2017). However, whether *PMCA4* also regulates other signalling pathways activated by VEGF in endothelial cells is unknown at present.

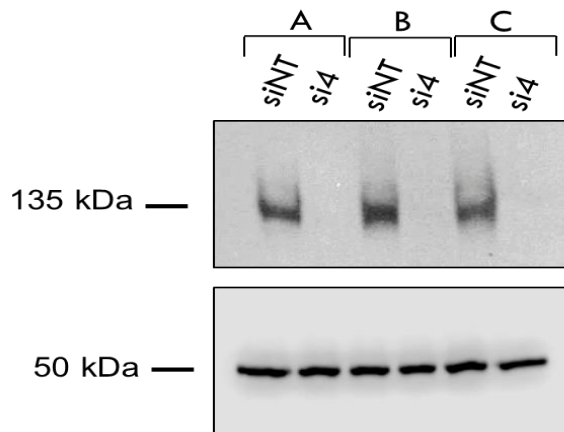
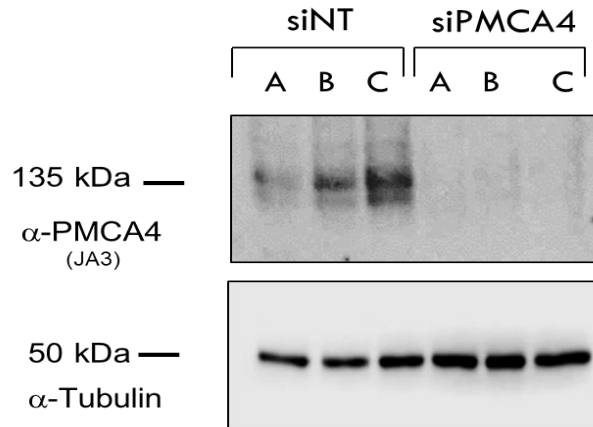
As a first step to investigate this question, this work has evaluated whether *PMCA4* silencing in endothelial cells affects to the expression of VEGF-induced critical angiogenic molecules such as components of the notch signalling pathway, extracellular matrix proteins (ECM) or cell adhesion molecules (CAM). Knock-down experiments were conducted in Human umbilical vein endothelial cells (HUVECs) by transfection of a siRNA targeting specifically human *PMCA4* (si-*PMCA4*). HUVECs transfected with a non-targeting siRNA (si-NT) were used as control. Four independent experiments with three replicates each were done (n=12) in total. Figure 4.1.1.i depicts a significant, strong reduction (around 90% reduction) in *PMCA4* RNA expression in HUVECs transfected with si-*PMCA4* compared to those transfected with control si-NT.



**Figure 4.1.1.i. siRNA-mediated PMCA4 silencing in endothelial cells leads to a strong reduction in PMCA4 expression at the RNA level.** Histograms show data as mean  $\pm$  SE, n=12. \*\*\*\* indicates  $p < 0.0001$ . Statistical analysis was done using unpaired, two-tailed, Student *t* test to verify deviation between the independent experiments when comparing siRNA-NT and siRNA-PMCA4 transfected cells.

#### 4.1.2 PROTEIN LEVEL DETECTION BY WESTERN BLOT

Consistent with the RNA data, western blot analysis of protein lysates isolated from HUVEC transfected with siRNA-NT (control) or siRNA-PMCA4 verified the knockdown of PMCA4 expression at protein level. As it can be seen in Figure 4.1.2.i, a band corresponding to a protein of the molecular weight expected for PMCA4 (135 KDa) was detected by western blot analysis in protein lysates isolated from siNT-transfected HUVECs using an anti-PMCA4 specific antibody. PMCA4 expression was, however, undetected in proteins from siPMCA4-transfected cells, confirming successful knockdown of PMCA4 expression at protein level. Triplicates of two independent experiments are shown. Protein expression of  $\alpha$ -tubulin (50 KDa) is shown as loading control, ruling out that lack of PMCA4 detection could be the consequence of differential protein levels in the samples.



**Figure 4.1.2.i. Western blot analysis of PMCA4 after siRNA-mediated silencing of *PMCA4* expression in human endothelial cells.** PMCA4 protein expression in whole lysates of HUVEC transfected with siRNA-NT or siRNA-PMCA4 was determined using an anti-PMCA4 mouse monoclonal antibody JA3 ( $\alpha$ -PMCA4). Levels of tubulin were analysed in parallel as loading control. Images show results generated from 2 independent experiments performed each time in triplicate.

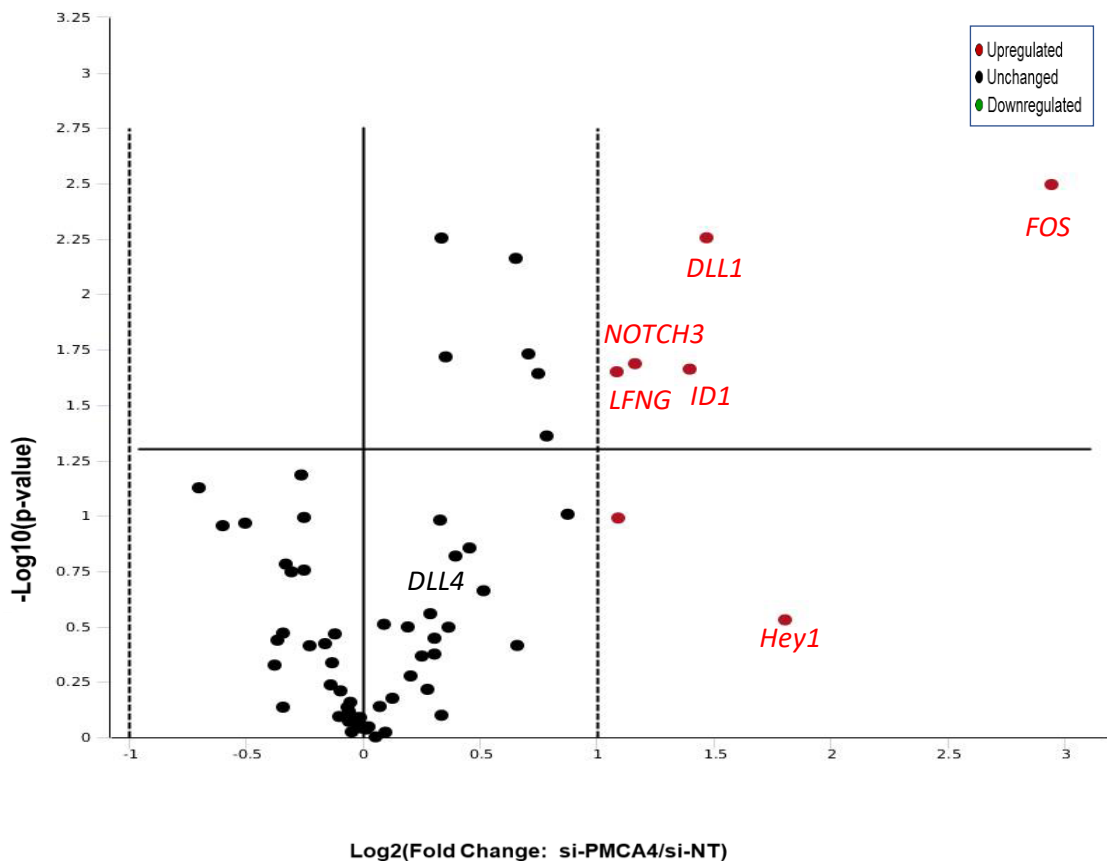
## **4.2 ANALYSES OF CHANGES IN GENE EXPRESSION CAUSED BY PMCA4 SILENCING IN ENDOTHELIAL CELLS**

Notch signalling activation, and degradation of the extracellular matrix are two critical events required during the onset of angiogenesis (described in section 1.3 of introduction). Therefore, potential changes in the expression of genes related to these cellular processes have been investigated in this study using endothelial cells where the expression of PMCA4 was significantly reduced by siRNA-mediated silencing.

### **4.2.1 SCREENING OF A GENE ARRAY RELATED TO NOTCH SIGNALLING PATHWAY**

In order to verify if *PMCA4* silencing regulates the Notch signalling pathway and the genes that code for proteins involved in Notch signalling during angiogenesis, a PCR-based gene array consisting of oligonucleotides to detect the expression of 84 genes important in notch signalling was screened with RNA isolated from HUVECs transfected with si-NT (control) or siPMCA4. Transfected cells were stimulated with VEGF (25 ng/mL) for 1 hour prior to RNA isolation. Expression of *DLL1* (Delta like ligand-1), *FOS*, *ID1* (Inhibitor of DNA binding 1), *NOTCH3* and *LFNG* (O-Fucosylpeptide 3-Beta-N-Acetylglucosaminyltransferase) was significantly enhanced by *PMCA4* silencing (figure 4.2.1.i). The higher increment was observed in the expression of *FOS* that was upregulated 7.68-fold. *DLL1* was upregulated 2.77-fold. Interestingly, the expression of *DLL4*, another key Notch ligand expressed in endothelial cells, remained unchanged. *ID1*, *NOTCH3* and *LFNG* were also upregulated just above the cut-off of 2-fold compared to control siNT transfected cells. *Hey1* (Hes Related Family BHLH Transcription Factor With YRPW Motif 1) which is a Notch-signalling target gene was also upregulated,

although differences with respect to control did not reach statistical significance. Knockdown of *PMCA4* did not result in any significant downregulation in the expression of the genes studied in the array (figure 4.2.1.i and Table 4.2.1.a).



**Figure 4.2.1.i. Volcano plot depicting changes in the expression of genes related to the Notch signalling pathway after *PMCA4* silencing in endothelial cells.** *PMCA4* was knocked down in HUVECs, and cells subsequently stimulated with VEGF (25 ng/mL) for 1 hour. Unchanged gene expression is denoted by a solid vertical line. Changes in gene expression lies on either side of this line, with dotted vertical lines indicating a cut-off in fold change expression of +2 (right dotted line) or 0.5 (left dotted line). Results are represented as mean of three independent gene array screening assays. The horizontal line marks statistical significance level  $p < 0.05$ . Red dots represent upregulation of gene expression above the cut-off. Changes in gene expression below cut-off are presenting by black dots.

