The role of Agrin in tumor angiogenesis

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THE ROLE OF AGRIN IN TUMOR ANGIOGENESIS

NJAH KIZITO NGWA

SCHOOL OF BIOLOGICAL SCIENCES

2019

THE ROLE OF AGRIN IN TUMOR ANGIOGENESIS

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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AUTHORSHIP ATTRIBUTION STATEMENT

This thesis contains material from the paper; [A role of Agrin in maintaining the stability of vascular endothelial growth factor receptor-2 during tumor angiogenesis] published in Cell Report peer-reviewed journal where I was the first author.

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The contributions of the co-authors are as follows:

- Dr. Sayan Chakraborty provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Prof Hong Wanjin and Dr. Sayan Chakraborty.
- I co-designed the study with Dr. Sayan Chakraborty and performed all the laboratory work at the Institute of Molecular and Cell Biology (IMCB), Agency for Science Technology and Research (A*STAR). I also analyzed the data, prepared the figures and models except otherwise stated.
- All microscopy, including sample preparation, was conducted by KN and SC in IMCB.
- Dr. Beiyin Qui performed and analyzed the fetal mouse metatarsal assay.
- Dr. Ajaybabu Pobbati generated the recombinant Agrin fragment used in rescue experiments.
- *In-vivo* experiments were performed with inputs from; Surender Arumugam, Anandhkumar Raju, Dr. Manikandan Lakshmanan, Prof Vinay Tergaonkar. All relevant data were analyzed by me.

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ABBREVIATIONS

Ab	Antibody
AchR	Acetylcholine receptor
α/β -DG	α/β -dystroglycan
Akt	Protein kinase B
β 1	Integrin beta 1
BSA	Bovine serum albumin
CD-31	Cluster of differentiation 31
cDNA	Complementary deoxyribonucleic acid
CHX	Cycloheximide
CM	Conditioned medium
CQ	Chloroquine
DAF2-DA	Diaminofluorescein-2 Diacetate
DAG1	Dystrophin-Associated Glycoprotein 1
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECD	Extracellular domain
ECM	Extracellular matrix
ECs	Endothelial cells
EGM-2	Endothelial growth media 2
eNOS	Endothelial nitric oxide synthase
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinases1/2
FA	Focal adhesion
FAK	Focal Adhesion Kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAEC	Human Aortic Endothelial Cells
HCC	Hepatocellular carcinoma
HDMEC	Human dermal microvascular endothelial cells
HPMEC	Human pulmonary microvascular endothelial cells
HRMEC	Human retinal microvascular endothelial cells
HSPGs	Heparan sulfate proteoglycans
HUVEC	Human umbilical vein endothelial cells
IF	Immunofluorescence

IHC	Immunohistochemistry
IP	Immunoprecipitation
L-NAME	N-nitro-L-arginine-methyl ester hydrochloride
Lrp4	Low-density Lipoprotein receptor-related protein 4
kDa	Kilo daltons
MG132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
mRNA	Messenger ribonucleic acid
MuSK	Muscle-specific Kinase
NMJ	Neuromuscular junction
OSCC	Oral squamous cell carcinoma
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Rh-Lrp4	Recombinant low-density lipoprotein receptor-related protein 4
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Real-time polymerase chain reaction
sAgrin	Soluble recombinant Agrin
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
UT	Untreated
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WCL	Whole cell lysate
YAP	Yes-associated protein

LIST OF PUBLICATIONS

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ABSTRACT

Angiogenesis is the process by which new blood vessels are formed from pre-existing capillaries. It is vital for tissue perfusion during fetal growth and organ development. In adults, angiogenesis is mostly quiescent and tightly regulated by a balance of pro/anti-angiogenic factors that modulate capillary formation and density. Solid tumors via a series of epigenetic alterations termed the angiogenic switch, hijacks this balance in favor of angiogenesis and tumor vascularization. Hence, the need for effective anti-angiogenic therapies is imperative in addressing tumor malignancies. However, the current lines of therapy, targeting single ligand-receptor(s) complexes such as vascular endothelial growth factor (VEGF) and its receptors (VEGFR1 and VEGFR2) have shown clinical limitations. Therefore, an effort to broaden the identification of key receptors and their underlying regulators as a therapeutic target(s) may offer improved clinical benefits. Though several proteoglycans have been implicated in cancer and angiogenesis, their roles in endothelial cell (EC) recruitment and vascularization during tumorigenesis remain poorly understood. Here we reveal that Agrin which is overexpressed and secreted in hepatocellular carcinoma (HCC) promotes vascularization during the early stages of tumor growth by recruiting ECs within localized and metastatic lesions. Moreover, Agrin facilitates adhesion of infiltrating cancer cells to ECs which is critical during the induction of tumor angiogenesis and metastatic dissemination. In ECs, Agrin stimulates endothelial proliferation, invasion, migration, and angiogenesis *in-vitro* and *in-vivo*. Agrin-induced angiogenesis and adherence to cancer cells are mediated via Integrin- β 1, Lrp4-MuSK signaling that also requires focal adhesion kinase (FAK) activity. Mechanistically, we uncovered that Agrin regulates VEGFR2 levels that sustain the angiogenic property of ECs and adherence to cancer cells. Agrin attributes an ECM stiffness-based stabilization of VEGFR2

by enhancing interactions with Integrin- β 1-Lrp4 and additionally stimulates endothelial Nitric-oxide synthase (e-NOS) signaling. Therefore, we propose that cross-talk between Agrin expressing cancer and endothelial cells favor angiogenesis via sustaining the VEGFR2 pathway. Therefore, we suggest that targeting Agrin in combination with existing therapies aimed at VEGF-VEGFR signaling may present an attractive modality for reducing tumor angiogenesis via down-regulation of VEGFR2.

