Understanding the interactions between Neuropilin-1 and Neuropilin-2 during developmental angiogenesis

Alfie Cardwell-Brown

Candidate ID: 100227862

Abstract

Angiogenesis is the process by which new vessels form from pre-existing vasculature and is a crucial process during embryogenesis. However, dysregulation of angiogenesis can lead to vasculature irregularities resulting in pathologies such as tumor formation. Angiogenesis is known to be stimulated by various pro-angiogenic factors, such as VEGF, that activates signaling cascades involved with endothelial cell angiogenic mechanisms. It is known that VEGF-VEGFR2 binding mediates the internalization of the integrins α5β1 and αVβ3, which are integral to angiogenic regulation through the recruitment of focal adhesion proteins. Two co-receptors, NRP1 and NRP2 have been identified to form complexes with VEGFR2, increasing the receptor proteins affinity for VEGF and further regulating angiogenesis. In this way, it is hypothesized that a deletion of either NRP1 or NRP2 may hinder regulatory mechanisms controlling angiogenesis. This study therefore sets out to further investigate the effect of a singular NRP1 or NPR2 deletion, and to newly establish the effect of a deletion of both NRPs on endothelial cell processes involved with angiogenesis. Evidence found from *in vitro* immunocytochemistry experiments seem to confirm that a depletion of both NRPs reduces focal adhesion recruitment and increases focal adhesion maturation. Furthermore, a reduction in levels of both NRPs may also reduce actin cytoskeleton remodeling in response to pro-angiogenic signals. On top of this, *in vitro* experiments involving a depletion of both NRPs hint towards the establishment of an angiogenic compensatory mechanism to cope with the loss of both co-receptors. Initial *in vivo* tumour sectioning experiments have also provided data showing that blood vessel formation is reduced in lung carcinoma cells of mice with a depletion of both NRP1 and NRP2.

Contents

List of abbreviations

Abbreviation	Meaning
BM	Basal Membrane
EC	Endothelial cell
ECM	Extracellular Matrix
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FN	Fibronectin
IM	Interstitial Matrix
IMMLEC	Immortalized Microvascular Mouse Lung Endothelial Cell
KO	Knock-out
MACS	Magnetic Activated Cell Sorting
MLEC	Mouse Lung Endothelial Cell
mLMEC	Mice Lung Microvascular Endothelial Cells
MMP	Metalloproteinase
NRP	Neuropilin
PNRC	Perinuclear Recycling Complex
PyMT	Polyoma-middle-T-antigen
TI	Tunica Intima
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2

1- Introduction

1.1 Foreword

Angiogenesis is the process by which new blood vessels form from pre-existing vasculature and is regulated by a signaling cascade. This process is crucial for embryonic and postnatal growth but also for adult physiological processes, such as wound healing. Dysregulation, however, can lead to pathological irregularities such as tumor formation and growth, retinopathy, and chronic wound healing. Two key co-receptors, Neuropillin-1 and Neuropillin-2 (NRP1 and NRP2) have been identified to play a role in the angiogenic signaling cascade in conjunction with vascular endothelial growth factor receptor 2 (VEGFR2). This report will extend on previous findings from the Robinson lab that investigated the role of NRPs in angiogenesis by providing preliminary data on the effect of a loss of both NRP1 and NRP2 on angiogenic regulation.

1.2 The vascular system

The vascular system is the first organ to develop during embryogenesis and is comprised of arterial, venous and capillary circuits [1]. These vessel circuits contribute to body blood circulation and control the exchange of nutrients and waste products into and out of the blood in bodily tissue. Normal cardiovascular maturation and growth during development is therefore crucial. In adult cardiovascular systems, blood circulates either through the systemic system, providing a blood supply to bodily tissue and removing waste products, or the pulmonary system, which regulates oxygen and carbon dioxide movement in the lungs [1]. Blood vessels involved with these systems have differing physiological functions suited for different tissues. As such, vasculature is made of three layers to benefit these roles: the inner tunica intima, the middle tunica media, and the outer tunica adventitia (Figure 1). The tunica intima (TI) is the innermost layer of an artery or vein and is comprised of one layer of endothelial cells (ECs) [2]. Capillaries possess only a tunica intima layer surrounded by pericytes. In this way, the tunica intima is of high importance to study in relation to angiogenesis as capillaries are the main vessel grown in response to angiogenic signals in the peripheral vascular system [2]. For instance, dysregulation of capillary growth can contribute to tumor growth and retinopathy [3]. The TI is comprised of ECs, which are highly dynamic and can undergo significant structural remodeling in the capillary vasculature in response to angiogenic cues such as hypoxia [3]. Morphologically, capillary endothelium is very similar across the body, although capillary EC populations when compared across different organs may have differing angiogenic potentials [4]. The endothelium is supported by an extracellular matrix (ECM) which is made up of 2 compartments: a basal membrane (BM) and interstitial matrix (IM) [5]. In turn, these compartments are made up of proteins and glycosaminoglycans that help regulate the vasculature environment. The BM forms thin, sheet-like structures between the epithelium and endothelium and this proves crucial for cellular function. The

BM contains structural proteins, such as collagen IV and laminin, that anchors the BM and acts as a structure for the endothelium [5]. The BM also acts as a base for EC movement and adhesions as the ECs can interact with proteins, such as fibrillar collagen and fibronectin, in the IM [6]. This can facilitate EC migration towards angiogenic stimuli.

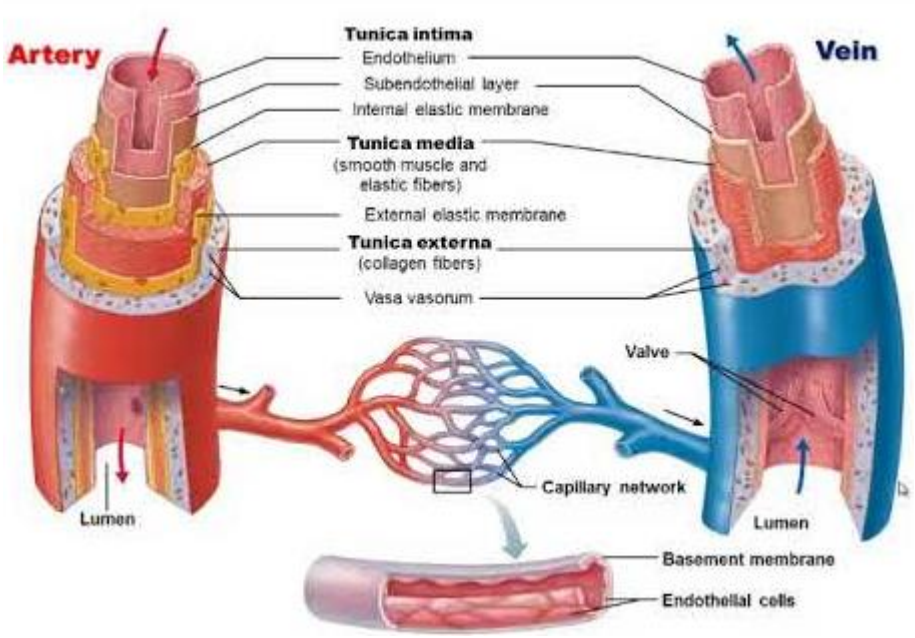

Figure 1: Vessel structure of the cardiovascular system [6]: Histologically, blood vessel walls are formed of three concentric layers: the tunica intima, the tunica media and the tunica adventitia. Large blood vessels contain all three layers, while capillary vessels consist of only the tunica intima.

1.3 Development of the vascular system

The vasculature system arises through the initial process of vasculogenesis and subsequently further develops through the process of angiogenesis. Vasculogenesis is the formation of *de novo* blood vessels in 'blood islands' from the EC precursors, hematopoietic and angioblast cells [7]. Angiogenesis is then the regulatory process by which blood pre-existing vessels formed during vasculogenesis proliferate and migrate to restructure vasculature (Figure 2).

During vasculogenesis, the EC precursors known as angioblast cells form early vessel tubes. These cells, of endodermal origin, go on to form the vasculature in organs such as the lung and in structures like the heart tube and dorsal aorta [8]. Vasculature networks set up during vasculogenesis can then be remodeled through the process of angiogenesis. This process of neovascularization is important for embryonic growth, with the vasculature for ectodermal and mesodermal derived organs such as the kidney and brain developed by angiogenesis, as well as the retinal vasculature. Angiogenesis also takes place during adult regulatory processes such as wound healing. However, dysregulation of

angiogenesis, in response to external factors such as hypoxia, can lead to the development of pathological disease such as tumor growth and chronic wound healing [9].

Recently, both the cellular and molecular mechanisms of angiogenesis have become progressively better understood. Angiogenesis is known to be initiated by a variety of protein factors that stimulate a signaling cascade. One such factor identified to regulate angiogenesis are members of the vascular endothelial growth factor (VEGF) protein family which, through VEGF receptor (VEGFR) binding, can regulate angiogenic mechanisms such as EC proliferation, migration, adhesion, and survival [10]. Through the establishment of a VEGF gradient, vessel growth direction can be determined by endothelial tip cells. Tip cells are characterized by long, dynamic filopodia and lamellipodia extensions that act to establish a route through the IM [11]. Initially, ECs involved with new blood vessel formation detach from the BM through proteolytic degradation and remodeling of the BM, mediated by metalloproteinases (MMPs) released by tip cells to allow for cell migration (Figure 2) [11]. Tip cells are followed by stalk cells which, in contrast, have a highly proliferative nature. This morphological characteristic allows for vessel outgrowth following EC detachment from the BM and leads to the establishment of a vessel lumen in response to VEGF signaling [11]. In addition to this, a mechanism mediated by NOTCH signaling, of which is yet to be established, differentiates individual stalk cells to facilitate the formation of separate vessels [12]. New vessels formed by tip and stalk cell migration then undergo anastomosis to finally fuse to surrounding vasculature [13]. Following further signals from ECs, the ECM is then remodeled, and the BM reformed to stabilize the new vasculature [13].