Position	Gene Symbol	AVG $\Delta C_t$		2 <sup>n</sup> $\Delta C_t$		Fold Change		p-value
		Group 1	Control Group	Group 1	Control Group	Group 1/Control	Comments	
A01	ADAM10	2.61	2.57	0.164241	0.167976	0.98		0.815225
A02	ADAM17	4.54	4.53	0.042933	0.043351	0.99		0.906054
A03	AES	2.42	2.36	0.187247	0.1947	0.96		0.695757
A04	AXIN1	6.1	6.05	0.014628	0.015142	0.97		0.844568
A05	CBL	5.69	5.71	0.019361	0.019041	1.02		0.896911
A06	CCND1	2.86	2.56	0.13769	0.170041	0.81		0.178900
A07	CCNE1	7.27	6.67	0.006479	0.009803	0.66		0.110800
A08	CD44	3.14	3.08	0.113263	0.11809	0.96		0.763666
A09	CDKN1A	2.06	2.77	0.239542	0.146848	1.63		0.016589
A10	CFLAR	4.49	4.23	0.044548	0.053171	0.84		0.178034
A11	CHUK	6.59	6.34	0.01039	0.012367	0.84		0.101771
A12	CTNNB1	3.65	3.55	0.0797	0.085489	0.93		0.806036
B01	DLL1	4.96	6.43	0.032063	0.011569	2.77		0.005557
B02	DLL3	14.76	15.61	0.000036	0.00002	1.81	B	0.263476
B03	DLL4	4.8	5.19	0.035897	0.027418	1.31		0.151972
B04	EP300	4.93	4.92	0.032832	0.033073	0.99		0.813745
B05	ERBB2	5.92	6.13	0.016478	0.014321	1.15		0.528945
B06	FIGF	11.84	10.92	0.000272	0.000515	0.53	A	0.122102
B07	FOS	5.84	8.79	0.017412	0.002267	7.68		0.003195
B08	FOSL1	3.57	3.86	0.083979	0.068866	1.22		0.276709
B09	FZD7	11.85	13.14	0.00027	0.000111	2.44	B	0.244575
B10	GLI1	13.19	14.02	0.000107	0.00006	1.78	B	0.245730
B11	GSK3B	4.42	4.19	0.046826	0.054794	0.85		0.386218
B12	HDAC1	3.63	3.98	0.080534	0.063186	1.27		0.019159
C01	HES1	4.03	4.33	0.061133	0.048551	1.23		0.421160
C02	HEY1	8.04	9.85	0.003794	0.001086	3.49		0.295049
C03	HOXB4	7.54	7.87	0.005366	0.004272	1.26		0.104560
C04	HR	11.95	13.32	0.000252	0.000098	2.58	B	0.244547
C05	ID1	4.4	5.8	0.04729	0.017953	2.63		0.021760
C06	IFNG	15.1	16.43	0.000029	0.000011	2.52	B	0.392096
C07	IL2RA	14.83	16.38	0.000034	0.000012	2.92	B	0.386674
C08	JAG1	4.54	5.29	0.043008	0.02591	1.68		0.022816
C09	JAG2	5.38	5.39	0.023967	0.023831	1.01		0.917466
C10	KRT1	15.13	16.43	0.000028	0.000011	2.45	B	0.393822
C11	LFNG	5.99	7.07	0.015749	0.007417	2.12		0.022380
C12	LMO2	2.68	3.33	0.156251	0.099189	1.58		0.006890
D01	LOR	15.59	16.43	0.00002	0.000011	1.78	B	0.435178
D02	LRP5	6.07	6.94	0.014892	0.008117	1.83		0.098470
D03	MAML1	6.06	5.94	0.015	0.016287	0.92		0.341729
D04	MAML2	7.24	6.53	0.006631	0.010791	0.61		0.074782
D05	MFNG	2.38	2.71	0.192724	0.152666	1.26		0.005573
D06	MMP7	10.98	10.63	0.000496	0.00063	0.79		0.731565
D07	NCOR2	4.59	4.25	0.041462	0.052583	0.79		0.338708
D08	NCSTN	3.28	3.41	0.102599	0.094197	1.09		0.668873

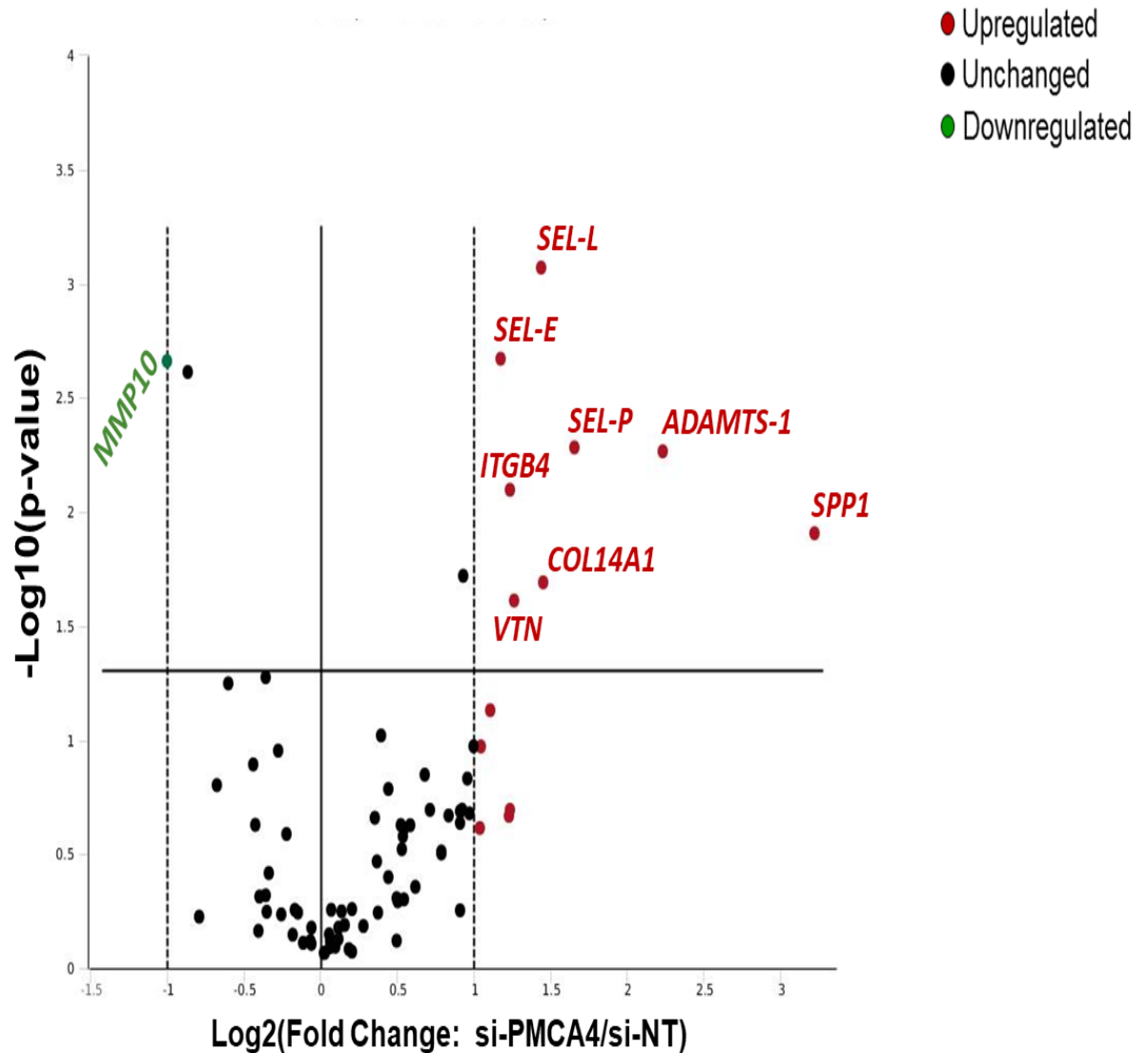
Position	Gene Symbol	AVG $\Delta C_t$		2 <sup>n</sup> $\Delta C_t$		Fold Change		p-value
		Group 1	Control Group	Group 1	Control Group	Group 1/Control	Comments	
D09	NFKB1	6.07	6.05	0.014885	0.015097	0.99		0.899494
D10	NFKB2	5.62	5.81	0.020302	0.017816	1.14		0.317530
D11	NOTCH1	5.76	6.09	0.018513	0.014705	1.26		0.795974
D12	NOTCH2	6.13	5.87	0.014245	0.017104	0.83		0.065438
E01	NOTCH3	8.8	9.96	0.002246	0.001003	2.24		0.020567
E02	NOTCH4	5.55	5.49	0.021309	0.022314	0.95		0.731428
E03	NUMB	4.08	3.94	0.059287	0.065073	0.91		0.460843
E04	PAX5	14.98	16.41	0.000031	0.000012	2.68	B	0.387710
E05	POFUT1	7.78	7.72	0.004542	0.00474	0.96		0.847856
E06	PPARG	8.31	9.09	0.00316	0.001831	1.73		0.043654
E07	PSEN1	4.57	4.47	0.042161	0.045023	0.94		0.617163
E08	PSEN2	6.4	6.85	0.011839	0.008548	1.37		0.139689
E09	PSENUM	4.89	4.87	0.033632	0.034145	0.98		0.870398
E10	PTCRA	14.61	15.76	0.00004	0.000018	2.21	B	0.394712
E11	RBPL1	13.67	15.2	0.000077	0.000027	2.89	B	0.363804
E12	RFNG	5.26	5.35	0.026137	0.024547	1.06		0.309091
F01	RUNX1	6.36	5.85	0.012195	0.017284	0.71		0.107952
F02	SELL1	3.33	3.32	0.099578	0.100038	1		0.885495
F03	SHH	13.01	13.38	0.000121	0.000094	1.29	B	0.621990
F04	SMO	6.59	6.89	0.010416	0.008418	1.24		0.357164
F05	SNW1	4.49	4.36	0.044359	0.048813	0.91		0.579797
F06	STAT6	4.09	4.34	0.058802	0.049498	1.19		0.430079
F07	TLE1	4.45	4.53	0.045654	0.043416	1.05		0.726399
F08	WISP1	13.81	15.26	0.00007	0.000026	2.72	B	0.261363
F09	ADA	5.17	5.01	0.027726	0.030986	0.89		0.377666
F10	AFAP1L2	8.41	11.47	0.002947	0.000353	8.36	A	0.005929
F11	DTX1	13.67	14.59	0.000077	0.000041	1.89	B	0.367367
F12	H19	12.09	13.68	0.000229	0.000076	3	B	0.090601
G01	Hes4	7.89	7.98	0.00422	0.003954	1.07		0.948521
G02	HES5	15.09	16.21	0.000029	0.000013	2.18	B	0.375668
G03	HEY2	10.31	10.97	0.000786	0.000497	1.58		0.385153
G04	HEYL	15.59	16.43	0.00002	0.000011	1.79	B	0.434478
G05	NRARP	6.07	6.58	0.014935	0.010445	1.43		0.217938
G06	SCGB1A1	15.14	16.01	0.000028	0.000015	1.82	B	0.468025
G07	SERPINA3	14.74	15.41	0.000037	0.000023	1.59	B	0.422411
G08	SLCGA12	15.54	16.43	0.000021	0.000011	1.85	B	0.427801
G09	SNAI2	9.37	8.99	0.001513	0.001965	0.77		0.472257
G10	TFF1	15.2	15.91	0.000027	0.000016	1.64	B	0.518078
G11	TNFSF10	3.22	4.32	0.107082	0.050218	2.13		0.102312
G12	UBD	12.07	14.01	0.000232	0.000061	3.84	B	0.073090
H01	ACTB	-2.66	-3.03	6.337898	8.154489	0.78		0.364451
H02	B2M	-0.13	0.24	1.092893	0.848194	1.29		0.318347
H03	GAPDH	-1.87	-1.82	3.653167	3.519156	1.04		0.995550
H04	HRPT1	5.6	5.27	0.020633	0.025961	0.79		0.165342
H05	RPLPO	-0.94	-0.66	1.915359	1.581287	1.21		0.607311