Keywords: Agrin; tumor angiogenesis; VEGFR2; cell adhesion; e-NOS; ECM stiffness; Angiogenesis, VEGFR2 stability

CHAPTER 1: INTRODUCTION

Angiogenesis Overview

Angiogenesis is the process by which new blood vessels are formed from pre-existing vasculature. It is initiated during embryonic development and orchestrates the formation of the vascular network of arteries, capillaries, and veins that transports nutrients, oxygen, and hormones critical for tissue metabolism and homeostasis (Adair and Montani, 2010; Hall, 2005).

Though angiogenesis occurs throughout life, it is mostly active *in-utero* during fetal growth and organ development. In adults, angiogenesis is mostly quiescent and tightly regulated by a balance of pro/anti-angiogenic factors that maintain tissue perfusion and capillary density (Carmeliet, 2003; Folkman, 1984; Risau, 1997). However, under pathophysiological conditions such as; wound healing and the female reproductive cycle angiogenesis is activated in adult tissues to facilitate wound repair and the vascularization of the endometrial wall respectively (Mariotti and Maier, 2006; Senger and Davis, 2011). Also, an increase in tissue metabolic stress such as that experienced during prolonged exercise and muscle building stimulates angiogenesis in adult tissues to increase muscle capillary perfusion and oxygen delivery (Bloor, 2005; Gustafsson et al., 1999). Further, increments in body weight during obesity enhances the vascularization of adipose tissue to address the increased metabolic requirements of the adipocytes (Cao, 2007; Lijnen, 2007).

Developmental Angiogenesis

Angiogenesis begins *in-utero* during the early stages of embryonic development. Pluripotent angioblast cells originating from the mesodermal germ layer differentiates to form committed endothelial cells (ECs) that cluster together to create the primitive blood islands of the yolk sac.

These blood islands are thereafter transformed into the primary vascular plexus by vasculogenesis; The *de-novo* formation of blood vessels from endothelial precursor cells (Risau and Flamme, 1995), and subsequently expanded by angiogenesis to form the vascular network of arteries, capillaries, and veins that represent the circulatory system (Veikkola and Alitalo, 1999; Veikkola et al., 2000).

Types of Angiogenesis

The formation of new blood vessels by angiogenesis requires the synergistic integration of different cellular processes such as; proliferation, invasion, directional migration and cytoskeletal reorganization of endothelial cells to form new capillaries. The newly formed vessels are subsequently remodeled by the recruitment of smooth muscle cells and pericytes that provide optimal tensile strength during blood flow (Folkman, 1984; Mariotti and Maier, 2006; Risau, 1997).

Angiogenesis can occur via sprouting of endothelial cells upon activation by angiogenic growth factors such as; vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF). Besides, new blood vessels can also be formed via interstitial tissue invasion of larger vessels leading to the formation of distinct subsidiaries via intussusceptive angiogenesis (Makanya et al., 2009; Ribatti and Crivellato, 2012; Williams et al., 2006).

Sprouting Angiogenesis

Sprouting angiogenesis is the most common form of angiogenesis encountered under standard physiological conditions (Carmeliet and Jain, 2000; Gerhardt et al., 2003). This is initiated when endothelial cells are activated by chemical or mechanical stimuli. Many cytokines and growth

factors such as VEGF or FGF have been implicated in the induction of sprouting angiogenesis. VEGF is expressed and secreted by endothelial cells (ECs) into the surrounding extracellular matrix (ECM), where it binds to its cell-surface receptors; vascular endothelial growth factor receptor 1-3 (VEGFR1-3) leading to the activation of signaling events that are critical for endothelial proliferation, survival, and angiogenesis (Eilken and Adams, 2010; Van Hinsbergh and Koolwijk, 2007).

Mechanistically, the binding of VEGF to VEGFR2 leads to receptor dimerization and conformational changes that lead to the activation of downstream effectors such as protein kinase B (Akt), extracellular signal-regulated kinase (ERK1-2), and endothelial nitric oxide synthase (eNOS) that cumulatively favor the development of new blood vessels by sprouting angiogenesis (**Figure 1.1**) (Graupera and Potente, 2013; Issbrucker et al., 2003; Olsson et al., 2006).