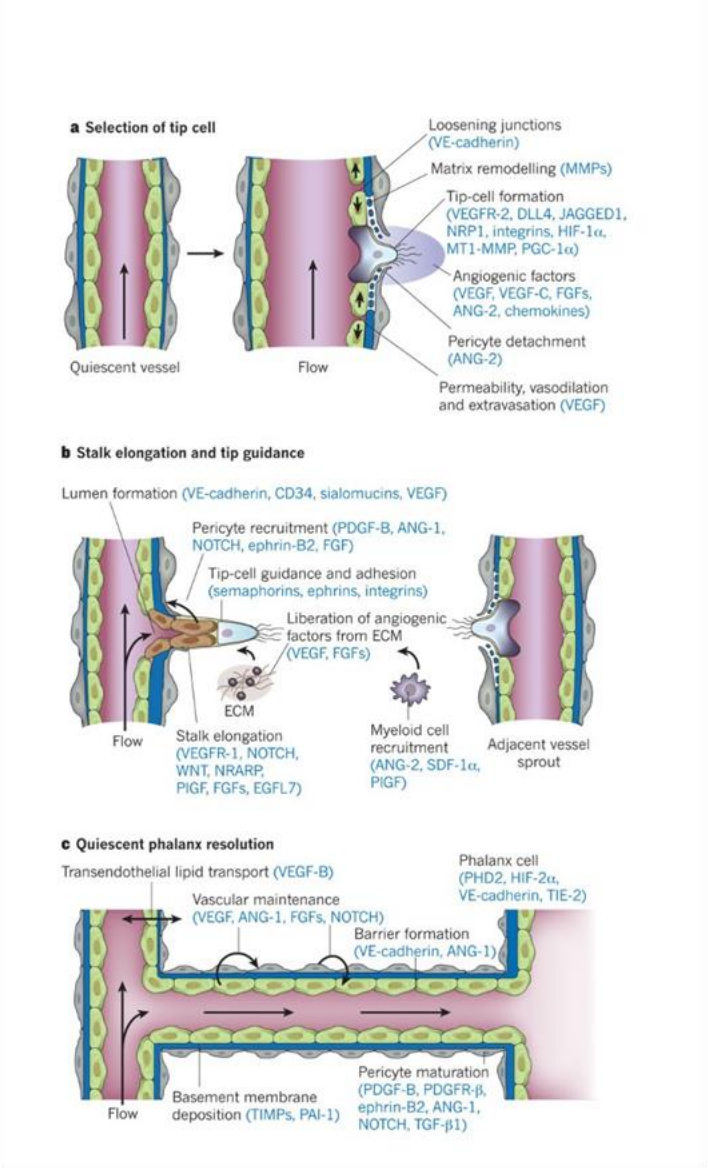

Figure 2: Angiogenic vessel remodelling [11]: A) Pro-angiogenic signals stimulate the proteolysis of the BM, allowing for tip cells to protrude from the IM. MMPs facilitate BM proteolysis with VEGFR2, NRP1 and NRP2 as well as NOTCH stimulation signalling for tip cell migration. **B)** Tip cells begin to adhere and migrate through the IM, guiding stalk cells as they migrate through further pro-angiogenic signalling. This is facilitated by the turnover of integrins such as α5β1 and αVβ3 that help FA maturation. **C)** Two tip cells meet in adjacent vasculature and the BM reforms to stabilise the newly formed vessel

1.4 Vascular Endothelial Growth Factors (VEGF) and Vascular Endothelial Growth Factor Receptors (VEGFR)

VEGF and VEGFR are two proteins that are highly important for angiogenic regulation and are thought to be the key drivers of angiogenesis [10]. The VEGF family comprises of 5 members (VEGF-A, VEGF-B, VEGF-C, VEGF-D and PIGF), each with their own function. VEGF-A, a growth factor protein, is a

widely studied pro-angiogenic regulator and has been found to interact and bind with VEGFR1 and VEGFR2, two tyrosine kinase transmembrane receptors. VEGFR2 has been found in various studies to be expressed in endothelial cells and cancer cells and has links to angiogenic regulation [14][15]. VEGFR2 appears to be the main receptor for VEGF-A mediated pro-angiogenic signalling, with VEGF-A to VEGFR2 binding found to stimulate the dimerization and autophosphorylation on the VEGFR2 tyrosine kinase domain [9]. This then initiates angiogenic downstream signalling responsible for EC proliferation, survival, migration and adhesion. For example, cascade pathways, such as the Ras/Raf/Erk/MAPK or the PI3k/Akt pathway, are stimulated by pro-angiogenic signals and have been identified in studies looking at tumorigenesis and cancer treatments [10].

1.5 The Neuropillin co-receptors

Neuropilins (NRPs) are transmembrane glycoproteins that are thought to act as co-receptors for the angiogenic receptor VEGFR. First identified in 1997, NRP1 and its ortholog NRP2 were reported to act as a receptor for SEMA3 [16]. As far as recent research, these are the only 2 confirmed forms of NRPs, sharing a 44% amino-acid similarity and are thus of a similar structure [17]. Structurally, NRPs are divided in to 4 domains: a CUB domain, a FC/FVIII domain, a MAM domain and a domain containing a transmembrane and short cytoplasmic tail region. Due to the truncation of the cytoplasmic tail, NRPs cannot transduce their own signals, with this conferring their identity as a co-receptor. In this way, the NRPs are recognised to be involved in angiogenic regulation and are identified as co-receptors for both class 3 semaphorins and VEGF family members, with the CUB and FV/FVIII domains of the NRPs acting as the binding sites for these receptors [18].

NRP1 has been identified to be expressed in multiple tumour types and upregulated on tumour associated vasculature [19]. Subsequent studies have confirmed the co-receptor capabilities of NRP1, working in conjunction with VEGFR2 and thus acting as a receptor for VEGFA isoforms, for example $VEGFA_{165}$ – the most prominent form of VEGF associated with pathological angiogenesis [19]. Numerous studies have associated both developmental and pathological angiogenesis with NRP1-VEGFR2-VEGFA binding, with dysregulation of the NRP co-receptor function found to promote tip cell function, vessel haemorrhaging and various other vessel regulatory abnormalities [20][21][24]. These studies highlight the importance of NRP1 regulation during vasculature development and hint towards a larger role as a VEGFR2 co-receptor. NRP1 is thus thought to play a large regulatory role during angiogenesis, enhancing the angiogenic functions of $VEGFA_{165}$ following VEGFR2 binding [21]. In this way, NRP1 could be a potential therapeutic target for vascular disorders in humans.

Although most research involving NRP angiogenic regulation have focussed mainly on NRP1 and VEGFR2 interactions, more recent studies have also established a similar role for NRP2, which is

perhaps not unsurprising considering the proteins' structural similarity. Most studies examining NRP2 have tended to focus on the role the co-receptor plays on tumorigenesis and cancer cell expression [22], with only more recently studies developing our knowledge of the involvement of NRP2 in angiogenic vasculature [23]. One such study found an upregulation of NRP2 in cancer cells and found a link between cell survival and either VEGF-A or VEGF-C stimulation in these cancer cells [24]. New data from the Robinson lab has also alluded more so to the role of NRP2 in angiogenesis, promoting EC cell adhesion and migration through the regulation of α5 integrin recycling [25].

1.6 The role of integrins and neuropilins in angiogenesis

Angiogenesis requires ECs to migrate over and adhere to the ECM to build new vasculature. For this to happen, cell-ECM interactions are mediated by proteins called integrins. Integrins are transmembrane receptors thatcan activate signal transduction pathways for cell adhesion, migration, proliferation, apoptosis and survival [26]. Structurally, integrins are α-β heterodimers, with each subunit possessing a large extracellular domain. These domains contain a headpiece needed for protein binding and a tail for receptor activity. ECs can regulate integrin function through changes to the integrin extracellular domain, allowing control of integrin activity and affinity for ECM ligands [26]. Importantly, integrins have both a transmembrane and cytoplasmic domain which allows for bi-directional inside-out and outside-in signal transduction [27]. This means that integrins can control signals into and out of the cell by selective pairing of various α to β subunits to extracellular and intracellular ligands. Integrins adopt an inactive 'bent' state and unbend once activated, allowing for the integrin to interact with various extracellular and intracellular proteins through elongation and separation of the integrin cytoplasmic domains [28]. Due to different α and β subunits, ECs can express at least 11 different integrins heterodimers (α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, αvβ1, αvβ3, αvβ5, α5β8 and α6β4), with each integrin able to bind different ligands and thus linked to a different cell function.. Two integrins, α5β1 and αVβ3 have been identified to contribute to angiogenic regulation [29].

Integrin signal transduction is initiated by ligand binding to the extracellular domain of integrins. Upon activation of the integrin, further binding and clustering of ligands take place that allows for enhanced signal propagation. For integrins associated with angiogenesis, these cascades can stimulate EC angiogenic mechanisms, such as cell migration, proliferation, and survival [28]. To further augment the transmission of intracellular signals, integrins can co-cluster with protein tyrosine kinases and adaptor proteins to form focal adhesion (FA) complexes. For instance, the angiogenic regulator integrin α5β1 can cluster with focal adhesion kinase (FAK) and the adaptor protein Shc [30]. FAK, having been identified to promote angiogenesis when overexpressed in mice ECs [30], is thought to be recruited and activated following EC VEGF-dependent integrin mediated adhesion to the ECM.

Following NRP1-VEGFR2-VEGF complex binding, FAK will undergo autophosphorylation which then potentiates the activation of further pro-angiogenic downstream signalling [31]. Other proteins can then be recruited at FAK FA sites. For instance, the signal transduction adaptor protein Paxillin can bind FAK and is thought to be involved with the control of EC cell migration and tumour angiogenesis [32]. Other such proteins include AKT, a protein kinase involved with cell proliferation and migration [33], and ERK, another protein kinase thought to promote angiogenesis [34]. In this way, upon ECs first adhering to a substrate, the site of integrin binding will assemble new FAs, which can develop and mature into larger FA complexes. Integrins, such as α5β1 and αVβ3, will be recycled from the trailing edge to the leading edge of the cell as it adheres and migrates along the ECM [34]. As such, FAs exist in a constant state of turnover to facilitate EC migration.

1.7 NRP and integrin interactions

Various studies have implicated multiple EC integrins with the regulation of angiogenesis. Two of these integrins, α5β1 and αVβ3, have been established to cross-talk with both NRPs and VEGFR2. The integrin αVβ3 was the first of the αV integrins to be associated with angiogenesis, with αVβ3 expression found in vessels of human tumour samples [35]. The αV integrin subunit is capable of heterodimerising with multiple beta subunits (β1, β3, β5, β6 and β8) to form several different integrins with different regulatory capabilities. When partnered these subunits act as an integrin receptor for proteins involved with EC-ECM adhesion, such as fibronectin (FN). Multiple studies have identified αVβ3 to be expressed in response to pro-angiogenic factors in various model organisms [36], with αVβ3 antagonists found to induce EC apoptosis in other studies [37]. In this way, it has been established that αVβ3 must be involved with the regulation of angiogenesis. More recently, it has been hypothesised that αVβ3 has interactions with the NRPs, with one study finding αVβ3 to enhance the activation of the NRP co-receptor VEGFR2 when also in the presence of VEGF [38]. The subsequent stimulation regulated by this interaction was found to promote EC proliferation and migration, suggesting the αVβ3 integrin and NRP-VEGFR2 complexes interact to regulate angiogenic events. To add to this, it is thought that recycling of αVβ3 is regulated by NRP1-VEGFR2 complex stimulation, with trafficking of the αV integrin from the trailing edge to leading edge of the cell allowing for FA to form, facilitating cell migratory events [39].