**Table 4.2.1.a. List of 84 genes screened in RT<sup>2</sup> profiler PCR array depicting changes in genes related to the Notch signalling pathway after PMCA4 silencing in endothelial cells.**

Analysis was performed using the GeneGlobe Data Analysis Center resource <https://geneglobe.qiagen.com/us/analyze/>. Genes upregulated more than 2-fold (cut-off) with *P* values <0.05 are highlighted in red boxes. Blue boxes indicate gene where expression was increased more than 2-fold but not significantly (*p*>0.05). The table shows comments; A, B, denoting that amplification of that gene required a high number of PCR cycles and the result is not reliable. Results with AVGDC<sub>t</sub> around 10 were not considered. Group 1 denotes results from si-PMCA4 transfected cells stimulated with VEGF for 1 hour. Control group denotes results from si-NT transfected cells stimulated with VEGF for 1 hour.

#### 4.2.2 SCREENING OF GENE ARRAY RELATED TO EXTRA CELLULAR MATRIX AND CELL ADHESION MOLECULES

An RT<sup>2</sup> profiler PCR array consisting of 84 genes related to extracellular matrix and cell adhesion molecules, was analysed by qPCR. Results shown in Figure 4.2.2.i and table 4.2.2.a demonstrate that the expression of 8 genes; *SELL* (Selectin L), *SELP* (Selectin P), *SELE* (Selectin E), *ADAMTS-1* (ADAM Metalloproteinase With Thrombospondin Type 1 Motif 1), *SPP1* (Secreted Phosphoprotein 1), *COL14A1* (Collagen 14A1), *VTN* (Vitronectin), *ITGB4* (Integrin Subunit Beta 4) increased significantly in PMCA4-silenced HUVEC stimulated with VEGF (25 ng/mL) for 3 hours. *MMP10* (Matrix metalloproteinase 10) was downregulated by 2-fold. *PMCA4* knockdown did not change the expression of other genes included in the array (Figure 4.2.2.i and Table 4.2.2.a). Highest changes were observed in the expression of *SPP1*, with an increment of 9.31-fold, and *ADAMTS-1*, with 4.68-fold increment. *ITGB4* with fold change 2.35, *VTN* with fold change 2.4, *VCAM-1* with fold change 2.07, *COL14A1* with fold change 2.73, *SELL* with fold change 2.71, *SELE* with fold change 2.26, and *SELP* with fold change 3.15, show lower but significant increments in expression in si-PMCA4 transfected cells (Figure 4.2.2.i and Table 4.2.2.a). These results suggest that PMCA4 is implicated in the regulation of these genes and the cellular processes involving these genes. Analysis of gene array using the “Qiagen GeneGlobe Data Analysis” resource <https://geneglobe.qiagen.com/us/analyze> indicated that the number of cycles required for PCR amplification of some genes was very high and the results are unreliable (genes with comment A or B in Table 4.2.2.a). Results from these genes were therefore disregarded for further studies.



**Figure 4.2.2.i. *PMCA4* gene knockdown in HUVEC shows changes in the expression of genes related to ECM-CAM.** *PMCA4* was knocked down in HUVEC, and cells subsequently stimulated with VEGF (25 ng/mL) for 3 hours. Unchanged gene expression is denoted by a solid vertical line. Changes in gene expression lay on either side of this line, with dotted vertical lines indicating a cut-off in fold change expression of +2 (right dotted line) or 0.5 (left dotted line). Results are represented as mean of three independent gene array screening assays. The horizontal line marks statistical significance level  $p < 0.05$ . Red dots represent upregulation of gene expression above the cut-off. Changes in gene expression below cut-off are presenting by black dots. Green dot represents downregulation of gene expression.

Position	Gene Symbol	AVG $\Delta C_t$		2 <sup>-<math>\Delta C_t</math></sup>		Fold Change		p-value
		Group 1	Control Group	Group 1	Control Group	Group 1/Control	Comments	
A01	ADAMTS1	2.78	5.01	0.145863	0.031137	4.68		0.005429
A02	ADAMTS13	10.66	11.24	0.000618	0.000413	1.5	B	0.235916
A03	ADAMTS8	12.09	12.63	0.00023	0.000158	1.46	B	0.499037
A04	CD44	3.3	3.44	0.101278	0.092063	1.1		0.563211
A05	CDH1	12.51	12.45	0.000171	0.000179	0.95	B	0.759514
A06	CLEC3B	7.65	10.68	0.004964	0.000608	8.16	A	0.080531
A07	CNTN1	12.66	13.81	0.000154	0.00007	2.21	B	0.248005
A08	COL11A1	12.67	13.81	0.000153	0.00007	2.2	B	0.181544
A09	COL12A1	8.7	8.3	0.002412	0.003183	0.76	A	0.485374
A10	COL14A1	9.34	10.79	0.001546	0.000555	2.73		0.020356
A11	COL15A1	12.51	13.59	0.000171	0.000081	2.11	B	0.301393
A12	COL16A1	10.63	11.17	0.000633	0.000434	1.46	B	0.243439
B01	COL1A1	11.91	12.91	0.00026	0.00013	1.99	B	0.106358
B02	COL4A2	1.72	0.85	0.303583	0.554461	0.55		0.002438
B03	COL5A1	3.23	3.42	0.106585	0.093582	1.14		0.824311
B04	COL6A1	5.1	6.21	0.029201	0.013551	2.15		0.073947
B05	COL6A2	8.66	9.62	0.00247	0.001272	1.94	B	0.147808
B06	COL7A1	11.92	12.83	0.000258	0.000138	1.87	B	0.205258
B07	COL8A1	4.69	4.53	0.03861	0.043386	0.89		0.554870
B08	CTGF	-0.68	-0.62	1.602145	1.531885	1.05		0.759403
B09	CTNNA1	1.8	2.71	0.287033	0.152932	1.88		0.557198
B10	CTNND1	3.76	3.83	0.073938	0.070427	1.05		0.554326
B11	CTNND1	3.81	3.91	0.071111	0.066575	1.07		0.804754
B12	CTNND2	12.68	13.59	0.000152	0.000081	1.88	B	0.230493
C01	ECM1	10.09	9.65	0.00092	0.001248	0.74	B	0.127819
C02	FN1	-0.84	-0.64	1.792673	1.555551	1.15		0.846112
C03	HAS1	12.03	12.31	0.000239	0.000197	1.21	B	0.653298
C04	ICAM1	4.1	5.34	0.058383	0.024757	2.36		0.202164
C05	ITGA1	7.28	8.83	0.00645	0.002195	2.44	A	0.021949
C06	ITGA2	4.66	5.15	0.039661	0.028095	1.41		0.756855
C07	ITGA3	3.23	3.61	0.106421	0.081976	1.3		0.570399
C08	ITGA4	9.44	9.56	0.00144	0.001326	1.09	B	0.744530
C09	ITGA5	1.28	1.78	0.411192	0.291432	1.41		0.493235
C10	ITGA6	3.1	3.88	0.116895	0.057801	1.72		0.308091
C11	ITGA7	11.95	13.34	0.000252	0.000097	2.61	B	0.182342
C12	ITGA8	11.89	12.95	0.000264	0.000126	2.09	B	0.348716
D01	ITGAL	11.6	12.1	0.000322	0.000227	1.42	B	0.508994
D02	ITGAM	11.73	11.85	0.000295	0.000272	1.09	B	0.663739
D03	ITGAV	2.63	3.42	0.161183	0.09332	1.73		0.313838
D04	ITGB1	0.38	0.23	0.768374	0.853027	0.9		0.570378
D05	ITGB2	11.45	11.47	0.000358	0.000351	1.02	B	0.853822
D06	ITGB3	4.58	5.62	0.041798	0.02039	2.05		0.242817
D07	ITGB4	5.86	7.1	0.017172	0.007296	2.35		0.008910
D08	ITGB5	4.13	3.79	0.057046	0.072165	0.79		0.382902