Figure 1.1: Model of sprouting angiogenesis

Since blood vessels are made of a single layer of endothelial cells that are interlinked by tight junctions (Bazzoni, 2006; Taddei et al., 2008), EC activation by VEGF and other pro-angiogenic factors induces a temporal disruption in the endothelial tight junctions ensuring vascular permeability and directional migration of ECs (Connolly et al., 1989; Dejana, 2004; Dvorak et al., 1995). During sprouting angiogenesis, the entire pool of endothelial cells along the capillary wall

is not uniformly activated due to intercellular communication between adjoining endothelial cells that result in differential responses to VEGF stimulation (Eilken and Adams, 2010; Hellström et al., 2007). Thus, creating two distinct groups of endothelial responders; tip cells and stalk cells. Though these cells have synergistic functions in sprouting angiogenesis, they elicit contrasting phenotypes upon VEGF activation. Some ECs assume a tip cell phenotype characterized as fast migrating but slow proliferating cells while others assume a stalk cell phenotype which is rather fast proliferating but slow migrating cells (Gerhardt et al., 2003; Tung et al., 2012). This differential activation is thought to be coordinated via the Delta-Notch signaling pathway. Activation of delta-like protein 4 (Dll4) abundant in tip cells confers the tip cell phenotype by enhancing the migratory potential of these cells. Further, cell-to-cell communication between ECs mediated by Jagged-1 inhibits the delta-notch signaling in adjoining cells. Thus, attributing a stalk cell phenotype (Benedito et al., 2009; Lobov et al., 2007). It is also proposed that the phosphorylation of different tyrosine residues within the kinase domain of VEGFR1-2 may contribute towards the characteristic tip and stalk cell phenotypes by the activation of different downstream pathways (Blanco and Gerhardt, 2013; De Smet et al., 2009).

Activation of tip endothelial cells causes cytoskeletal reorganization that facilitate the formation of locomotive filopodia required for the directional migration in response to VEGF gradient. Additionally, the tip cells secrete large amounts of proteolytic enzymes into the surrounding extracellular matrix (ECM) that degrades the basement membrane and facilitates tip cell migration (Jakobsson et al., 2010; Sainson et al., 2005). Following the tip cells are the fast-proliferating stalk cells that rapidly increase the endothelial cell population to ensure the availability of adequate cellular material for new capillary formation. Once the tip cells reach the source of the angiogenic stimuli, the ECs realign to form tube-like structures that are subsequently remodeled by pericytes

and smooth muscle cells to form a functional new capillary (Ausprunk and Folkman, 1977; Friedl and Gilmour, 2009; Ribatti and Crivellato, 2012).

Intussusceptive Angiogenesis

Alternatively, new blood vessels can be formed from pre-existing capillaries via intussusception also known as splitting angiogenesis. Here, a parent vessel splits into smaller distinct capillaries due to interstitial invasion by surrounding tissue (**Figure 1.2**) (Makanya et al., 2009). This form of angiogenesis was first confirmed by electron micrograph observations of trans-capillary tissue pillars in postnatal lung samples of rats and humans and has been subsequently demonstrated experimentally in chick choroid allantoic membrane (CAM) assay upon stimulation by VEGF-A (Djonov et al., 2003; Kurz et al., 2003). Physiologically, this sort of angiogenesis is common in vessels undergoing significant mechanical stress, especially in regions of high blood flow (Makanya et al., 2009).

Figure 1.2: Model of intussusceptive angiogenesis

Functionally, the invasive properties of endothelial sprouts are vital for tumor vascularization as it provides an extensive reach for tissue perfusion beyond the capillary bed thereby creating new

blood vessels where none existed (Adams and Alitalo, 2007; Ausprunk and Folkman, 1977; Carmeliet, 2003; Ferrara, 2000; Ribatti and Crivellato, 2012; Risau, 1997). Intussusceptive angiogenesis on the other hand, though primarily localized around the parent vessel, significantly increases the capillary bed thus, creating a larger surface area for gaseous exchange and diffusion of metabolic nutrients and waste products (Djonov et al., 2003; Kurz et al., 2003; Makanya et al., 2009). Given that sprouting angiogenesis is highly reliant on endothelial cell proliferation, it is metabolically demanding and relatively slow compared to splitting angiogenesis which only requires cytoskeletal reorganization of ECs.

Tumor Angiogenesis

Tumors are metabolically active tissues that also require a steady supply of nutrients, oxygen and waste removal from their microenvironment (Folkman, 1975; Kerbel, 2008). Though tumors begin as avascular clumps of fast-proliferating cancer cells, subsequent vascularization is critical for tumor growth and cancer progression. The acquisition of tumor dedicated capillaries initiates the vascular phases of tumor growth (Folkman, 1984, 2002; Risau, 1997). Tumors below 2-3mm in diameter are mostly avascular as they can be nourished by simple diffusion from nearby capillaries. However, as the tumor size increases, the inner core becomes increasingly unreachable by simple diffusion (Folkman, 1971, 2002). This results in hypoxia, nutrient deprivation and tissue necrosis from cytotoxic signals generated by the tumor cells. The recruitment of surrounding capillaries by induction of the angiogenic switch is therefore critical for tumor survival and cancer progression (Liao and Johnson, 2007; Shweiki et al., 1992).

The Angiogenic Switch

During tumorigenesis, cancer cells undergo various genetic and epigenetic alterations that allow the uncontrolled proliferation and survival of these cells. In addition to suppressing apoptotic signals, growing lesions initiate vascularization by disrupting the balance between pro/anti-angiogenic factors in favor of tumor angiogenesis (**Figure 1.3**) (Bergers and Benjamin, 2003; Hanahan and Folkman, 1996).

Figure 1.3: Model of the pro/anti-angiogenic factors employed in the angiogenic switch

The increasing hypoxic environment of the tumor core stimulates the expression of hypoxia-inducible factor α/β (HIF α/β); that facilitates the production of VEGF which is constantly secreted into the tumor microenvironment (Pugh and Ratcliffe, 2003; Semenza, 2002).