Another integrin identified to be involved with angiogenic regulation is α5β1. Similarly to αVβ3, although the β1 subunit has a few potential binding partners, the α5 subunit can pair only with β1. For this reason, the α5 integrin is the main target when analysing α5β1 in relation to angiogenesis. In a few studies, the α5β1 integrin has been found to be upregulated on angiogenic vasculature [40] and, like αVβ3, also interacts with the key ECM protein FN [41]. It has been found that, during both developmental and pathological angiogenesis, FN is secreted by ECs and any deletion of FN regulatory

genes proves to either correspond to a vascular defect or result in embryonic lethality [41]. In this way the β1 integrins, specifically α5β1, are thought to play a key role in angiogenesis by acting as FN receptors and controlling EC adhesion to FN. Interestingly, one study found that a depletion of both αv and α5 subunits completely prevents the formation of FAs, lowering the level of FN adhesions by ECs, suggesting regulatory cross-talk between the α5β1 and αVβ3 integrins [42].

Although α5β1 expression is not induced by VEGF stimulation, it is thought that NRP1 and NRP2 both interact with the a5 integrin. It is thought that NRP1 interactions with α5β1 allows for the internalisation and recycling of the integrin, which in turn initiates FA complex formation of the leading edge of ECs [43]. In this way, FA turnover is thought to be linked to NRP1-α5β1 interactions whereby FA turnover rate is controlled by the speed at which α5β1 can be trafficked by the cell. It is then understood that, following signals to do so, α5β1 is internalised from disassembling FAs by endocytosis to be recycled from the trailing edge to the leading edge of the cell to from new adhesions [44]. This recycling is facilitated by endosomes that regulate cytoskeletal changes to drive cell migration. It has previously been established that α5β1 is driven by Rab-11 positive vesicular endosomes through a 'long-loop' recycling pathway. Through this pathway, α5β1 is transported along the cell actin cytoskeleton to the perinuclear recycling compartment (PNRC) and then back to developing adhesions. Interestingly, it has been found that NRP1 mediated stimulation of α5β1 endocytosis takes place through Rab5-positive vesicular endosome 'short-loop' trafficking [44], suggesting that NRP1 mediated recycling takes place at a faster rate, elucidating to a faster cell migration rate. More recent data also suggests that NRP2 shares similar interactions with α5β1, however trafficking through NRP2 mediated endocytosis relies on the aforementioned Rab11 'long-loop' pathway [45].

1.8 Research aims and Objectives

Following on from previous data from the Robinson lab, my research will continue to further study the role of both NRPs in angiogenic regulation. My research will set out to further characterise the effect of a depletion of either NRP1 or NRP2 on angiogenesis in mouse ECs *in vitro*. However, my primary aim is to identify the effects of a depletion of both NRPs on angiogenesis by looking at FA recruitment and actin remodelling in mouse ECs *in vitro*, as well as investigating how a double depletion affects the phosphorylation of protein kinases involved with focal adhesion maturation, such as FAK. These investigations will be undertaken by performing various experiments, such as immunocytochemistry and VEGF-stimulated protein production analysed with western blotting. Furthermore, a NRP1/NRP2$^{fl/fl}$ KO mouse line has been generated to undertake *in vivo* retinopathy and tumour experiments assessing the effect of a depletion of both NRPs on vascular formation.

1.9 Immortalised cell lines

As a side project, I also helped assess the morphology of immortalised microvascular mouse-lung endothelial cells (IMMLECs). Both mine and other projects in my lab made use of IMMLECs throughout our experiments involving studies in to angiogenic regulation. In general, studies tend to make use of primary cells for experiments. Primary cells are isolated and used directly from the animal, for example from mice lung tissue, and therefore retain their original characteristics [46]. In contrast, immortalised cells undergo a process of immortalisation from primary cells and are then passaged to continue cell proliferation for an extended period. This provides large populations of homogenous and genetically identical cells. In this sense, immortalised cells provide benefits in our studies that primary cells cannot. For example, by obtaining large cell quantities, we can extract large amount of protein for use in western blotting experiments analysing the proteins involved in angiogenic regulation when cells are depleted of NRPs. This provides useful in repeated experiments involving a very high number of cells to provide reproducible results [46]. On top of this, as immortalised cells can be kept for a much longer duration that primary cells, *in vitro* experiments can be continued and repeated over larger durations. In essence, immortalised cells allow us to be more cost effective, less wasteful, and more efficient when undertaking long studies.

However, some researchers are rightfully dubious when it comes to the characteristics of immortalised cells, with research suggesting immortalised cells may not be similar enough to primary cells to allow for a high reliability in experimental use [48]. The fact that immortalised cells can be passaged indefinitely means these cells may lack functionality when compared to primary cells, potentially losing cell 'normality' through passages as cell characteristics change. To limit problems associated with loss of cell normality, our immortalised cells were passaged a maximum of 25 times, with ECs expected to pick up genomic abnormalities at a passage of 30 or higher [49]. By undertaking experiments assessing the characteristics of FAs in our IMMLECs, we hope to establish that immortalised cells are a plausible alternative to primary cells in relation to research involving the mechanisms of angiogenesis.

2- Materials and Methods

2.1 Chemicals and antibodies

All chemicals and antibodies used for fluorescence staining and western blotting were purchased from ThermoFisher scientific and are detailed in Table 1 and Table 2.

Table 1- List of Primary antibodies. WB: Western Blotting, ICC: Immunocytochemistry.

Antigen	Reactivity	Host	Application	Supplier	Cat#Clone#
NRP2	Mouse	Rabbit	WB	CST	D39A5
NRP1	Mouse	Rabbit	WB	CST	3725S
GAPDH	Mouse	Mouse	WB	Proteintech	60004-1-1g
FAK	Mouse	Rabbit	WB	CST	3285
P-FAK	Mouse	Rabbit	WB	Abcam	Ab4814
HSC70	Mouse	Mouse	WB	SCB	b-6/sc-7298
AKT	Mouse	Rabbit	WB	CST	9272
P-AKT	Mouse	Rabbit	WB	CST	4060
Paxillin	Mouse	Rabbit	ICC	Abcam	Ab32084
α5 integrin	Mouse	Rabbit	ICC	Abcam	Ab150361
VEGFR2	Mouse	Rabbit	WB	CST	55B11
P-VEGFR2	Mouse	Rabbit	WB	CST	Y1175
Phalloidin	N/A	N/A	ICC	CST	9535

Table 2- List of conjugated Secondary antibodies.

Host	Anti-	Application	Conjugate	Supplier	Cat#Clone#
Donkey	Rabbit	ICC	Alexa®-488	Invitrogen	A-21206
Donkey	Rabbit	ICC	Alexa®-555	Invitrogen	A-31572
Goat	Rabbit	ICC	Alexa®-647	Invitrogen	A-32728

2.2 Animal breeding and generation

Animals were bred on a mixed C57BL6/129 background. All experiments were performed in accordance with UK home office regulations and the European Legal Framework for the Protection of Animals used for Scientific Purposes (European Directive 86/609/EEC). Breeding was performed prior to my research by Chris Benwell of Stephen Robinson's lab.

2.3 Mice lung EC isolation and immortalization

Mice lung microvascular ECs (mLMECs) were isolated by Chris Benwell of Stephen Robinson's lab prior to the start of my research at an age of between 3-6 weeks old as described in L.Reynolds *et al.* 2006 [66]. Lungs were removed from 3 animals at a time, collected in mouse-lung EC (MLEC) media (1:1 mixture of Ham's F-12-DMEM medium (low glucose) (Invitrogen) supplemented with 20% fetal bovine serum (FBS) (Invitrogen), penicillin/streptomycin (pen-strep): 100 units/ml (Invitrogen), glutamax: 2 mM (Invitrogen), heparin: 50 µg/ml, and endothelial mitogen: 25 mg (AbD Serotech) before being washed in 70% ethanol and returned to fresh MLEC media. Lungs were then pulped using scalpels and digested at 37°C for 1 hour in a PBS + Ca^{2+} and Mg^{2+} (both at 1mM concentrations) solution containing 0.1% collagenase I (Invitrogen) and 0.01 % DNase I (Invitrogen). Cell digests were then channelled through a 19G needle 3 times and a 21G needle once before being filtered through a 70 µm strainer and the filtrate subsequently centrifuged for 3 minutes at 260g. The resulting pellet was then resuspended in MLEC media and seeded onto 0/1% gelatine containing 10 µg/ml fibronectin (FN) and 10 µg/ml collagen in a T75 flask and incubated at 37°C overnight. Red blood cells were then removed with 2 lots of PBS washes. ECs were then positively selected for through expression of endomucin by magnetic activated cell sorting (MACS). These selected ECs were then washed with PBS and subsequently incubated with sheep-anti-rat IgC coated magnetic beads (Invitrogen) at 4°C for 30 minutes. After 3 PBS washes, cells were detached from the gelatine with 0.25% trypsin-EDTA, resuspended in MLEC media, collected in a 15ml falcon tube, and placed on a magnet for 3 minutes. The supernatant was then discarded and all ECs that were attached to beads were resuspended in fresh MLEC media and seeded into a T25 flask. A second endomucin selection was undertaken to enhance EC purity. The selected cells were then immortalised through polyoma-middle-T-antigen (PyMT), to ensure cells were immortalised without interference by transformation events, by retroviral transfection as previously described in Robinson, *et al.* (2009). Cells were then selected through Endomucin-2 positive selection, before being treated with PyMT conditioned media accompanied by polybrene (8 µg/ml) for 6 hours at 37°C, before being returned to MLEC media. The same procedure was then repeated the next day and ECs were culture in immortalised microvascular mouse-lung EC (IMMLEC) media.

2.4 Tissue culture

ECs were grown in tissue culture flasks (T25, T75 and T175) coated with 0.1% gelatine in IMMLEC media. Upon ECs reaching confluency, the IMMLEC media was removed, the cells washed with PBS and 0.25% trypsin-EDTA added to the flask for 3 minutes while kept at 37°C in a 5% CO^2 incubator to detach cells from the gelatine. The trypsin-EDTA was then neutralised with IMMLEC media, and the cell suspension was moved up to a T75 and successively a T175 flask until ECs were confluent. At each

step, the cells were stored at 37°C in a 5% CO^2 incubator. These immortalized cells were then used for future experiments between passages 5-25.

2.5 siRNA nucleofections

Transfection of ECs was performed by nucleofection, according to the Lonza general nucleofection protocol, using the Lonza 4D Core/X Unit Nucleofector™. ECs were resuspended in a nucleofection buffer (HEPES:200 mM, NaCl:137 mM, KCl:5 mM, D-glucose:6 mM, Na^2HPO^4:7 mM), (100 µl per $1x10^6$ ECs). ECs were nucleofected with siRNA duplexes (SMARTpool), with NRP1 cre positive and negative cells added to either a control or NRP2 siRNA to produce artificial control, NRP1 KO, NRP2 KO and double NRP1+NRP2 KO cells. The siRNA duplexes were then resuspended as described by the manufacturer at a concentration of 40 µM, with 5.6 µl being used per $1x10^6$ ECs.