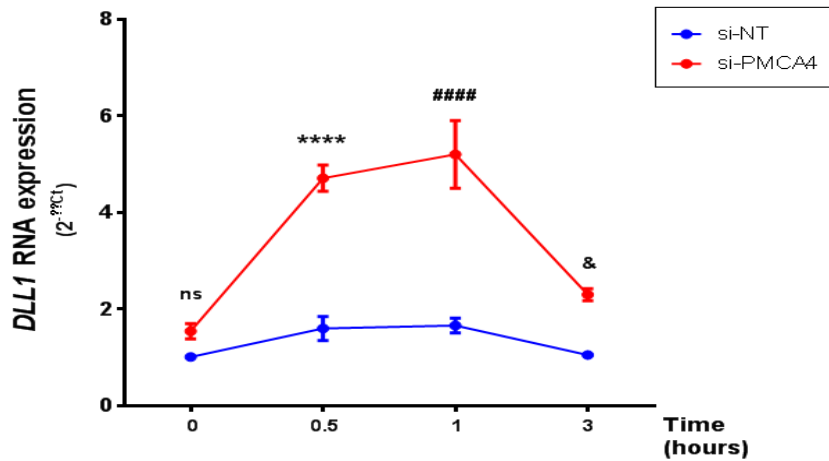
Position	Gene Symbol	AVG $\Delta C_t$		2 <sup>-<math>\Delta C_t</math></sup>		Fold Change		p-value
		Group 1	Control Group	Group 1	Control Group	Group 1/Control	Comments	
D09	ANOS1	12.18	12.8	0.000215	0.00014	1.54	B	0.440055
D10	LAMA1	13.28	13.81	0.000101	0.00007	1.45	C	0.301066
D11	LAMA2	10.06	9.63	0.000909	0.001262	0.74	B	0.235281
D12	LAMA3	8.06	8.58	0.003751	0.002607	1.44		0.235793
E01	LAMB1	1.21	1.27	0.432578	0.414512	1.04		0.807834
E02	LAMB3	6.91	6.5	0.00832	0.011028	0.75		0.684825
E03	LAMC1	0.98	1.42	0.507288	0.374032	1.36		0.183844
E04	MMP1	0.26	0	0.834215	0.997213	0.84		0.581086
E05	MMP10	6.23	5.23	0.013295	0.026845	0.5		0.002182
E06	MMP11	6.75	6.15	0.009275	0.014063	0.66		0.056388
E07	MMP12	12.88	13.81	0.000132	0.00007	1.9	B	0.201670
E08	MMP13	13.09	13.81	0.000114	0.00007	1.64	B	0.202096
E09	MMP14	2.8	2.74	0.14383	0.150006	0.96		0.781504
E10	MMP15	10.27	9.91	0.000811	0.001038	0.78	B	0.478983
E11	MMP16	5.73	6.67	0.018776	0.009853	1.91		0.019054
E12	MMP2	0.93	0.95	0.525873	0.519069	1.01		0.858585
F01	MMP3	11.83	11.65	0.000275	0.000311	0.88	B	0.12812
F02	MMP7	10.22	9.87	0.000641	0.00107	0.79	B	0.565118
F03	MMP8	12.06	11.27	0.000234	0.000404	0.58	B	0.593966
F04	MMP9	12.88	13.72	0.000132	0.000074	1.78	B	0.214175
F05	NCAM1	12.84	13.81	0.000136	0.00007	1.96	B	0.209397
F06	PECAM1	2.09	2.24	0.235373	0.210971	1.12		0.645951
F07	SELE	6.07	7.25	0.014873	0.00656	2.28		0.002134
F08	SELL	6.41	7.85	0.011756	0.004343	2.71		0.000650
F09	SELP	5.45	7.1	0.022934	0.007278	3.15		0.005220
F10	SGCE	4.67	4.72	0.039323	0.03788	1.04		0.710112
F11	SPARC	-0.38	-0.5	1.300202	1.409567	0.92		0.774004
F12	SPG7	4.98	5.51	0.031752	0.021875	1.45		0.263844
G01	SPP1	8.83	12.05	0.002201	0.000236	9.31		0.012401
G02	TGFB1	6.87	6.2	0.006535	0.013638	0.63		0.157528
G03	THBS1	-2.84	-3.2	7.183756	9.219016	0.78		0.052984
G04	THBS2	11.97	12.41	0.000249	0.000183	1.36	B	0.398676
G05	THBS3	9.07	9.44	0.001859	0.001438	1.29	B	0.340072
G06	TIMP1	4.03	4.71	0.06119	0.038141	1.6		0.141711
G07	TIMP2	1.01	1.41	0.496285	0.377345	1.32		0.095547
G08	TIMP3	7.9	9.85	0.004188	0.001081	3.88	A	0.001429
G09	TNC	12.58	13.81	0.000163	0.00007	2.34	B	0.214881
G10	VCAM1	7.38	8.43	0.006013	0.002908	2.07		0.166650
G11	VCAN	9.81	8.43	0.001117	0.002892	0.39	A	0.139455
G12	VTN	10.25	11.51	0.000823	0.000343	2.4		0.024484
H01	ACTB	-2.7	-2.98	6.498688	7.881883	0.82		0.111241
H02	B2M	-0.13	-0.35	1.090599	1.270377	0.86		0.258096
H03	GAPDH	-1.4	-1.2	2.644058	2.29838	1.15		0.550186
H04	HPRT1	5.9	5.84	0.016727	0.017425	0.96		0.663425
H05	RPLP0	-1.67	-1.32	3.191139	2.492781	1.28		0.219193

**Table 4.2.2.a. List of 84 genes screened in RT<sup>2</sup> profiler PCR array depicting changes in genes related to ECM-CAM after *PMCA4* silencing in endothelial cells.** Analysis was performed using the GeneGlobe Data Analysis Center resource <https://geneglobe.qiagen.com/us/analyze/>. Genes upregulated more than 2-fold (cut-off) with *P* values <0.05 are highlighted in red boxes. Green box indicates a gene where expression was reduced 50%. Comments “A” or “B” denote that amplification of that gene required a high number of PCR cycles and the result is not reliable. Results with AVGDC<sub>t</sub> around 10 were not considered. Group 1 denotes results from si-*PMCA4* transfected cells stimulated with VEGF for 3 hours. Control group denotes results from si-NT transfected cells stimulated with VEGF for 3 hours.

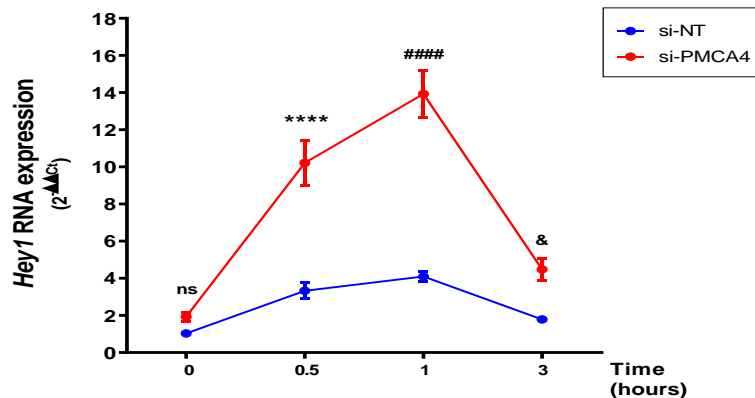
### **4.3 VALIDATION OF DIFFERENTIAL GENE EXPRESSION OF PMCA4-TARGET GENES AFTER PMCA4 SILENCING IN ENDOTHELIAL CELLS ISOLATED FROM LARGE VESSELS (HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS, HUVEC) OR THE MICROVASCULATURE (HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS, HDMEC)**

#### **4.3.1 VALIDATION OF GENES IMPLICATED IN NOTCH SIGNALLING PATHWAY USING TAQMAN qPCR TECHNIQUE.**

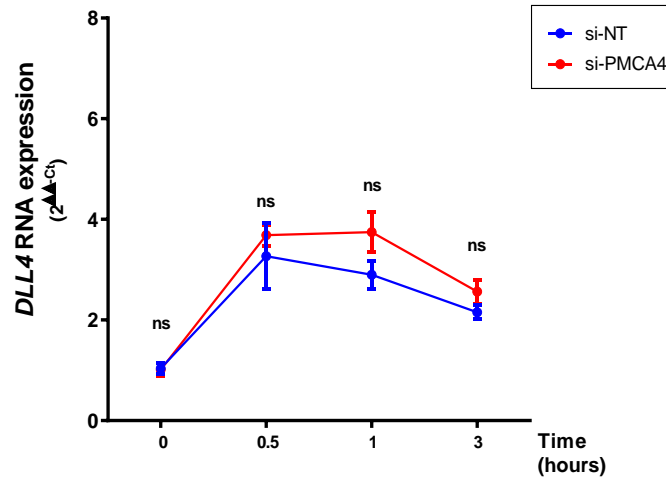
To verify the results obtained from the screening of the array of genes implicated in notch signalling we performed TaqMan-based qPCR using RNA isolated from control or PMCA4-silenced HUVECs. The figures 4.3.1.i - 4.3.1.iii show the RNA expression of *DLL1*, *Hey1*, *DLL4* in transfected cells at resting conditions and after stimulation with VEGF (25 ng/mL) at various time intervals. Data were analysed for statistical significance using 2-way ANOVA, with *post-hoc* multiple comparison Bonferroni's test. Expression of RNA levels for *DLL1* and *Hey1* was not affected by PMCA4 silencing in resting cells, However, expression of these genes was strongly enhanced in PMCA4-lacking cells after stimulation with VEGF for 30 and 60 minutes. Consistent with the data obtained from gene array experiments, expression of *DLL4* was unaffected by PMCA4 silencing in HUVEC cells. Figure 4.3.1.iv. show that RNA levels of *cFOS* were highly upregulated in HUVECs that were silenced for *PMCA4* and stimulated with VEGF for 1h. Altogether, these results further reinforce the involvement of PMCA4 as a regulator of the expression of genes implicated in notch signalling.