Chemoattraction and Endothelial Recruitment

The continual secretion of VEGF builds up a concentration gradient in the tumor microenvironment that is sensed by surrounding endothelial cells thus, inducing EC recruitment by chemotaxis and sprouting angiogenesis (Hicklin and Ellis, 2005; McMahon, 2000). Additionally, tumor cells also secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) that attract CD-34 positive cells from the bone marrow and stimulates their differentiation into committed endothelial cells that clustered to form a primitive vascular plexus in the vicinity of the growing lesion. These early vessels are subsequently expanded by angiogenesis to form tumor dedicated capillaries that perfuse the inner core (Gutschalk et al., 2013; Natori et al., 2002; Shojaei et al., 2009; Sunderkötter et al., 1994).

Mediators of Tumor Angiogenesis

The functional characterization of VEGF in the context of tumor angiogenesis and cancer progression has been well elucidated. Besides VEGF, tumor cells also secrete fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) which have complementary roles in tumor vascularization (Cao et al., 2008; CzuBAYKO et al., 1997; Dunn et al., 2000).

VEGF overview

VEGF is a large family of related growth factors with about seven known members that include, VEGF-(A-E), and placental growth factor (PlGF) (Holmes and Zachary, 2005; Li and Eriksson, 2001). Though they are encoded by different genes, they bear structural and functional similarities in the context of vascular homeostasis and angiogenesis (Clauss, 2000). VEGF-A is thought to be the most potent of these factors in eliciting an angiogenic response. Currently, about nine different

isoforms of VEGF-A have been identified. Among these variants, VEGF-A₁₆₅ is the most abundantly expressed in mammalian cells and critical for developmental and tumor angiogenesis (Risau, 1997).

Vascular endothelial growth factor receptors

The vascular endothelial growth factor receptors 1-3 (VEGFR1-3) are tyrosine kinase receptors that bind ligands of the VEGF family, inducing conformational changes in the receptor that allows the phosphorylation of specific tyrosine residues in their respective kinase domains. This receptor activation facilitates the induction of downstream signaling events that are critical for EC homeostasis and functions. VEGF signaling via VEGFR1-2 mediates pro-angiogenic responses during organ development and tumor vascularization. VEGF signaling via its cognate receptor VEGFR3, that is highly expressed in lymphatic vessels, is critical for the process of lymphangiogenesis (Cross et al., 2003; Matsumoto and Claesson-Welsh, 2001; Olsson et al., 2006).

VEGFR1

VEGF receptors exhibit ligand specificity to different members of the VEGF family. Fms-like tyrosine kinase 1 (Flt1), also known as VEGFR1, binds VEGF-A, VEGF-B, and PlGF with high specificity. VEGFR1 is expressed as a full-length transmembrane receptor or a truncated soluble form (sVEGFR1) that is secreted into the extracellular matrix (Hornig et al., 2000; Wu et al., 2010). In primary angiogenesis, VEGFR1 functions as a negative regulator of the angiogenic phenotype. Secreted sVEGFR1 competes for VEGF binding in the ECM and sequesters VEGF thereby decreasing ligand availability for VEGFR2 activation and angiogenesis (Cao, 2009; Korpelainen and Alitalo, 1998; Shibuya and Claesson-Welsh, 2006). Membrane-bound VEGFR1

also functions as a decoy to VEGFR2 by competing for VEGF-A binding given its higher affinity for the ligand when compared to VEGFR2 (Boucher et al., 2017; Ferrara et al., 2003).

Under quiescent conditions in ECs, membrane-bound VEGFR's form homodimers that are necessary for ligand recognition and activation. In this regard, homodimer formation favors VEGFR2 which is more abundantly expressed in ECs (Karkkainen and Petrova, 2000; Mac Gabhann and Popel, 2007). However, VEGFR1 levels are up-regulated during active angiogenesis leading to the formation of heterodimers between VEGFR1 and VEGFR2 that hampers angiogenic signaling by mitigating ligand recognition and activation (Lohela et al., 2009; Zhang et al., 2009).

VEGFR2

Vascular endothelial growth factor receptor 2 (VEGFR2) is a type III receptor tyrosine kinase that is encoded by the KDR gene (Parast et al., 1998). This is the main VEGF receptor during primary angiogenesis that can bind VEGF-A, VEGF-C, VEGF-D, and VEGF-E (Otrock et al., 2007). It is highly expressed in endothelial cells and elicits strong angiogenic responses to VEGF-A stimulation during developmental and tumor angiogenesis. Phosphorylation at different tyrosine residues within the kinase domain of VEGFR2 induces the activation of different signaling pathways that are vital for EC proliferation, migration, and endothelial permeability (Lamallice et al., 2007; Shibuya, 2012; Weis and Cheresh, 2011).

Vascular Permeability and Tumor Angiogenesis

The permeability of surrounding capillaries is critical for EC recruitment during tumor vascularization. Disruptions of endothelial tight junctions allow for directional migration of ECs

by sprouting angiogenesis (Connolly et al., 1989; Dvorak et al., 1995). VEGF is implicated in vascular permeability via endothelial nitric oxide synthase (eNOS) signaling and nitric oxide production (Boopathy et al., 2017; Church and Fulton, 2006; Fleming, 2010; Nakashima et al., 1999; Otrock et al., 2007; Valastyan and Weinberg, 2011; Zhang et al., 2009).