2.6 Signalling assays

ECs were subjected to nucleofection as described in section 2.5. Nucleofected cells were seeded into 6-well plates ($2x10^5$ cells per well) coated with 10 µg/ml FN and incubated for 48 hours at 37°C in a 5% CO^2 incubator. ECs were then washed once with PBS and serum starved in serum free media (OptiMEM®; Invitrogen) for 3 hours. ECs were then incubated with VEGF stimulation for required timepoints with 30 ng/ml/well (3 µl), with VEGF activity suspended by putting EC coated plates on ice. The serum starve media was then removed along with the VEGF media and ECs were washed twice with PBS. Cells were then lysed with 60µl electrophoresis sample buffer (ESB). ECs were then used for western blotting experiments.

2.7 Western blotting

Cells were nucleofected and lysed according to section 2.5 and 2.6 respectively to obtain EC protein samples. Lysed cells were scrapped from wells using pipette tips and collected in safe-lock Eppendorf tubes containing acid washed glass beads. Cells were spun in a tissue lyser for 2 minutes at 50Hz and subsequently spun in a centrifuge at 16500g for 10 minutes. Protein quantification was then performed to quantify lysate protein concentrations using the BioRad DC protein assay. Calculated protein volumes for each cell line were then added to NuPage 10X sample denaturing agent and 4X LDS sample buffer (Life Technologies). All samples were then boiled at 95°C for 5 minutes on a dry block thermostat and centrifuged at maximum RPM for 1 minute. Protein samples of 20 µl were then added to 8% acrylamide gels along with a 5 µl loading ladder and subjected to SDS-PAGE at 100V for 2 hours. Gels were then transferred to a 0.45 µm Amersham Protran® nitrocellulose membrane (GE Healthcare, Amersham) while in a transfer buffer (700 ml H^2O, 200 ml MeOH and 100 ml of 10X transfer buffer), run at 30V for 3 hours and afterward stained with Ponceau S for 2 minutes. Any Ponceau S residue was then gently washed off with water before blocking the nitrocellulose

membrane in a 5% milk powder and 0.1% PBS Tween-20 (PBST) solution for 5 minutes and repeated three times. A primary antibody (a 1:1000 dilution in 5% BSA/PBST 0.1% solution) was then added to the strips and left on a shaker for 2 hours at room temperature, before being washed three times in 0.1% PBST for 5 mins. A 5% milk powder PBST solution was added to the strips with a horseradish peroxidase (HRP)-conjugated secondary Ab (at a 1:2000 dilution in 5% milk/PBST 0.1% solution) and left on a shaker for 2 hours at room temp. The strips were again washed in PBST as previously mentioned and treated with a 1:1 solution of Pierce ECL Western Blotting Substrate (Thermo Scientific). Chemiluminescence was detected on a ChemiDoc™ MP Imaging System darkroom (BioRad) and densitometric readings of band intensities for blots were obtained using Image J.

2.8 Immunocytochemistry

ECs were subjected to nucleofection as described in section 2.5. ECs were then seeded into 4 sets of 10cm tissue culture dishes coated with FN (10 µg/ml) and incubated for 24-48 hours at 37°C in a 5% CO^2 incubator. ECs were then PBS washed, detached from FN with 0.25% trypsin-EDTA, and seeded on to acid washed coverslips in wells coated with FN (10 ug/ml), at a density of $2.5x10^4$ cells per well, and incubated at 37°C in a 5% CO^2 incubator for 90- and 180-minute timepoints. The coverslips were then washed with PBS and 4% paraformaldehyde (PFA) was added for 10 minutes at room temperature, before being washed again with PBS and subsequently stored in PBS overnight at 4°C. Coverslips were then blocked in 0.5 ml goat serum and PBS 0.3% triton (at a 1:100 dilution) for 1 hour at room temperature and stained in a suitable primary antibody at a 1:1000 dilution and stored in PBS overnight at 4°C. The primary antibody was then washed off in PBS three times and stained with a suitable alexa-fluorescent antibody solution in PBS at a 1:200 dilution for 1 hour at room temperature in the dark. Actin staining was also undertaken at this stage by adding 5 µl phalloidin-555 and 200 µl PBS at a 1:40 dilution for each coverslip. The secondary antibody was then removed with PBS three times and the coverslips were mounted on microscope slides using prolong® gold antifade reagent (Invitrogen) with DAPI and stored in the dark until microscope imaging. ICC imaging was undertaken using a Zeiss AxioImager M2 fluoresence microscope (AxioCam MRm camera) at 63x. FA was quantified using ImageJTM software as previously described using a FA size lower detection limit of 0.8 microns [25][50].

2.9 Tumour growth and sectioning

A NRP1$^{fl/fl}$NRP2$^{fl/fl}$ mice line was established prior to my research by Chris Benwell of the Robinson lab to achieve endothelial specific deletion of both NRPs. The NRP1$^{fl/fl}$NRP2$^{fl/fl}$ mice received intraperitoneal injections of tamoxifen (75 mg/kg body weight, 2mg/mL stock). This was administered three times a week from D4 to D17 to induce cre-recombinase activity. CMT19T lung carcinoma cells (1 X 10^6) were

implanted subcutaneously into mice at D0 and grown until D18. Mice were killed on D18, and tumors extracted and fixed in 4% PFA for blood vessel density analysis. Extracted tumors were placed in Eppendorf tubes and frozen in liquid nitrogen until tumor sectioning. Tumors were sectioned to 5 µm thin using a Cryostar NX70 Cryostat machine, and sections subsequently placed on positively charged microscope slides.

2.10 Immunofluorescence analysis of tumour sections

Tumor sections fixed in 4% PFA for 10 mins at RT. Sections were then washed twice in PBS 0.3% triton-X100, then twice in PBLEC (1x PBS, 1% Tween 20, 0.1 mM CaCl2, 0.1 mM MgCl2, 0.1 mM MnCl2) and incubated in Dako protein block serum free (Agilent). Sections were then incubated overnight at 4°C, in primary antibodies against both NRP1, NRP2 and endomucin. Sections were washed again twice in PBS 0.3% triton-X100 and PBLEC. Sections were then incubated in appropriate Alexa flour secondary for 2 hours at RT. Sections were blocked in Sudan black for 5 mins, mounted with flouromount and imaged at 10X magnification using a Zeiss AxioImager M2 microscope. Blood vessel density was assessed by counting the number of endomucin-positive vessels per mm^2 from 3 images per section and averaged.

2.11 Statistical analysis

Statistical analysis and produced graphs were generated using Students T-tests in GraphPad Prism 7 software. Bar charts show mean values and the standard error of the mean (±SEM). Asterisks indicate the statistical significance of P values: $P > 0.05$ = ns (not significant), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to the control data and whereby any lines linking bars above the graph compare corresponding data.

3- Results

3.1 A depletion of both NRPs may stimulate a compensatory mechanism to cope with disruption of the actin cytoskeleton

During angiogenesis, migrating ECs rely on the stimulation of various signaling networks for cell movement and regulation. For instance, in response to a pro-angiogenic signaling, ECs will begin FA assembly to migrate across the ECM [34]. For this to happen, the cell actin cytoskeleton must undergo remodeling into filopodia and lamellipodia to traffic various regulatory proteins, allowing for cell motility [11][29]. Given that previous evidence suggesting either a NRP1 or NRP2 depletion disrupted the actin cytoskeleton, we assessed the effect of a double NRP depletion on the actin cytoskeleton of our ECs.

ECs were isolated from the lungs of NRP1 floxed mice and subsequently immortalised before nucleofection with tatCre to generate a NRP1 KO mouse line (NRP1 KO). ECs that had not been transfected with tatCRE provided wild-type cells as controls in my experiments. These wild-type cells were transfected with non-targeting siRNA to act as the control for siRNA treated EC experiments (Ctrl), and with NRP2-targeting siRNA to provide a temporary knockout of NRP2 and thusly NRP2 depleted ECs (siNRP2). To examine the effects of a depletion of both NRPs, the isolated NRP1 KO ECs were nucleofected with NRP2 siRNA to produce a temporary knockdown of NRP2 alongside the permanent knockout of NRP1 (NRP1 KO + siNRP2).

These EC types were subsequently stained with phalloidin-555 for analysis of actin through ICC fluorescence microscopy. Fluorescent microscopy was then undertaken to analyze the amount of lamellipodia protrusions (categorised as elongated structures sprouting from ECs) and filopodia microspikes (categorised as much smaller, thinner arrangements) in each of our control, NRP1 KO, siNRP2 and NRP1 KO+ siNRP2 lines (figure 2A). Microspikes and protrusions are formed during cell migration over a matrix whereby FAs must undergo recycling from the trailing to leading edge of cells, changing cell morphology as migration occurs over a longer time. Therefore, data was collected after seeding cells on FN at a 90 and 180-minute timepoints- to assess EC migration during different parts of the cell migratory cycle. Unfortunately, microspike and protrusion data was not collected for the 180-minute timepoints.

Whilst protrusions were reduced in NPR1 KO and siNRP2 cells, NRP1 KO+siNRP2 ECs were more similar in morphology to ECs of our control group at 90-minutes (Figure 2B). In contrast, microspike numbers at 90 minutes were reduced in all three NRP depleted cell lines, although there seemed to be a small recovery in microspike numbers in NRP1 KO+siNRP2 ECs compared to NPR1 KO and siNRP2 cells. At 180 minutes, no significant differences were exhibited in protrusions or microspikes across the lines I examined. These data also suggests that if siNRP2 ECs were examined at 180-minutes it is unlikely that

there would be any differences in these either. These findings suggest that a compensatory mechanism restores angiogenic regulation and cell motility in the absence of both NRPs, at least at an early time point. The data may also suggest that the cellular processes I examined are independent of NRPs at later time points. For example, I observed no differences in any ECs at 180 minutes.