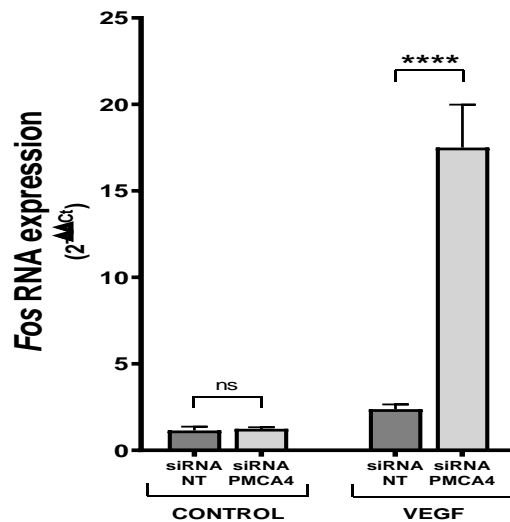
**Figure 4.3.1.i. Validation of *DLL1* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances VEGF-induced upregulation of *DLL1* gene expression in HUVEC. HUVEC transfected with si-NT (blue line) or si-*PMCA4* (red line) were stimulated with VEGF (25ng/mL) at the times indicated on the x-axis. Expression of *DLL1* was normalised to expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by two-way ANOVA with *post-hoc* multiple comparison Bonferroni's test. &, indicates  $p < 0.05$  at 3 hours, \*\*\*\* indicates  $p < 0.0001$  at 0.5 hours, #### indicates  $p < 0.0001$  at 1 hour, and ns, indicates non-significant differences, when the values of *DLL1* expression in si-*PMCA4* transfected cells were compared to those in si-NT transfected cells at a particular time of stimulation.



**Figure 4.3.1. ii. Validation of *Hey1* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances VEGF-induced upregulation of *Hey1* gene expression in HUVEC. HUVEC transfected with si-NT (blue line) or si-*PMCA4* (red line) were stimulated with VEGF (25ng/mL) at the times indicated on the x-axis. Expression of *Hey1* was normalised to expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by two-way ANOVA with *post-hoc* multiple comparison Bonferroni's test. &, indicates  $p < 0.05$  at 3 hours, \*\*\*\* indicates  $p < 0.0001$  at 0.5 hours, #### indicates  $p < 0.0001$  at 1 hour, and ns, indicates non-significant differences, when the values of *Hey1* expression in si-*PMCA4* transfected cells were compared to those in si-NT transfected cells at a particular time of stimulation.



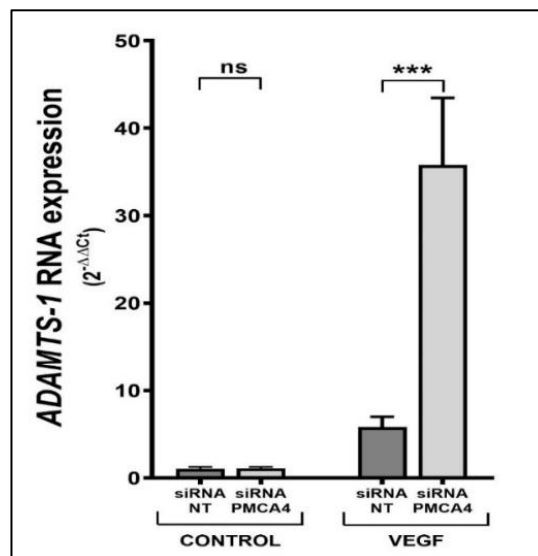
**Figure 4.3.1.iii. Validation of *DLL4* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown does not alter the expression of *DLL4* in HUVEC. HUVEC transfected with si-NT (blue line) or si-*PMCA4* (red line) were stimulated with VEGF (25ng/mL) at the times indicated on the x-axis. Expression of *DLL4* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by two-way ANOVA with *post-hoc* multiple comparison Bonferroni's test. ns, indicates non-significant differences, when the values of *Hey1* expression in si-*PMCA4* transfected cells were compared to those in si-NT transfected cells at a particular time.



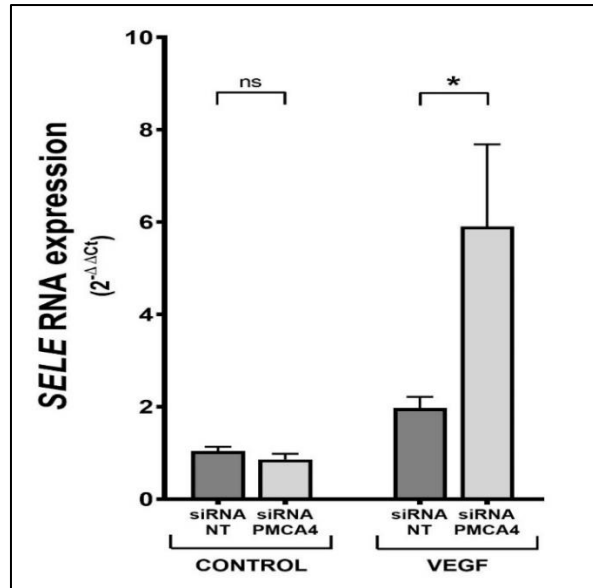
**Figure 4.3.1.iv. Validation of *c-Fos* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances VEGF-induced upregulation of *c-Fos* gene expression in HUVEC. HUVEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25 ng/mL) for 1 hour. Expression of *c-Fos* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with *post-hoc* Tukey's multiple comparison test. \*\*\*\* indicates ( $p < 0.0001$ ). ns = non-significant.

#### 4.3.2 RNA EXPRESSION OF GENES RELATED TO ECM-CAM SIGNALLING PATHWAY IN HUVEC

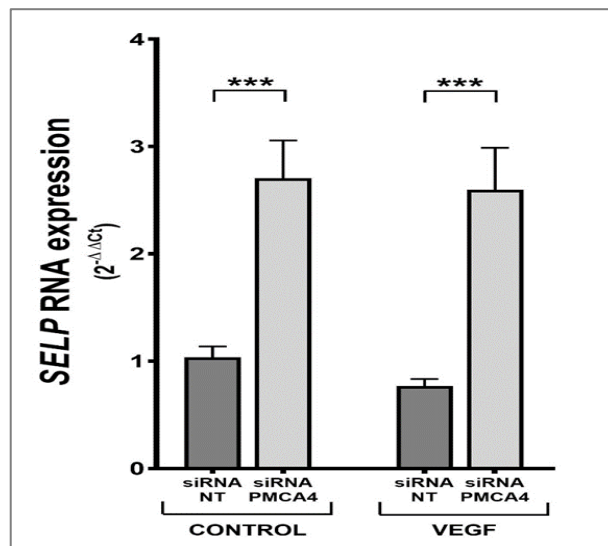
To verify the results obtained from the screening of the array of genes encoding components of the extracellular matrix and cell adhesion proteins, we performed TaqMan-based qPCR using RNA isolated from control or *PMCA4*-silenced HUVEC. Figures 4.3.2.i - 4.3.2.v shows RNA expression of *ADAMTS-1*, *VCAM-1*, *SELE*, *SELL*, and *SELP* in resting cells and after stimulation with VEGF (25 ng/mL) for 3 hours. *PMCA4* gene knockdown enhanced the VEGF-dependent upregulation of *ADAMTS1*, *SELE* and *VCAM1* in HUVEC. In the case, of *SELL* and *SELP*, *PMCA4*-knockdown increased the expression of these genes under basal conditions independently of any further stimulation.



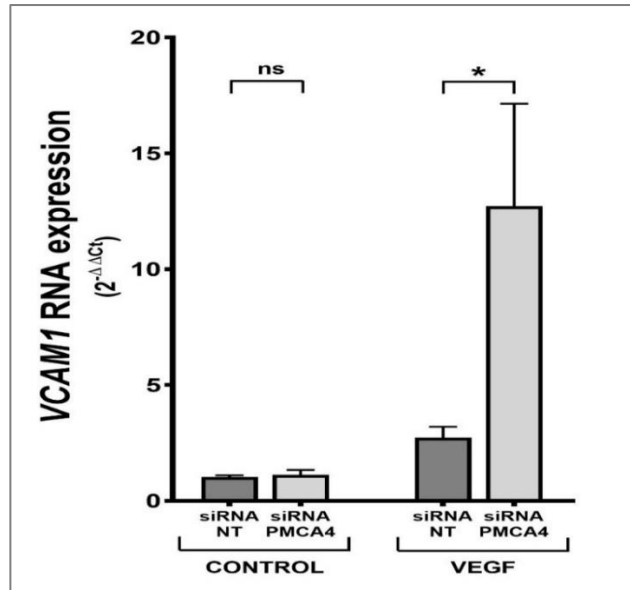
**Figure 4.3.2.i. Validation of *ADAMTS-1* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances VEGF-induced upregulation of *ADAMTS-1* gene expression in HUVEC. HUVEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25 ng/mL) for 3 hours. Expression of *ADAMTS-1* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \*\*\* indicates ( $p < 0.005$ ), ns = non-significant.



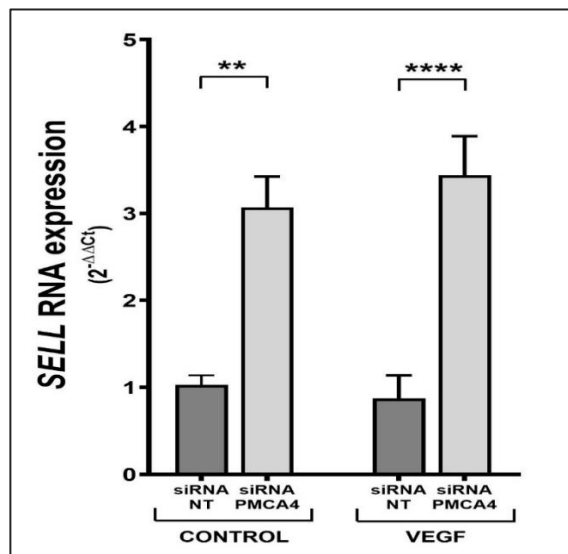
**Figure 4.3.2.ii. Validation of *SELE* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances VEGF-induced upregulation of *SELE* gene expression in HUVEC. HUVEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25ng/mL) for 3 hours. Expression of *SELE* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \* indicates ( $p < 0.05$ ). ns= non-significant.



**Figure 4.3.2.iii. Validation of *SELP* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances upregulation of *SELP* gene expression under basal condition in HUVEC. HUVEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25ng/mL) for 3 hours. Expression of *SELP* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \*\*\* indicates ( $p < 0.005$ ). ns = non-significant.