The constitutive secretion of high amounts of VEGF by growing lesions sustains eNOS signaling and vascular permeability. Consequently, tumor blood vessels appear ambiguous and often leaky (Duda et al., 2004; Dvorak, 2002; Lala and Orlucevic, 1998; Morbidelli et al., 2004). The tortuous nature of tumor vessels and the persistent angiogenic response allows the incorporation of tumor cells within the capillary wall that increases the likelihood of metastatic dissemination to secondary locations (Claffey et al., 1996; García-Román and Zentella-Dehesa, 2013; Maeda et al., 2000).

Given that tumor cells and ECs secrete a host of growth factors and cytokines into their microenvironment, the extracellular matrix (ECM) therefore serves as a reservoir for various ligands that regulate cellular processes including angiogenesis (Hynes, 2009; Paltridge et al., 2013; Taipale and Keski-Oja, 1997).

Extracellular matrix and Angiogenesis

The ECM and its constituent proteins/polysaccharides have long been recognized for their mechanical roles in maintaining tissue integrity where they serve as molecular scaffolds (Badylak et al., 2009; Grenier et al., 2005; Muiznieks and Keeley, 2013). However, given that the ECM is also a reservoir for many growth factors and cytokines secreted by various cells, cross-talk between ECM components and cell surface receptors represent a major link for extracellular to intracellular communication (Bonnans et al., 2014; Yue, 2014). Recently, we have begun to appreciate the roles

of several ECM components in mediating signaling events that regulate cellular processes including angiogenesis (Eliceiri and Cheresh, 2001; Senger and Davis, 2011; Stupack and Cheresh, 2002). Notably, heparan sulfate proteoglycans (HSPGs) such as Perlecan and collagen XVIII have been implicated in angiogenesis and tumor growth (Jiang and Couchman, 2003; Marneros and Olsen, 2005; Zhou et al., 2004). Perlecan binds and sequesters VEGF, preventing its degradation in the ECM and facilitates the presentation to its cell-surface receptors that sustain angiogenic signaling (Zoeller et al., 2009). Contrastingly, Perlecan and Collagen XVIII also elicit anti-angiogenic responses under certain physiological conditions. Proteolytic cleavage of the C-terminal domains of Perlecan and collagen XVIII generate endogenous anti-angiogenic factors endorepellin and endostatin respectively (O'Reilly et al., 1997; Poluzzi et al., 2016).

Another abundant proteoglycan in the ECM is Agrin which together with Perlecan and collagen XVIII are the most abundant HSPGs in the basement membrane (Halfter et al., 1998). However, the functional characterization of Agrin in the context of angiogenesis has not been expounded. Recently we reported that Agrin which is secreted in hepatocellular carcinoma (HCC) mediates tumorigenesis and cancer progression. Silencing Agrin in HCC severely hampered tumor growth and the invasive properties of HCC cells (Chakraborty et al., 2015; Chakraborty et al., 2017). Given that cancer progression is angiogenesis dependent, and the fact that Agrin bears structural similarity to Perlecan which is known to play diverse angiogenic roles, we envisage that Agrin may drive tumor progression in HCC by enhancing angiogenesis (Folkman, 2002; Patthy and Nikolics, 1993).

Agrin overview

Agrin is a secreted proteoglycan in the ECM that forms an integral part of the basement membrane. It was first identified in tissue extracts of the *Torpedo californica* (Nitkin et al., 1987). Agrin is best known for its role in synaptic transmission at the neuromuscular junction.

Neuromuscular Junction

Agrin expression is critical for the development of the neuromuscular junction (NMJ) during embryogenesis. Agrin facilitates the aggregation of acetylcholine receptors (AChR) at the synaptic clefts during neural transmission (Gautam et al., 1996; Ruegg and Bixby, 1998). In humans, Agrin is encoded by the *AGRN* gene which transcribes into the seven isoforms of Agrin currently identified. These variants are expressed as transmembrane or secreted forms which include muscle and neural Agrin. Alternative splicing of the *AGRN* transcript allows the insertion or deletion of amino acids at specific spliced sites; X, Y, and Z that is unique to the various isoforms and responsible for their characteristic differences (**Figure 1.4**) (Burgess et al., 1999). Insertion of 8-19 amino acids at the Z-spliced site of neural Agrin facilitates high-affinity binding to Agrin receptor; low-density lipoprotein receptor-related protein 4 (Lrp4) a member of the low-density lipoprotein receptor (LDLR) family. This interaction leads to the phosphorylation of muscle-specific tyrosine kinase (MuSK) which is critical for the aggregation of acetylcholine receptors (AChR) at the neuromuscular junction during synaptic transmission (Kim et al., 2008; Zhang et al., 2008; Zong and Jin, 2013; Zong et al., 2012). These Z⁺ variants are the predominant form expressed and secreted by neuronal cells, though many other cells including liver hepatocytes also express neural Agrin. This differs from muscle Agrin which lacks the Z-insertion (Z⁰) and is the predominant form expressed by muscle cells and other non-neuronal cells (Burgess et al., 1999).

Amino acid insertions at the Z-splice site though not absolute for Agrin function, enhances Agrin-Lrp4 binding by ~1000 fold (Kim et al., 2008).