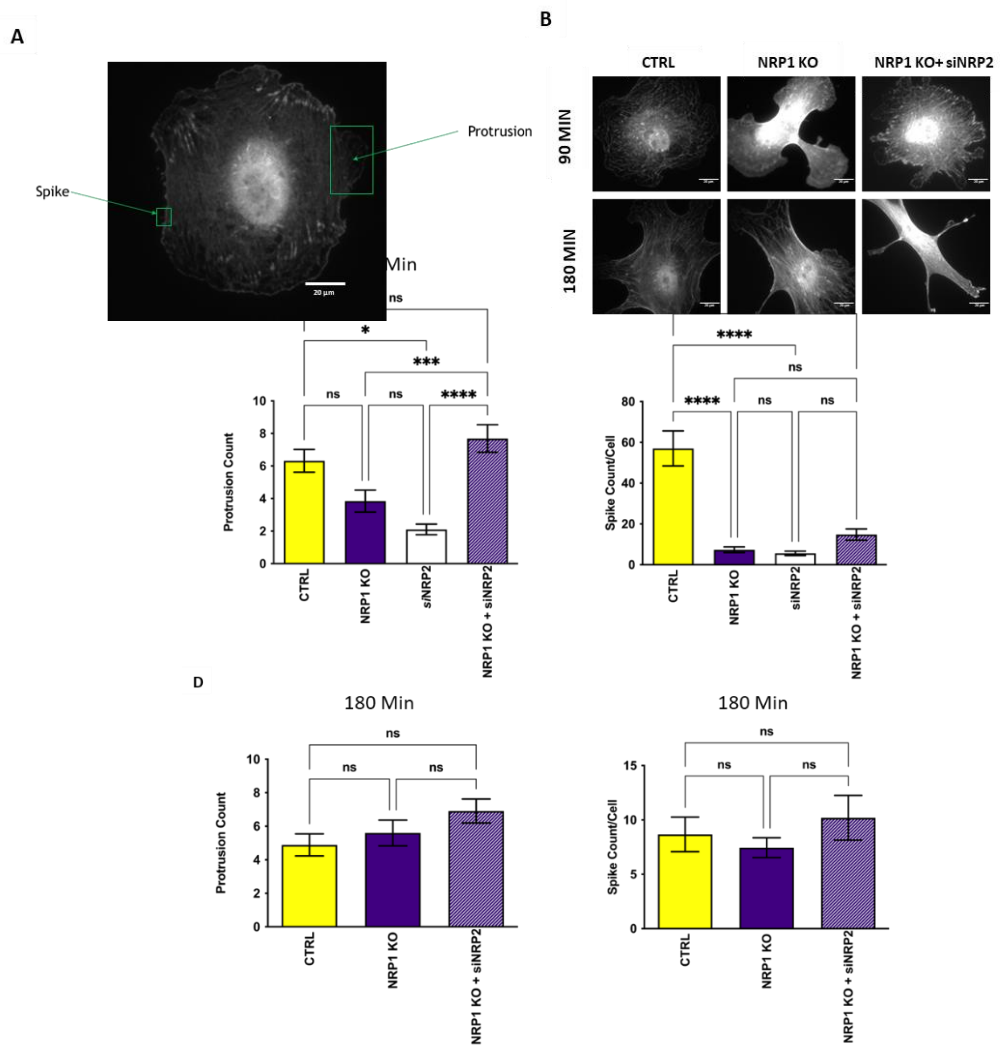

Figure 3: A depletion of both NRP1 and NRP2 could enact a compensatory mechanism to re-establish actin cytoskeletal regulation. $1x10^6$ NRP1 KO and $1x10^6$ NRP1 wild-type cells were nucleofected with either Ctrl or NRP2 siRNA and mounted on to FN-coated coverslips. After 24 hours, ECs were fixed and incubated with Phalloidin-555 to stain for actin. **A)** Cell images demonstrating cell lamellipodia protrusions and filopodia microspikes. Protrusions were categorised as elongated structures sprouting from ECs, whereas spikes were much smaller, thinner arrangements. **B)** Representative images of each cell line at 90-minute and 180-minute migration timepoints. **C and D)** Protrusion and microspike quantification, respectively, in control, NRP1 KO and NRP1 KO+siNRP2 ECs for 180-minute timepoints, and for all plus siNRP2 cells for the 90-minute timepoints. Bars represent mean (± SEM) protrusion and microspike numbers per cell, with N=3 (n=45 per condition). *=*P*<0.05 ***=*P*<0.001 ****=P<0.0001 and ns= not significant

3.2 While a NRP2 depletion impairs FA formation and maturation, depletion of just NRP1 or both NRPs promotes FA maturation

When binding to the ECM, integrins recruit proteins to regulate EC regulatory changes. One such protein is paxillin and its associated angiogenic regulator focal adhesion kinase (FAK), which are recruited at FA sites to aid cell adhesion to the ECM following VEGF stimulated pro-angiogenic signalling. Upon binding paxillin, FAK becomes autophosphorylated and subsequently promotes cell migratory events through activation of several signalling cascades [31]. In this way, paxillin acts as a marker for FAK. For ECs to migrate, FA proteins must be recruited for FAs to assemble and disassemble at the leading edge of the cell and thus provide FA complex turnover to mediate cell migration [34]. Therefore, paxillin-positive FA number and size data was collected, and ImageJ software was used to analyse FA recruitment and maturation in the cell lines previously described above. Cells were plated on FN-plated glass coverslips at a density of $2.5x10^4$ for each cell line. ECs were then stained with an anti-paxillin primary antibody before being washed and subsequently stained green with a donkey anti-rabbit Alexa 488 secondary antibody to assess paxillin-containing FAs (figure 3A). All cell lines were, again, analysed at both a 90- and 180-minute timepoint to assess FA maturation at different stages of the cell migratory cycle.

Whilst a significant decrease in the number of paxillin-containing FAs was observed in siNRP2 cells when compared to controls, both NRP1 KO and NRP1 KO+siNRP2 ECs reported no significant changes in this regard at 90-minutes (figure 3B). In contrast, FA size at 90-minutes for siNRP2 cells also decreased compared to control cells, while NRP1 KO and NRP1 KO+siNRP2 ECs elicited an increased FA size. FA size increase was not as significant in NRP1 KO+siNRP2 cells when compared to NRP1 KO cells. At 180-minutes, the only statistically significant difference noted when examining FA number was a large decrease in NRP1 KO cells. However, more differences between cell lines were observed at this time point when measuring FA size. FAs were smaller when comparing control cells to siNRP2 ECs, but larger when comparing control cells to NRP1 KO and NRP1 KO+siNRP2. These findings demonstrate that both NRP1 and NRP2 contribute to FA recruitment and maturation but when neither can do so, their absence is compensated for by an unknown NRP-independent mechanism or pathway.

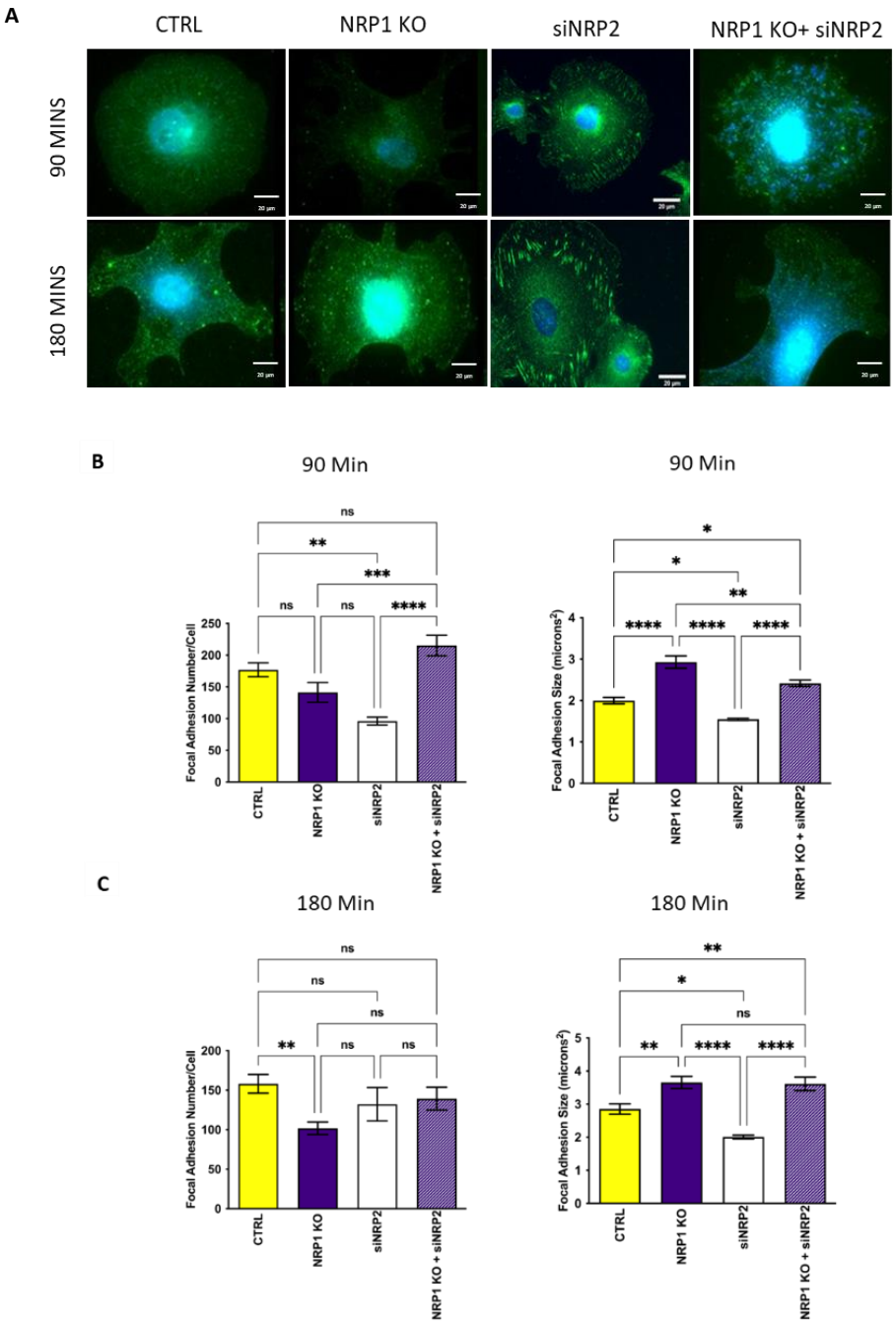

Figure 4: A depletion of both NRP1 and NRP2 does not impair and may enhance EC FA formation and maturation. $1x10^6$ NRP1 KO cells and $1x10^6$ wild-type cells were nucleofected with either Ctrl or NRP2 siRNA and mounted on to FN-coated coverslips. After 24 hours, ECs were fixed and incubated with goat anti-rabbit Alexa-647 secondary to stain for paxillin focal adhesions. **A)** Cell images demonstrating staining of each condition cell line at 90- and 180-minute timepoints. Paxillin FAs are stained green. Coverslips were mounted with Prolong Gold with DAPI and stain nuclei blue. **B and C)** Mean FA number and size (μm^2) per cell in control, NRP1 KO, siNRP2 and NRP1 KO+siNRP2 cell lines at 90-minute and 180-minute adhesion timepoints. Bars represent mean (± SEM) FA number and size per cell; with N=3 (n=45 cells per condition). *=P<0.05 **= P<0.01 ***=P<0.001 ****=P<0.0001 and ns= not significant.