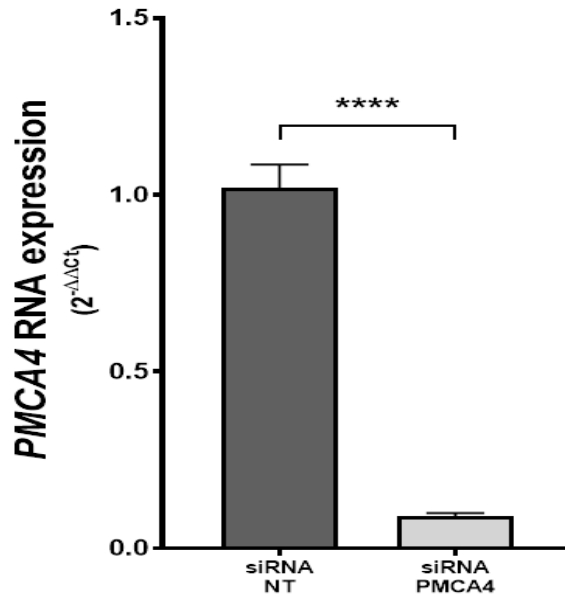
**Figure 4.3.2.iv. Validation of VCAM1 RNA expression during PMCA4 gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances VEGF-induced upregulation of *VCAM1* gene expression in HUVEC. HUVEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25ng/mL) for 3 hours. Expression of *VCAM1* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean ± SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \* indicates ( $p < 0.05$ ). ns = non-significant.



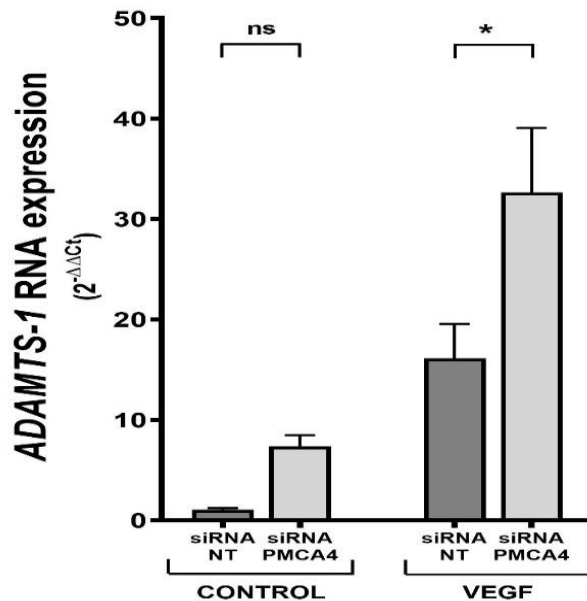
**Figure 4.3.2.v. Validation of SELL RNA expression during PMCA4 gene silencing in VEGF-stimulated HUVEC.** *PMCA4* gene knockdown strongly enhances upregulation of *SELL* gene expression under basal conditions in HUVEC. HUVEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25ng/mL) for 3 hours. Expression of *SELL* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean ± SE, n=9. Data were analysed for statistically significant differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \*\* indicates ( $p < 0.01$ ), \*\*\*\* indicates ( $p < 0.0001$ ).

### 4.3.3 RNA EXPRESSION OF GENES RELATED TO ECM-CAM IN HDMECs

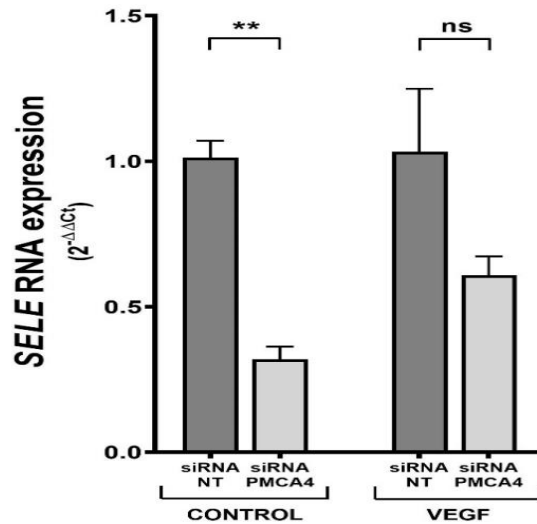
To elucidate whether the results observed in HUVEC were reproducible in other cell types of endothelial cells, we silenced *PMCA4* gene expression in Human Dermal Microvascular Endothelial cells (HDMECs) (figure 4.3.3.i). The figures 4.3.3.ii – 4.3.3.vi shows RNA expression of *ADAMTS-1*, *SELE*, *SELL*, *SELP*, and *VCAM-1* in HDMEC after *PMCA4* gene silencing, and treatment with or without the pro-angiogenic stimulus VEGF (25ng/mL) for 3 hours. Comparison of the results in the two cell types showed that the expression of genes related to ECM-CAM is varying between different cell types. We found that the gene *ADAMTS-1* is upregulated in both HUVEC and HDMEC after *PMCA4* silencing (figure 4.3.2.i and figure 4.3.3.i). We have also seen that expression of *SELE* in figure 4.3.2.ii is upregulated in HUVEC when stimulated with VEGF as expected, whereas in HDMECs the expression of *SELE* did not change after VEGF stimulation of the cells (figure 4.3.3.ii). Figure 4.3.2.v shows that *SELL* levels are up-regulated by *PMCA4* silencing even under unstimulated conditions in HUVEC, which is the same case as we observe in HDMECs (figure 4.3.3.iv). HUVEC *PMCA4*-silenced cells showed upregulated expression of *SELP* under basal conditions (Figure 4.3.2.iii) whereas in HDMEC (figure 4.3.3.v). *PMCA4* silencing did not alter *SELP* level in basal conditions or after stimulation VEGF. Finally, *PMCA4* silencing leads to upregulated levels of *VCAM1* gene expression in VEGF-stimulated HUVEC (figure 4.3.2.iv) whereas *PMCA4* knockdown does not affect expression of this gene in HDMEC (figure 4.3.3.vi).



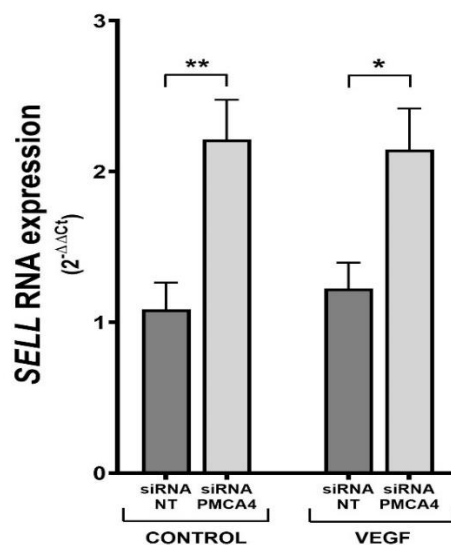
**Figure 4.3.3.i. siRNA-mediated *PMCA4* silencing leads to a strong reduction in *PMCA4* expression at the RNA level in HDMECs.** Histograms show data as mean  $\pm$  SE, n=9. \*\*\*\* indicates  $p < 0.0001$ . Statistical analysis was done using unpaired, two-tailed, Student t test to verify deviation between the independent experiments when comparing siRNA-NT and siRNA-*PMCA4* transfected cells.



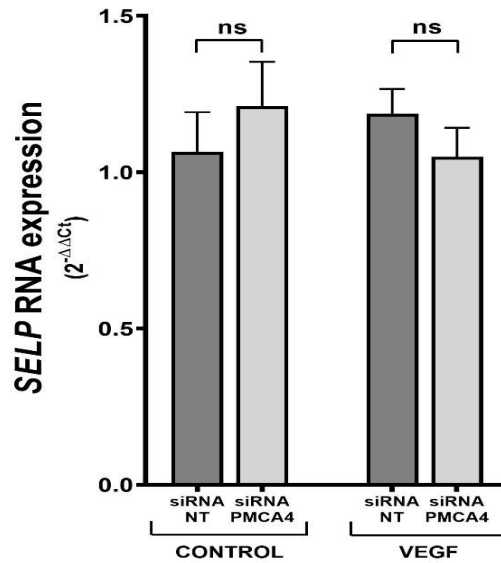
**Figure 4.3.3.ii. Validation of *ADAMTS-1* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HDMEC.** *PMCA4* gene knockdown strongly enhances VEGF-induced upregulation of *ADAMTS-1* gene expression in HDMECs. HDMEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25ng/mL) for 3 hours. Expression of *ADAMTS-1* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9.. Data were analysed for statistical significance differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \* indicates ( $p < 0.05$ ). ns = non-significant.



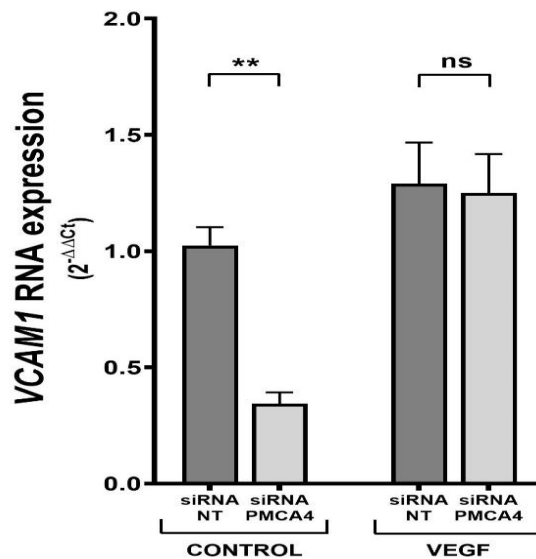
**Figure 4.3.3.iii. Validation of *SELE* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HDMEC.** *PMCA4* gene knockdown remains unchanged under basal conditions in VEGF-induced *SELE* gene expression in HDMECs. HDMEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25 ng/mL) for 3 hours. Expression of *SELE* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \*\* indicates  $p < 0.01$ ; ns= non-significant.



**Figure 4.3.3.iv. Validation of *SELL* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HDMEC.** *PMCA4* gene knockdown remains upregulated in basal conditions in VEGF-induced *SELL* gene expression in HDMECs. HDMEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25 ng/mL) for 3 hours. Expression of *SELL* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with post-hoc Tukey's multiple comparison test. \*\* indicates  $p < 0.01$ , \* indicates  $p < 0.05$ .