Additionally, Agrin also binds α -Dystroglycan a transmembrane protein that is part of the dystrophin/utrophin complex which connects the ECM to the cytoskeleton (**Figure 1.4**). The dystroglycan complex is also thought to play a role in neural transmission as it co-localizes with AChR's at the post-synaptic membrane of the synaptic cleft during neural transmission (Fallon and Hall, 1994; Sugiyama et al., 1994). Besides the NMJ, Agrin also plays a critical role in bone mineralization and cartilage formation. Disruptions of Agrin function in this system is implicated in osteoarthritis (Eldridge et al., 2016). Moreover, Agrin interacts with other ECM proteins such as Laminin via the N-terminal Agrin domain (NtA) that enhances the structural integrity of the tissue microenvironment (**Figure 1.4**) (Denzler et al., 1997; Yamada et al., 1996).

Figure 1.4: Cartoon model of Agrin highlighting various domains and the respective binding partners essential for Agrin signaling. Alternative splice sites labeled X, Y, Z.

Embryogenesis and fetal development

The expression of Agrin begins early during embryogenesis and is critical for neuromuscular development. However, a comprehensive functional characterization of Agrin in this system has not been achieved due to the embryonic lethality of Agrin knockout mice. Heterozygous deletion of Agrin causes severe neuromuscular defects that decrease skeletal muscle activity including the diaphragm (Bogdanik and Burgess, 2011; Gautam et al., 1999; Ruggiu et al., 2009).

Inhibition of Agrin function by antibody-targeted therapy presents with symptoms of myasthenia gravis (MG). An autoimmune disease characterized by skeletal muscle weakness, fatigue, and breathing difficulties. Moreover, antibodies against key effectors of the neuromuscular system including; Agrin, Lrp4, MuSK, and AChR have been detected in serum samples from MG patients. Experimentally, these anti-Agrin positive samples were shown to severely hamper Agrin-mediated phosphorylation of MuSK when exposed to skeletal muscle cells *in-vitro* (Gasperi et al., 2014; Zhang et al., 2014).

Agrin in hematopoiesis

During hematopoiesis which is the process by which blood cells are formed from multipotent hematopoietic stem cells (HSC), Agrin plays a vital role in maintaining the hematopoietic stem cell niche. Also, Agrin promotes the proliferation and survival of various blood cells of the hematopoietic lineage such as neutrophils, eosinophils, basophils, monocytes, macrophages, megakaryocytes, platelets, and erythrocytes (Mazzon et al., 2011; Mazzon et al., 2012). As a result, Agrin knockout mice reveal severe anemic phenotype owing to impaired formation of red blood

cells (Anselmo et al., 2016). Additionally, Agrin promotes innate immunity beyond the hematopoietic stem cell stage by stimulating the development and functional maturation of myeloid cells via α -dystroglycan signaling that activates the extracellular signal-regulated kinases (ERK 1/2) and cytoskeletal reorganization of actin filaments (Mazzon et al., 2012).

Cardiovascular regeneration

Agrin is also implicated in cardiomyocyte regeneration post-myocardial infarction in adult mice. Unlike neonatal hearts that are capable of self-regeneration, adult cardiac muscles lack regenerative abilities due to the post-mitotic dynamics in cardiomyocytes that prevent their proliferation. In neonatal heart muscles, Agrin is identified as a key component that attributes their regenerative abilities. Moreover, injection of Agrin into adult heart muscle in mice revealed significant tissue regeneration and proliferation of cardiomyocytes. Additionally, Agrin also promotes revascularization of the cardiac wall post-myocardial infarction (Bassat et al., 2017).

Agrin in Cancer

Though Agrin is expressed in healthy hepatocytes and oral squamous epithelial cells, the relative levels are considerably lower when compared to tumor cells of the same origin (Chakraborty et al., 2015; Rivera et al., 2018). Variations in Agrin expression have been linked to many diseases including cancer. The overexpression of Agrin is implicated in tumorigenesis and cancer progression of hepatocellular carcinoma (HCC) and oral squamous cell carcinoma (OSCC) respectively (Chakraborty et al., 2015; Rivera et al., 2018).

Agrin in oral squamous cell carcinoma (OSCC)

Head and neck cancers are the 6th most common form of cancers diagnosed across the world. Oral squamous cell carcinoma represents approximately 95% of all head and neck carcinomas with about 500,000 new cases diagnosed each year. In OSCC, Agrin expression is significantly upregulated compared to non-tumorigenic cells. These high levels of Agrin enhance tumor cell proliferation, survival and invasive properties that drive tumor progression and metastatic dissemination. Silencing Agrin in OSCC xenograft models significantly mitigated tumor growth and vascularization (Rivera et al., 2018).

Agrin in hepatocellular carcinoma (HCC)

In hepatocellular carcinoma (HCC), the most common form of primary liver cancers, Agrin is implicated in tumorigenesis and cancer progression. Agrin is overexpressed and secreted in the extracellular matrix of tumor hepatocytes and promotes tumor cell proliferation and their invasive properties via maintaining focal adhesion (FA) integrity (Chakraborty and Hong, 2018; Chakraborty et al., 2015). Moreover, Agrin facilitates epithelial to mesenchymal transition (EMT) and extracellular matrix (ECM) dynamics that enhance tumor progression. Silencing Agrin in HCC xenograft models revealed a dramatic decrease in tumor sizes and relative vascularization. Furthermore, in HCC, Agrin signaling via its receptors Integrin β 1-Lrp4/MuSK attributes matrix stiffness and sustains the mechanoactivity of Yes-associated protein (YAP) leading to cancer progression (Chakraborty and Hong, 2018; Chakraborty et al., 2015; Chakraborty et al., 2017).