3.3 Protein kinase phosphorylation activity is comparable to control ECs following depletion of both NRPs

A host of protein kinases, such as AKT, ERK and FAK are involved with cell angiogenic regulatory processes- namely EC survival, proliferation and migration. These proteins undergo phosphorylation to activate further signalling cascades involved with their specific regulatory mechanisms. Protein kinases are recruited at the leading edge of the cell following α5β1 trafficking stimulated by NRP-VEGFR-VEGF complex formation. Previous studies by the Robinson lab have found that a NRP2 depletion impairs FAK phosphorylation (pFAK), confirming the involvement of NRP2 with the autophosphorylation of FAK and subsequent formation of FAs needed to facilitate EC migration. For this reason, the amounts of these phosphorylated proteins, along with phosphorylated VEGFR2 (pVEGFR2), were analysed in relation to a single NRP1 and double NRP depletion.

Isolated NRP1 KO ECs and control ECs were again nucleofected with either non-targeting siRNA or a NRP2-targeting siRNA to produce 4 cell lines- a control, NRP1 KO, siNRP2 and NRP1 KO+ siNRP2. The cell lines were then stimulated with VEGF to trigger angiogenic signalling. This VEGF stimulation was carried out for 0-, 5- and 15-minute timepoints to provide a greater description of protein activity involved in angiogenesis. Cells were then lysed and used for Western Blotting experiments to collect expression level data for total and phosphorylated levels of VEGFR2, NRP1 and NRP2, FAK, ERK and AKT (figure 3A). Relative levels of expression were then compared to the 0-minute stimulation time point for each cell type, as well as comparing to an overall loading control protein (HSC70).

Although there were some fluctuations in protein expression levels across EC types, there were no significant changes between any cell type at any stimulation point. However, although not significant, it is still useful data to pick apart. For instance, when comparing to the phosphorylated proteins 0 stimulation timepoint, we can see that phosphorylated protein expression is highest for the NRP1+siNRP2 cell type at all stimulation points (figure 3B). There is also a high fluctuation of phosphorylated protein expression for the Western blots compared to the control across those Western blots that were compared to the HSC70 control protein (figure 3C). The NRP1+siNRP2 cell type exhibits a decreased pERK expression compared to each of the other cell types as well as a decreased expression of pAKT and pFAK at the 15-minute time points.

It is hard to further dissect this data due to inconsistencies when comparing phosphorylated protein expression in my Western blot analysis. However, it would appear from these data sets and a lack of any significant changes in the data sets, that a depletion of both NRP1 and NRP2 together would have no noticeable effect regarding the phosphorylation of proteins involved in angiogenic regulation when stimulated by the VEGF angiogenic regulator protein.

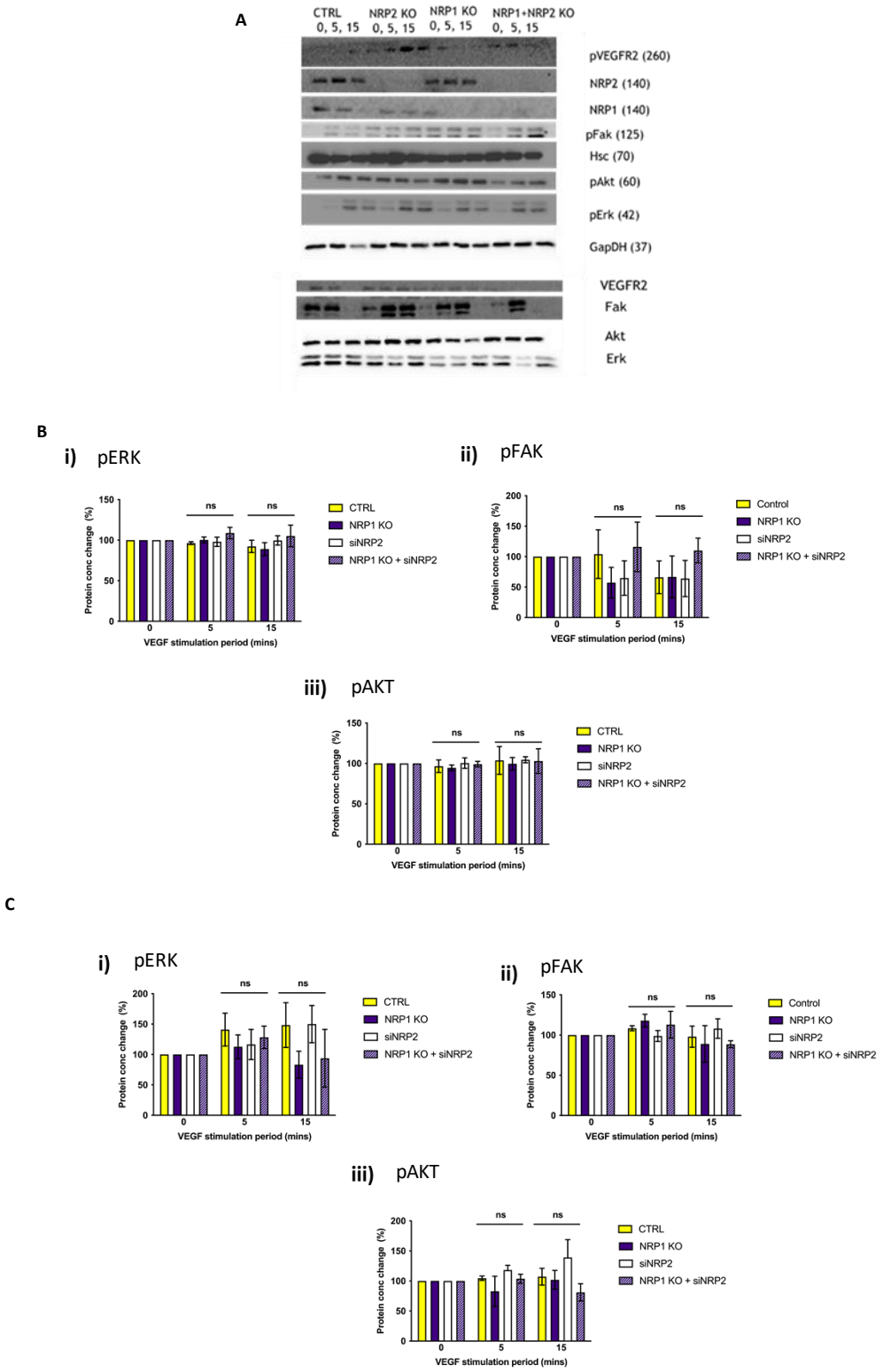

Figure 5: A depletion of both NRPs does not regulate Erk, Akt or Fak phosphorylation and has no significant effect on Erk, Akt or Fak recruitment. 3x10^5 NRP1 Cre positive cells were nucleofected with either Ctrl or NRP2 siRNA and NRP1 Cre negative cells were nucleofected with Ctrl siRNA to produce 4 cell lines: control, NRP1 KO, siNRP2 and double NRP1 KO+siNRP2. After a 48-hour incubation period, VEGF was added to the ECs to attempt stimulation of protein recruitment and phosphorylation in the cell lines at 0-, 5- and 15-minute timepoints. Protein concentrations were quantified by Western blots and protein bands and analysed using ImageJ. **A)** Representative Western blots of phosphorylated and non-phosphorylated proteins associated with NRP1 and NRP2 mediated angiogenesis. **B)** Densitometric analysis of mean band intensities for each of the pErk **(B, i),** pFak **(B, ii)** and pAkt **(B, iii)** protein concentrations respectively compared to total protein concentrations and made relative to the individual cell type VEGF stimulation 0-minute timepoint. **C)** Densitometric analysis of mean band intensities for each of the pErk **(C, i)** pFak **(C, ii)** and pAkt **(C, iii)** protein concentrations respectively compared to the Hsc70 loading control concentration and made relative to the individual cell type VEGF stimulation 0-minute timepoint. Bars represent mean (± SEM) phosphorylated protein concentrations, with N=3 per cell line. ns = not significant.

3.4 A depletion of both NRP1 and NRP2 reduces tumour blood vessel formation

As aforementioned, NRPs have a key role in angiogenic regulation through modulation of various angiogenic pathways. I therefore speculated that a depletion of both NRP1 and NRP2 would have an impact on the formation of blood vessels during angiogenesis. Thus, it was of interest to examine the effects of a depletion of both NRPs on tumour blood vessel formation.

A NRP1+NRP2 KO mouse line was established to examine the effects of a depletion of both NRPs *in vivo*, with target depletion induced by tamoxifen injection. Intraperitoneal injections of tamoxifen were administered 3 times weekly, starting at D4 through to D17, to induce cre-recombinase activity. CMT19T lung carcinoma cells (1×10^6) were implanted subcutaneously into the mice at D0, and the mice killed at D18. Tumours were subsequently removed from the NRP1+NRP2 KO mice, frozen in liquid nitrogen and sectioned before being added to positively charged microscope slides. The tumour sections were then stained with anti-endomucin before being examined using fluorescence microscopy to enumerate the blood vessel density per mm^2 of tumour sections, with data being averaged from 3 images per tumour section from 3 different sections. These data sets were then compared to tumour sections from a control mouse line. The control group was littermate controls that had still been administered with tamoxifen, however these mice were cre-negative and so did not express the NRP1+NRP2 KO phenotype.

There was a significant difference in blood vessel density between the control and NRP1+NRP2 KO tumour sections, with the blood vessel density per mm^2 reducing by almost half in the NRP1+NRP2 KO tumours. These data sets suggest that regulation of angiogenic signalling pathways involved in the growth of irregular blood vessel formation is decreased when neither of the NRPs are available for use in said regulation. This strongly hints towards the need for at least one of the NRPs for fully functioning tumour angiogenesis. This is promising data when compared to our *in vitro* experiments as it seems to confirm that a depletion of both NRPs has an effect *in vivo* on angiogenic regulation.