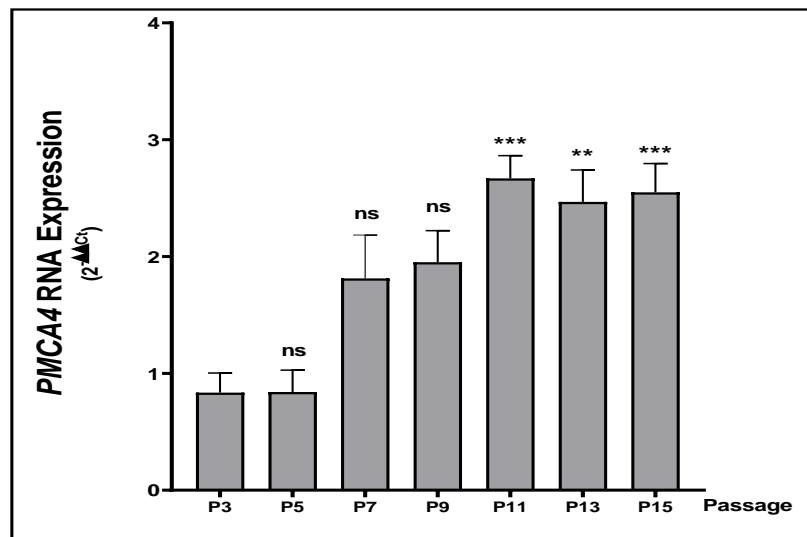
**Figure 4.3.3.v. Validation of *SELP* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HDMEC.** *PMCA4* gene knockdown remains unchanged in VEGF-induced *SELP* gene expression in HDMECs. HDMEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25 ng/mL) for 3 hours. Expression of *SELP* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with *post-hoc* Tukey's multiple comparison test. ns= non-significant.



**Figure 4.3.3.vi. Validation of *VCAM1* RNA expression during *PMCA4* gene silencing in VEGF-stimulated HDMEC.** *PMCA4* gene knockdown remains unchanged in VEGF-induced *VCAM1* gene expression in HDMECs. HDMEC transfected with si-NT or si-*PMCA4* were left unstimulated (control) or stimulated with VEGF (25 ng/mL) for 3 hours. Expression of *VCAM1* was normalised to the expression of the *Hprt-1* housekeeping gene in the same samples. Data are presented as mean  $\pm$  SE, n=9. Data were analysed for statistical significance differences by One-way ANOVA with *post-hoc* Tukey's multiple comparison test. \*\*, indicates ( $p < 0.01$ ), ns= non-significant.

#### 4.4. QUANTIFICATION OF *PMCA4* RNA EXPRESSION IN AGING HUVEC

Advanced aging of cells leads to sensitisation of endothelial cells to apoptosis and therefore cell death causing atherosclerosis due to the impairment within the vessel walls (Hoffmann *et al.*, 2001). *PMCA4* has been implicated as a regulator of apoptosis and thus we decided to analyse the RNA expression of *PMCA4* in primary cells along different passages of *in vitro* culture. RNA isolated from HUVEC cultured *in vitro* at various passages showed a progressive increase in the levels of *PMCA4*, reaching maximal levels after 11 passages where the expression of *PMCA4* increased more than 2.5-fold compared to that in young cells cultured for low passage numbers (Figure 4.4.1.i). These results indicate that *PMCA4* RNA levels increase in cell culture along with passage number, suggesting that there might be a link between expression of *PMCA4* and endothelial cell senescence.



**Figure 4.4.1.i. Bar chart representing increase in levels of *PMCA4* in aging HUVECs.** RNA levels of *PMCA4* were obtained from aging HUVECs cultured from passage 3-15. Statistical analysis was done using one-way ANOVA, Tukey's Multiple comparison test, comparing the RNA levels of day 3 with that of others. \*\*\* denotes the statistical significance  $p < 0.001$ , \*\* denotes statistical significance of  $p < 0.01$ , \* denotes  $p < 0.05$  relative to the P3 group

## **5. DISCUSSION**

Our previous findings have demonstrated that the protein PMCA4 plays a crucial role as a negative regulator of angiogenesis by downregulating the activity of intracellular signalling pathways (such as the calcineurin-NFAT pathway) involved in angiogenesis progression (Baggot *et al.*, 2014; Kurusamy *et al.*, 2017). However, we hypothesised in this study that PMCA4 might as well regulate angiogenesis by controlling the activity of other signalling pathways in endothelial cells stimulated with VEGF. A prominent regulator of VEGF-induced signalling in endothelial cells is the Notch signaling pathway, that plays a central coordinator role in endothelial cell proliferation, migration, and tubular morphogenesis during blood vessels formation in response to VEGF stimulation. The Notch pathway is involved in selection of endothelial tip cell versus stalk cells establishing its importance in the response to VEGF activation and in the fate of the nascent blood vessel. A study by Adam *et al.*, 2013, demonstrated that when HUVECs were subjected to a time dependent exposure to VEGF, there was a steady increase in the mRNA levels of *DLL4* and *Hey1* at 30 minutes and 1-hour of stimulation, decreasing after 3 hours of adding the stimulus. Here, we show that time-point analysis of *DLL1*, *Hey1*, and *DLL4* gene expression in HUVEC stimulated with VEGF showed transient increased expression of these genes peaking at 0.5h and 1h, and decay after 3h post-stimulation (Figure 4.3.1.i-4.3.1.iv). These results are consistent with the results observed in the study by Adam *et al.*, 2013. Our study has also revealed that knockdown of PMCA4 results in a significant enhancement on the VEGF-induced upregulation of *DLL1* (2.77-fold enhancement, Figure 4.3.1.ii), whereas *DLL4* expression remained unaffected by PMCA4 loss (Figure 4.3.1.iii). Limbourg *et al.*, 2007

showed that heterozygous loss of *DLL1* resulted in impaired arteriogenesis but, not microvascular angiogenesis in the ischemic limbs of mice subjected to experimentally induced hindlimb ischemia. The study by Limbourg *et al* suggests that DLL1 is an important notch ligand which regulates arteriogenesis and is shown to be predominantly expressed in arterial endothelium. They successfully demonstrated in *in vivo* models that Dll1 regulates arteriogenesis via Ephrin B2 receptor expressed on the EC and increased the expression of arterial markers *Hes1* and *Hey1*. This is also consistent with our results which shows enhancement VEGF-dependent upregulation of *Hey1* in cells where PMCA4 expression was knocked-down. Interestingly, work by our laboratory has shown that blood reperfusion in the ischaemic limbs of *Pmca4<sup>-/-</sup>* mice is higher than in those of littermate wild-types (Baggott *et al.*, 2014). Further research is necessary to ascertain whether increased blood reperfusion in the ischaemic limbs of *Pmca4* KO mice is the result of enhanced arteriogenesis and/or angiogenesis. Moreover, it would be very interesting to determine the expression of *DLL1* in the arteries of PMCA4 KO animals and compare it to that in wild-type mice.

DLL4 plays a vital role in sprouting angiogenesis by maintaining the tip cell characteristics in the vascular beds thereby driving proliferation and migration of blood vessels in a VEGF dependent manner and functions by repressing the expression of *Nrp1/Vegfr2* and maintains notch signalling. *DLL1<sup>+/-</sup>* EC specific ablation models survived until birth in which, DLL4 was expressed but was unable to sustain notch activity or maintain arterial identity and its markers such as *Hey1*, Ephrin B2, *Nrp1* thus permitting the expression of COUP-TF II which is a venal marker (Gessler *et al.*, 2009). *Hey1<sup>-/-</sup>* alone did not show any

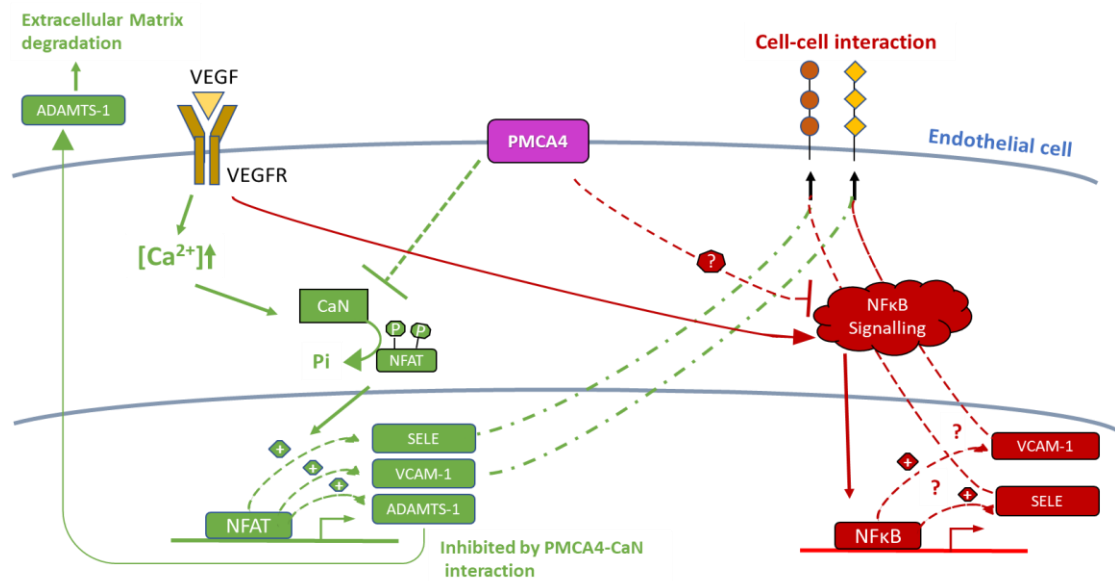
morphological defects in mouse models (Fischer *et al.*, 2004). *Hey1* and *Hey2* genes together are required for embryonic vascular development and are essential in mediating arterial fate decision (Fischer *et al.*, 2004). Therefore, DLL1-notch signalling, but not DLL4, is important in expression of arteries (Gessler *et al.*, 2009). Although DLL1 and DLL4 show structural similarities, they are having functional differences and their ectodomains play a vital role in receptor selectivity (Tveriakhina *et al.*, 2018). It will be very interesting to analyse whether PMCA4 plays any role on post-natal development of blood vessel in mouse retina. Our results show that lack of PMCA4 in endothelial cells does not alter VEGF-induced expression of *DLL4*, which is a pivotal regulator of physiological post-natal retinal angiogenesis (Suchting *et al.*, 2007). However, we have seen that lack/inhibition of PMCA4 function interferes with pathological angiogenesis processes (Baggot *et al.*, 2014; Kurusamy *et al.*, 2017). Therefore, the exact role of PMCA4 in physiological versus pathological angiogenesis deserves further investigation.