Agrin expression overlaps key angiogenic signatures in cancer

Angiogenesis is crucial for tumor growth and cancer progression. Many solid tumors including HCC are highly vascularized. The up-regulation of pro-angiogenic factors in cancer is consistent

with the induction of the angiogenic switch that initiates tumor vascularization (Folkman, 1971, 1975; Kimura et al., 1998; Semela and Dufour, 2004). Analysis of protein expression profiles across a multi-cancer dataset from Oncomine reveals an overlap between Agrin expression and various key angiogenic signatures (**Figure 1.5**) which may suggest the role of Agrin in tumor vascularization. Moreover, given that Agrin silencing mitigated tumor growth and vascularization in hepatocellular carcinoma and oral squamous cell carcinoma further highlights the potential of Agrin in tumor angiogenesis.

Figure 1.5: Agrin expression in multi-cancer panel and HCC. Agrin expression overlaps with pro-angiogenic factors across multiple cancers as represented by Ramaswamy multi-cancer dataset generated by Oncomine.

Anti-angiogenic therapy

The interlink between tumor angiogenesis and cancer progression provides an attractive milieu for targeting tumor growth via anti-angiogenic therapy. Given that endothelial cells in the vessel wall are more readily accessible to chemotherapeutic agents compared to the tumor mass, targeting tumor blood vessels thus, presents an attractive modality in cancer treatment. Moreover, the relative genomic stability of endothelial cells when compared to tumor cells further supports the benefits of targeting angiogenesis in cancer treatment. Besides, endothelial cells do not exhibit the diverse cellular heterogeneity that is observed in tumors thus ensuring that a single anti-angiogenic agent might effectively mitigate the progression of multiple cancers (Bergers and Hanahan, 2008; Jung et al., 2000; Vasudev and Reynolds, 2014).

However, despite the foreseen advantages of anti-angiogenic therapy over conventional anticancer therapy, it has failed to meet the anticipated clinical expectations owing to a host of factors including tumor resistance to the said therapies. Current approaches in anti-angiogenic therapy are mostly targeting single ligand-receptor(s) complexes such as vascular endothelial growth factor (VEGF) and its receptors (VEGFR1 and VEGFR2). Hence, an effort to broaden the identification of the underlying regulators of these key receptors as a therapeutic target(s) may offer improved clinical benefits (Abdollahi and Folkman, 2010; Gee et al., 2003; Moserle et al., 2014).

Targeting VEGF and its associated receptors have been at the forefront of anti-angiogenic therapy owing to its critical role in developmental and tumor angiogenesis (Ellis and Hicklin, 2008; Gacche and Meshram, 2014; Sitohy et al., 2012). Monoclonal antibodies such as Bevacizumab (Avastin), tyrosine kinase inhibitors (TKIs) like sorafenib and sunitinib, VEGF-traps, and soluble VEGFR's/hybrids are some of the approaches targeting VEGF signaling (Jain, 2001; Norden et al., 2008; Wagner et al., 2009). However, many of these strategies, though effective against other angiogenesis-dependent diseases, have not met the anticipated clinical benefits as first-line approaches or single therapies in early cancer treatment (Bergers and Hanahan, 2008). Nonetheless, in combination with anti-tumor agents, Bevacizumab is approved for metastatic colorectal carcinoma (CRC), non-small cell lung cancer (NSCLC) and metastatic breast cancer (Ferrara et al., 2005; Gordon and Cunningham, 2005; Molina et al., 2008; Sandler et al., 2006; Tol et al., 2009). Thus, demonstrating that anti-angiogenic therapies can be effective in cancer treatment when used in combination with other anti-tumor agents. The reason for this improved benefit might stem from the multi-target effect of various oncogenic factors and their associated pathways that mitigates tumor resistance to these antiangiogenic therapies.

Moreover, some researchers and clinicians argue that the limited clinical efficacy of targeting single ligand-receptor(s) complexes might result from the activation of alternate angiogenic pathways and underlying feedback mechanisms that compensate for the targeted inhibition of these complexes (Gacche and Meshram, 2014; Meyerhardt and Mayer, 2005; Moserle et al., 2014; Norden et al., 2008; Sitohy et al., 2012). This may be justified by the better clinical outcomes observed in combination therapies which may suggests that a multitarget approach against key angiogenic receptors and oncogenic factors might undermine tumor resistance to anti-angiogenic therapies, alternative angiogenic pathways and/or potential feedback mechanisms thus, favoring

better clinical outcomes (Albini et al., 2012; Giuliano and Pagès, 2013; Shojaei, 2012). Hence, the identification of novel angiogenic regulators with targetable potential might offer improved clinical benefits in anti-angiogenic therapy via a multi-target approach.

HYPOTHESIS AND STUDY AIMS

We hypothesize that Agrin plays a role in angiogenesis and tumor vascularization by facilitating the recruitment of surrounding endothelial cells to the tumor foci. To validate this hypothesis, we have set the following study aims to investigate the potential of Agrin in blood vessel recruitment and tumor vascularization:

1. Ascertain the role of Agrin in endothelial recruitment and angiogenesis during localized or metastatic tumor growth.
2. Examine if Agrin regulates endothelial behavior and pro-angiogenic phenotype in primary endothelial cells.
3. Determine the potential mechanism(s) of Agrin mediated angiogenesis.