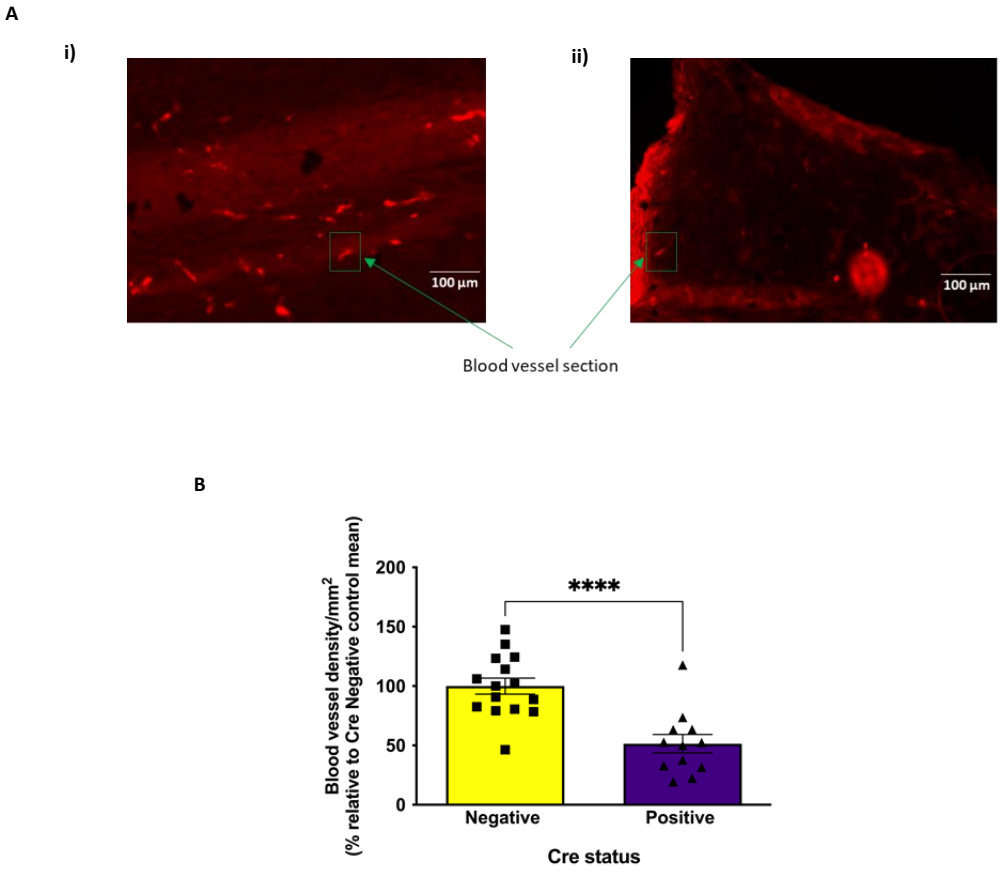

Figure 6: A depletion of both NRPs drastically reduces blood vessel formation in angiogenic ECs. CMT19T lung carcinoma cells (1 X 10^6) were subcutaneously implanted in to the mice at D0, with cre-recombinase activity induced in our mice by intraperitoneal tamoxifen injection between D4 and D17. Tumors were then extracted from each of the Cre-negative and Cre-positive Nrp1+Nrp2 KO mouse cell line. After liquid nitrogen freezing, tumors were sectioned and subsequently stained with anti-endomucin on positively charged microscope slides. **A)** Representative images of tumor sections for **(A, i)** Cre Negative and **(A, ii)** Cre Positive tumors respectively. **B)** Mean blood vessel density per mm^2 of tumor made relative to Cre negative control vessel density mean for both Cre negative and Cre positive mouse lines. Bars represent mean (± SEM) vessel density for a cell line, with individual tumor mean vessel density plotted as points on bars. ****=P<0.0001

3.5 Immortalising IMMLECs maintains similar cell morphology as primary IMMLECs

By immortalising cells, we can hope to be more efficient and less wasteful when undertaking *in vitro* studies. However, despite the Robinson Lab repeatedly showing IMMLEC's behaving similarly to primary MLECs, there is a perception in the field that IMMLEC's may not be a valid model for *in vitro* experiments. It would therefore be of good value to undertake further studies of IMMLECs to demonstrate that they can represent a good model for studying cell adhesion and migration.

To obtain primary and immortalised ECs, mice lungs were extracted from mice at an age of 3-6 weeks, minced and the tissue enzymatically digested before undertaking an endomucin selection for ECs. Selected ECs were then put through a second endomucin selection to enhance EC purity. Selected cells were then immortalised through PyMT by retroviral transfection. Cells were then selected through Endomucin-2 positive selection, treated with PyMT conditioned media, and returned to MLEC media. This procedure was then repeated, and ECs were cultured in IMMLEC media. A non-immortalised primary cell line was also maintained to compare results. Both cell types were then added to FN-covered coverslips and allowed to adhere for either a 90-minute or 16-hour timepoint to compare cell morphology and different timepoints of the cell adhesion cycle.

It was found that there is some slight variation between IMMLECs and MLECs, with this being exhibited between the 90-minute timepoint primary ECs and the 90-minute immortalised ECs, with the immortalised cells displaying significantly more FAs. There was also a small difference in cell size between the 90-minute primary ECs and the 16-hour immortalised ECs, and quite a large difference between the 16-hour primary ECs and 16-hour immortalised ECs. Thesel data suggests that immortalised cells left to adhere for longer than 90 minutes may adopt different cell characteristics to primary ECs. However, the comparable nature of the 90-minute immortalised ECs to primary ECs is a promising start towards supporting a comparable nature between the two cell types.

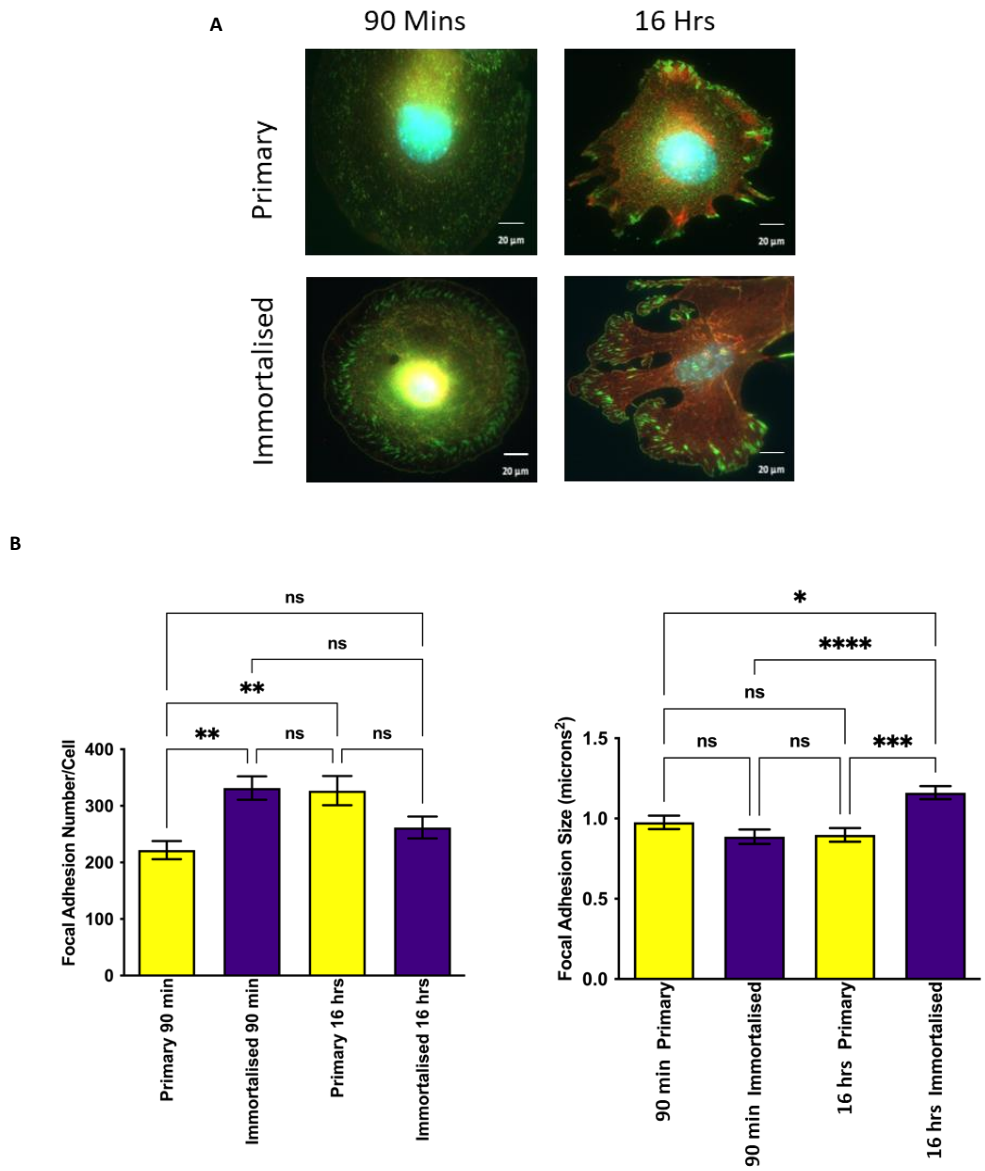

Figure 7: Immortalizing IMMLECs may change cell FA morphology. IMMLECs were isolated and immortalized and seeded on to FN-coated coverslips for 90- and 180-minute adhesion periods before being stained with goat anti-rabbit Alexa-647 (far-red) secondary to stain for paxillin focal adhesions. A) Representative images of each cell type B) Mean FA size per microns^2 in primary cells compared to immortalized cells at 90- minute and 16-hour adhesion timepoints. C) Mean FA count in primary cells compared to immortalized cells at 90- minute and 16- hour adhesion timepoints. *=P<0.05 **= P<0.01 ***=P<0.001 ****=P<0.0001 and ns= not significant.

4- Discussion and future plans

4.1 Discussion

As research into angiogenesis expands, the mechanisms behind the regulation of angiogenic events have become clearer. It is uniformly understood that the phosphorylation of VEGFR2 by binding VEGF is a core driver of the angiogenic cascade [9, 11, 12]. In addition, it is increasingly regarded that the co-receptors, NRP1 and NRP2, have a role in the regulation of angiogenesis, with their larger role relying on VEGF-independent functions [20, 22, 62]. Both NRP1 and NRP2 have been shown to regulate EC migration through their complex interactions with α5β1 and αVβ3 integrins, mediating cell migration by enhancing FA turnover [38, 42]. To further define the VEGF-independent of both NRP1 and NRP2 we examined proteins involved with FA development and the regulation of the actin cytoskeleton during angiogenesis in immortalised ECs depleted for NRP1, NRP2 and both NRP1 and NRP2. We subsequently substantiated our findings *in vivo* by analysing blood vessel formation in NRP1 and NRP2 depleted tumour cells.