AP-1 is a heterodimeric transcription factor that combines cFOS and JUN proteins critical for regulating cellular growth and differentiation (Lee *et al.*, 1998). In a study by Armesilla *et al.*, 1999, *cFOS* expression was upregulated by short exposure of HUVEC to VEGF stimulation. Similarly, in a study by Holmes and Zackary 2004, when HUVECs were stimulated with 25 ng/ml VEGF an increase in mRNA levels of *cFOS* for a short exposure up to 45 minutes was observed which gradually decreased after 90 minutes of stimulus, which is also coincident with our data showing upregulation of *cFOS* at short VEGF stimulation of 1h. Also, in agreement with our results, Aung *et al.*, 2009, showed that *PMCA4b* overexpression in colon cancer cells resulted

in reduction of *cFOS* RNA expression. Our results show an increase in *cFOS* levels in HUVECs when we silence *PMCA4* expression. *cFOS* is an immediate responsive transcription factor controlling the expression of many target genes. Further experiments in *PMCA4*-silenced cells are required to determine the effects of enhanced *c-Fos* expression on the transcriptional regulation of target genes and the endothelial cell response to VEGF stimulation. Increasing evidence from literature shows that the process of angiogenesis begins with degradation of extracellular matrix resulting in new capillary sprout formation which is guided by hypoxia induced growth factors (Houck *et al.*, 1992). For this reason, we decided to analyse the effect of *PMCA4* silencing on the endothelial expression of metalloproteinases, on components of the extracellular matrix, and on cell adhesion molecules. Our results in HUVEC show upregulated *ADAMTS1* expression in cells stimulated with VEGF for 3 hours (Figure 4.3.2.i). These results are consistent with data previously published by Oller *et al.*, 2015. Further to VEGF-dependent upregulation, our results demonstrate that lack of *PMCA4* enhances *ADAMTS-1* expression in endothelial cells (HUVEC and HDMEC). Our previous work indicates that lack of *PMCA4* promotes angiogenesis (Baggott *et al.*, 2014; Kurusamy *et al.*, 2017) and in this study we demonstrate that lack of *PMCA4* enhances the expression of *ADAMTS1*. Therefore, it would be logical to think that *PMCA4*-mediated enhanced expression of *ADAMTS1*, would participate in increased degradation of the ECM, and therefore promote angiogenesis. However, several reports have indicated a negative role for *ADAMTS1* in the regulation of angiogenesis (Iruela-Arispe *et al.*, 2003; Luque *et al.*, 2003; Lambert *et al.*, 2020). It is thought that several factors co-operate with *ADAMTS1* to exert this inhibitory effect on angiogenesis (Lambert *et al.*, 2020). Thus, although we have only studied in this work the effect of *PMCA4* silencing on the expression of a small selection of endothelial genes, it would be very interesting to expand this study to the analysis of the whole

transcriptome, for example by performing RNA-Seq experiments, to determine whether the expression of *ADAMTS1* co-factors is also altered by lack of PMCA4.

Our results also show that PMCA4 silencing enhances the VEGF-induced upregulation of the cell adhesion molecules E-Selectin and VCAM-1 in HUVEC cells. E-Selectin and VCAM-1 are well-known endothelial mediators of leukocyte adhesion to the endothelium during inflammation (Ley *et al.*, 2007). Additionally, several groups have reported that expression of these molecules in endothelial cells in response to VEGF play an important role in angiogenesis (Nguyen *et al.*, 1993; Luo *et al.*, 1999; Koch *et al.*, 1995). Our results suggest that enhancement in the expression of these molecules in *PMCA4*-silenced cells, would support the increase in angiogenic processes previously reported in cells lacking PMCA4 (Baggott *et al.*, 2014; Kurusamy *et al.*, 2017). Consistent with a positive role as inducers of angiogenesis, we were expecting that our results using HDMEC would yield similar results to those observed in HUVEC. However, stimulation with VEGF did not upregulate *E-Sel* or *VCAM-1* expression in HDMEC (Figure 4.3.3.iii). Furthermore, PMCA4 knockdown in HDMEC neither enhanced the expression of these genes (Figure 4.3.3.iii). It will be necessary to repeat these experiments using different batches of HDMEC, and also to use different microvascular cell types in our experiments to reach a clear conclusion of these results.



**Figure 5.1. Graphical representation of CaN/NFAT and NFκB signalling pathway which leads to increase of *ADAMTS-1*, *SELE* and *VCAM-1* regulated by VEGF/VEGFR signalling.** Pathway indicated in green denotes the CaN/NFAT signalling, and pathway in red denotes NFκB signalling. It is unknown whether PMCA4 regulates NFκB signalling, and this is highlighted by “?” in the figure.

Various studies showed that VEGF increased the mRNA and protein level expression of metalloproteinase and cell adhesion molecules like ADAMTS-1, SELE and VCAM-1 in a CaN/NFAT or NFκB signalling pathway (Oller *et al.*, 2015, Stannard *et al.*, 2007, Kim *et al.*, 2001). In our experiments we successfully demonstrated that PMCA4 silencing and stimulation with VEGF upregulates the expression of *ADAMTS-1*, *SELE*, and *VCAM-1* (Fig 5.1). Stannard *et al.* (2007) showed that preincubation of ECs with VEGF activates the endothelial cells to enhance SELE upregulation by TNF-α or IL-1β in a CaN/NFAT dependent pathway. However, Kim *et al* (2001) showed that VEGF induces *SELE* and *VCAM-1* expression through the NFκB pathway. It will be interesting to see if PMCA4 affects NFκB signalling leading to regulation of *VCAM-1* and *SELE* expression (Fig 5.1), to determine if the enhancement in VEGF-induced expression of these genes observed in cells lacking PMCA4 involves the effect of the pump in CaN/NFAT and/or NFκB signalling. It has

been previously demonstrated that VEGF stimulation is necessary for upregulation of *ADAMTS-1* via CaN/NFAT pathway (Oller *et al.*, 2015; Baggott *et al.*, 2014). The authors also showed in their work that cyclosporin A (a well-known inhibitor of calcineurin signalling) significantly reduced *ADAMTS-1* gene expression in response to VEGF. We have previously established that PMCA4 inhibits the CaN/NFAT pathway in endothelial cells (Kurusamy *et al.*, 2017). Therefore, in future experiments, it will be interesting to inhibit CaN/NFAT activity using CsA, and to check whether the enhancement in the VEGF-mediated expression of *ADAMTS-1* due to PMCA4 knockdown is eliminated. In our results we see that *P-Selectin* levels are basally overexpressed in HUVEC silenced for PMCA4 expression. Auvinen *et al.*, 2014, showed that P-Selectin was present under physiological conditions in embryonic endothelial cells in mouse models starting from week 11, suggesting the involvement of P-Selectin in developmental angiogenic processes. Moreover, expression of P-selectin is elevated in the vitreous of patients with neovascular ocular diseases such as proliferative diabetic retinopathy (McLeod *et al.*, 1995) suggesting a positive role in pathological angiogenesis. In this sense, lack of P-selectin impaired platelet deposition in tumors, and thus decreased secretion of VEGF by adherent platelets leading to a reduction in tumor angiogenesis (Qi *et al.*, 2015). These works indicate that P-Selectin plays a positive role as a mediator of angiogenesis and therefore, elevated levels in PMCA4-silenced cells are concurrent with the increased angiogenesis previously reported by us in cells lacking PMCA4 (Baggot *et al.*, 2014; Kurusamy *et al.*, 2017). L-Selectin, is a cell surface molecule majorly present on the leucocytes, aiding in its recruitment into the

endothelium during inflammation (Tedder *et al.*, 1995). Their levels tend to increase during post-ischemic conditions on the endothelial cells to induce adaptive migratory response to external stimulus (Ley., 2003). Simon *et al.*, 1999 in their paper described that in response to inflammatory stimulus, the endothelial cells express L-Selectin to capture the circulating leukocyte. Our study demonstrates that suppression of PMCA4 in both HUVEC and HDMEC results in an increase in the basal expression of L-Selectin. Yukami *et al.*, 2007 have reported that mice deficient in E-Selectin, P-Selectin, L-Selectin, and ICAM-1 show suppressed angiogenesis and significant delays in the process of wound healing, indicating that these molecules all co-operate as positive mediators of angiogenesis and wound healing. It would be interesting to evaluate in further works whether suppression of PMCA4 can be used therapeutically to increase wound healing.

Cardiovascular diseases are common with increasing age. Thus, we were interested to quantify the expression of *PMCA4* during aging. As an initial step we analysed the *PMCA4* RNA expression in HUVECs along various passages. We demonstrated from two independent experiments that there was an increase in *PMCA4* RNA levels which co-related with increasing cell passage. However, we have only seen the data of *PMCA4* RNA levels in aging HUVECs and it would be interesting to quantify *PMCA4* protein levels along increasing passages. In agreement with our results, Angenendt *et al.*, 2020, have recently reported that expression of *PMCA4* increases in the T-Cells of elderly mice. More experiments are needed to validate the expression of *PMCA4* in other cell types and investigate the functional consequences of changes in *PMCA4* levels during aging.

## **6. CONCLUSION**

- Knockdown of *PMCA4* in HUVECs enhanced the VEGF-induced expression of genes related to the Notch signalling pathway, like *DLL-1* and *HEY1* suggesting that *PMCA4* regulates VEGF-induced Notch signalling by controlling the expression of gene encoding different molecules integrated in this pathway.
- Lack of *PMCA4* leads to upregulation of *SELE*, *SELP*, *SELL*, *ADAMTS-1* and *VCAM1* in HUVEC which are important genes related to ECM and cell adhesion biology. Upregulation of *SELP* and *SELL* in response to *PMCA4* silencing occurred in HUVEC cultured in basal conditions, whereas upregulation of *ADAMTS-1*, *SELE*, or *VCAM-1* required stimulation with VEGF indicating that *PMCA4* regulates the different pathways controlling the expression of these genes in basal or VEGF-stimulated conditions.
- Analysis of gene expression changes in HDMEC lacking *PMCA4* showed different results to those observed in HUVEC, indicating that *PMCA4* might control different signalling pathways in different cellular types.
- *PMCA4* RNA levels significantly increased along passage number in cultured HUVEC, suggesting that *PMCA4* might be involved in cellular senescence of endothelial cells.

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