EC migration heavily relies on their ability to adhere and interact with the ECM, facilitated by cell actin remodelling. This remodelling is made possible by FA protein formation and turnover, regulated by integrin binding to ECM proteins stimulating the recruitment of FA proteins. Upon VEGF stimulated autophosphorylation of VEGFR2, FAK accumulates at FAs and activates signalling cascades that promote cell adhesion and remodelling of the actin cytoskeleton [29, 30]. Through the assessment of filopodia-like actin microspikes, a previous member of the Robinson Lab identified that NRP2 depleted ECs exhibited impaired actin remodelling and reduced microspike formation compared to control ECs. On top of this, other research has shown NRP1 to promote filopodia formation in endothelial tip cells during angiogenesis [61] It was therefore of interest to further assess the impact of a depletion of NRP1 and NRP2, and newly assess the effect of a depleting both NRP1 and NRP2 on the actin cytoskeleton. As such, protrusions (elongated structures sprouting from ECs) and microspike counts were assessed in NRP1 KO, siNRP2 and NRP1+NRP2 KO cell lines. It was found that, in NRP1 depleted cells, protrusion number was not reduced at either a 90- or 180-minute timepoint, whereas the number of microspikes were reduced at a 90m-minute timepoint before seeing a recovery in number at a 180-minute timepoint. This suggests that a NRP1 depletion may initially impair actin remodelling before a compensatory mechanism is activated. This supports previous data showing that NRP1 promotes EC migration during angiogenesis [53, 55]. In comparison, NRP1KO + siNRP2 ECs also saw no significant change in protrusion count at either timepoint. Similar to NRP1 KO ECs, microspike count at the 90-minute timepoint was significantly decreased, while at the 180-minute timepoint microspike count had returned to comparable amounts to the control line. This again suggests a compensatory mechanism is in place when both NRP1 and NRP2 are lost, with this system establishing similar actin

remodelling mechanisms regulated by NRP stimulation. It is of note, however, that data for microspike count at the 90-minute timepoint may have been inaccurately pooled with previous data collected by previous research of the Robinson lab, with microspike count for the control line at this timepoint seeming exceedingly high compared to data for the 180-minute timepoint. If these experiments were repeated, it may be the case that results would still not be significant, but protrusion count would be reduced in NRP1+NRP2 KO cells. In a similar vein, although no data for siNRP2 was obtained for the 180-minute timepoint, comparable results for microspike and protrusion count at the 90-minute timepoint to the NRP1 KO and NRP1+NRP2 KO cells would suggest that results would be similar at a 180-minute timepoint. Therefore, it is presumed that a compensatory mechanism would also restore cell normality in cells with a depletion of NRP2 only. For future experiments it would therefore be of use to speculate what compensatory mechanism could be resorting cell angiogenic normality. For example, could it be that VEGFR2 uses another co-receptor other than NRP1 and NRP2 to continue the angiogenic signalling cascade, or maybe another receptor independent of VEGFR2 continues to regulate angiogenesis when both NRPs are depleted.

To further mediate cell adhesion and migratory events, NRPs regulate integrin trafficking and recycling [24]. This, in turn, leads to FA protein recruitment and maturation throughout the cell, more-so at the leading edge of the cell during cell migration. To date, over 60 focal adhesion proteins have been identified. One such protein recruited at FA sites that is associated with angiogenesis is the FAK protein, which is typically associated with paxillin. For this reason, paxillin-positive FAs and FA size was analysed in relation to a NRP1, NRP2 and a NRP1 and NRP2 depletion. Previous studies have found NRP1 depletion to correspond with a decrease in β3-integrin internalisation and trafficking, with αVβ3 thought to be more present in ECs at a later migratory timepoint than α5β1 [56]. It was therefore of no surprise that a depletion of NRP1 saw no decrease in the number of FAs per cell at the 90-minute timepoint but saw a significant decrease at the 180-minute timepoint. This is thought to be because, with a depletion of NRP1 and subsequently less internalisation of αVβ3, there would be a reduced rate of nascent FA recruitment. A depletion of NRP1 also saw an increase in FA size at both the 90-minute and 180-minute timepoints. This supports past findings that a depletion of NRP1 reduces the rate of angiogenic regulation, as without NRP1 present, there would be a decrease in FA turnover. Without NRP1 regulating FA protein recycling, the FA must continue to mature and grow when ECs are subject to a NRP1 depletion. In contrast, ECs with a depletion of both NRP1 and NRP2 saw no significant changes in FA number at either timepoint. Similar to the potential compensatory mechanism seen with protrusion and microspike count, a double KO of both NRPs may also enact a compensatory mechanism with the number of FAs formed. Interestingly however, there was a distinct increase in FA size when both NRPs were depleted. This, similarly, to ECs with only a single NRP1 depletion, may

mean that there could be an increase in FA maturation in NRP1+NRP2 KO cells. This may suggest that, although FA recruitment levels stay the same as in control cells, cells with a KO of both NRPs may have a slower FA turnover. This suggests that any compensatory mechanism ratified by a loss of both NRPs may result in a 'slow-loop' recycling of integrins and thus associated FA protein recycling to continue facilitating 'normal' angiogenic cell migration. To assess this, it would be appropriate to undertake recycling assays, to determine the rate at which the α5β1 and αVβ3 integrins are recycled during cell angiogenesis in response to a depletion of both NRPs. Finally, siNRP2 cells reported similar FA number to NRP1+NRP2 KO cells, with FA number showing no difference to control cells. Interestingly, cells with a depletion of NRP2 saw a decrease in FA size at both 90-minute and 180-minute timepoints when compared to the control cells. In contrast to cells with a depletion of NRP1 or both NRPs, these data suggests that a depletion of only NRP2 may reduce FA maturation.

Proteins recruited at FAs during EC migration, such as FAK, and other angiogenesis related proteins, such as AKT and ERK, undergo phosphorylation at FA complexes to activate angiogenic signalling cascades that stimulate downstream angiogenic cell mechanisms. For this reason, the amounts of these proteins and their phosphorylated forms were analysed in relation to VEGFR2 angiogenic regulation by stimulating our different ECs with VEGF. Previous studies have found that a depletion of either NRP1 or NRP2 depletion impairs FAK phosphorylation following VEGF stimulation [45][52], confirming the involvement of the NRPs with VEGFR2 and subsequent FA protein phosphorylation. I therefore set out to analyse the effects of a depletion of either NRP1, NRP2 or both NRPs on the levels of total and phosphorylated FAK, ERK and AKT. It was found that data sets were uniform throughout, with all of the NRP1KO, siNRP2 and NRP1KO+siNRP2 ECs exhibiting comparable pFAK, pERK and pAKT levels to the control groups. It could be expected that, when both NRPs are depleted, there would be a reduction in phosphorylated protein expression as a lack of NRPs would disrupt the angiogenic signalling cascade. This in turn would mean that any integrin-mediated signals leading to the phosphorylation of the FAK, ERK and AKT protein would be at least momentarily disabled, leading to disruption of angiogenesis. In this sense, it may have been expected that the expression of phosphorylated proteins would be reduced at a 5-minute timepoint and as previously speculated, returned to normal expression levels at a later time point due to a compensatory mechanism. I believe that this may be the case and that the data sets may have inaccuracies as Western blots were repeated multiple times, however they did not always transfer protein correctly. It may therefore be beneficial to continue with Western blot analysis in regard to a depletion of both NRPs, and maybe increase the number of repeats to help reduce the error in my data.

As it has been established that NRPs have a key role in the regulation of angiogenic pathways, it is inferred that a depletion of the NRPs will affect blood vessel formation during tumour growth. If

neither NRP1 nor NRP2 are present during the growth of a tumour, it is plausible that the number of blood vessels would be significantly decreased due to the disruption of angiogenic signalling cascades. This data shows that a depletion of both NRPs does reduce blood vessel formation and thus disrupts the process of angiogenesis *in vivo*. However, when paired with data from my previous experiments, the outcome of a depletion of both NRPs still is not fully clear, with a compensatory mechanism still only implied with angiogenic 'normality' restored at later timepoints of *in vitro* experiments. This still proves a very promising piece of data that further confirms the role of both NRPs in the regulation of angiogenesis and it would be interesting to start to delve deeper into the mechanisms that may regulate angiogenesis after the depletion of the NRPs. To further advance these findings, it would be of interest to measure the difference in size between tumours in WT mice compared to NRP1+NRP2 KO mice. This would help us further understand how NRPs control tumour growth, and whether decreased blood vessel formation in the NRP1+NRP2 KO mice would also affect the growth of tumours.

As a side experiment to my project, I helped to compare the characteristics of immortalised cells to primary cells. Experiments revolving around immortalised cell migration had been undertaken prior to my data analysis. Data from this experiment was analysed to help further initial studies looking into the capability of immortalised cells [24, 62, 63, 64] and help show that IMMLECs are a viable source for *in vitro* experiments, in the sense that immortalised cells are comparable enough to primary cells. Through using immortalised cells, our lab hopes to be less wasteful with resources, as well as providing a more efficient cell source for experiments. Immortalised cells are particularly useful for experiments used in this study as these IMMLECs allow us to produce large amounts of homogenous cells. This, in turn, helps to extract large protein quantities for use in, for example, Western Blotting experiments. For the Robinson lab to continue the use of immortalised cells, it is hoped that the cells will have similar characteristics to that of the primary cells. Our data found that there is a slight variation between the immortalised and primary cell lines. At a 90-minute timepoint, immortalised cells display significantly more FAs than primary cells. On top of this, a difference in FA size was also found between the 90-minute primary cells and 16-hour immortalised cells, and also between the 16-hour primary cells and 16-hour immortalised cells. In contrast, all other data sets for the immortalised cells had a comparable FA number and size to that of the primary cells. In essence, this data helps us to understand that there may be slight differences between the two cell types depending on the amount of time allowed to adhere to FN. However, compared to previous data from the Robinson Lab, and some similarities between the FA size and amount of the two cell types, it is plausible that immortalised cells should be continued to be used for angiogenesis experiment. However, this data does have limitations. For example, the experiments did not take into consideration the migratory

characteristics of the immortalised cells, or other cell functions. Experiments assessing the migratory speed of the cells could be useful to compare similarities of a characteristic linking to angiogenesis. On top of this, cell signalling experiments would be useful to delve into the differences between VEGF signalling in primary and immortalised cells.

4.2 Future work

To further our understanding of the effects of a depletion of both NRPs, it would be appropriate to perform *in vitro* studies focussing on scratch wound assays to look at directional migration as another measure of FA turnover. Scratch wound assays have been developed as a technique to help measure EC migration and help mimic *in vivo* cell reactions in response to removal of part of the blood vessel endothelium [65]. Similar to experiments in this report, NRP1 and NRP2 could be depleted in isolated ECs through siRNA nucleofection. This will help further our understanding of cell-cell and cell-matrix interactions after a depletion of both NRPs.

On top of this, experiments involving analysis of the internalisation and recycling of the integrins α5β1 and αVβ3 could be undertaken through surface biotinylation assays. As acknowledged, cell migration during angiogenesis relies on the recycling of these integrins to allow for the formation of FA at the leading edge of the cell. By undertaking these biotinylation assays, it will help us to further understand how the NRPs regulate integrin trafficking and confirm the hypothesis of a potential compensatory mechanism enacted in response to a depletion of both NRPs. To add to this, omics analyses would help to further internalisation experiments. By using omics analyses experiments, it would allow us to approach the genetic or molecular profiles of ECs after a depletion of NRPs. For example, we could analyse binding partners for NRP1 and NRP2, or identify genes that could be upregulated after a NRP1+NRP2 KO. This will further our current understanding of the role of the NRPs and help establish what other genes or proteins may be involved in any compensatory mechanisms established after a depletion of the NRPs.

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