CHAPTER 2: MATERIALS AND METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Agrin (D-2)	Santa Cruz Biotechnology	Cat#sc-374117; RRID: AB_10947251
Mouse monoclonal anti-Agrin	Millipore	Cat#MAB5204; RRID: AB_2225272
Rabbit polyclonal anti-Lrp4 (H-98)	Santa Cruz Biotechnology	Cat#sc-98775; RRID: AB_2281510
Goat polyclonal anti-Lrp4	Abcam	Cat#ab122923; RRID: AB_10902970
Rabbit polyclonal anti-Lrp4	St. John's Laboratory	Cat#STJ112211
Rabbit polyclonal anti-Integrin β 1	Cell Signaling Technology	Cat#4706
Mouse monoclonal anti-Integrin β 1 (A-4)	Santa Cruz Biotechnology	Cat#sc-374429; RRID: AB_11012020
Mouse monoclonal anti-Integrin β 1 (P5D2)	Abcam	Cat#ab24693; RRID: AB_448230
Mouse monoclonal anti- α Dystroglycan (VIA41)	Santa Cruz Biotechnology	Cat#sc-53986; RRID: AB_831190
Mouse monoclonal anti- β Dystroglycan (4F7)	Santa Cruz Biotechnology	Cat#sc-33702; RRID: AB_627292
Mouse monoclonal anti- β Tubulin (D-10)	Santa Cruz Biotechnology	Cat#sc-5274; RRID: AB_2288090
Mouse monoclonal anti- β Actin (C4)	Santa Cruz Biotechnology	Cat#sc-47778; RRID: AB_2714189
Rabbit polyclonal anti-GAPDH (FL-335)	Santa Cruz Biotechnology	Cat#sc-25778; RRID: AB_10167668

Rabbit polyclonal anti-CD31	Abcam	Cat#ab28364; RRID: AB_726362
Rabbit polyclonal anti-VEGFA	Abcam	Cat# ab46154; RRID: AB_2212642
Rabbit monoclonal anti-VEGF Receptor 1 [Y103]	Abcam	Cat#ab32152; RRID: AB_778798
Rabbit polyclonal anti-VEGF Receptor 3	Abcam	Cat#ab27278; RRID: AB_470949
Rabbit monoclonal ant-VEGFR2 (55B11)	Cell Signaling Technology	Cat#2479; RRID: AB_2212507
Rabbit monoclonal anti-Phospho VEGF Receptor 2 (Tyr951) (15D2)	Cell Signaling Technology	Cat#4991
Rabbit monoclonal anti-Phospho-VEGF Receptor 2 (Tyr1059) (D5A6)	Cell Signaling Technology	Cat#3817; RRID: AB_2132351
Rabbit monoclonal anti-Phospho-VEGF Receptor 2 (Tyr1175) (19A10)	Cell Signaling Technology	Cat#2478; RRID: AB_331377
Rabbit polyclonal anti-FAK	Cell Signaling Technology	Cat#3285; RRID: AB_2269034
Mouse monoclonal anti-Phospho FAK (pY397)	BD Biosciences	Cat#611807
Rabbit monoclonal anti-eNOS (D9A5L)	Cell Signaling Technology	Cat#32027; RRID: AB_2728756
Rabbit polyclonal anti-Phospho eNOS (Ser1177)	Cell Signaling Technology	Cat#9571
Mouse monoclonal anti-eNOS (pS1177)	BD Biosciences	Cat#612393; RRID: AB_399751
Rabbit polyclonal anti-Akt	Cell Signaling Technology	Cat#9272; RRID: AB_329827
Rabbit polyclonal anti-Phospho Akt (Ser473)	Cell Signaling Technology	Cat##9271
Rabbit polyclonal anti-p44/42 MAPK (Erk1/2)	Cell Signaling Technology	Cat#9102; RRID: AB_330744
Rabbit polyclonal anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology	Cat#9101
Rabbit polyclonal anti-MuSK	Abcam	Cat#ab5510; RRID: AB_304946
Goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology	Cat#sc-2030; RRID: AB_631747

Donkey anti-goat IgG-HRP	Santa Cruz Biotechnology	Cat#sc-2056; RRID: AB 631730
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology	Cat#sc-2005; RRID: AB 631736
Goat anti-mouse IgM-HRP	Santa Cruz Biotechnology	Cat#sc-2973; RRID: AB 650513
Goat anti-Rabbit IgG (H+L) Alexa Flour 488	Invitrogen	Cat#A11034
Goat anti-Mouse IgG (H+L) Alexa Flour 555	Invitrogen	Cat#A21422
Goat anti-mouse IgM Alexa Flour 594	Invitrogen	Cat#A21044
Bacterial and Virus Strains		
Human VEGFR2 with V5 and tGFP tag (pCMV vector- mammalian expression)	Raikwar et al., 2018	Cat#83436, Addgene
pET28 vector	Genscript	Genscript
Biological Samples		
Human Hepatocellular carcinoma and normal liver tissue array	US Biomax., Inc.	Cat#BC03116a
Chemicals, Peptides, and Recombinant Proteins		
L-NAME (N(ω)-nitro-L-arginine methyl ester)	Sigma-Aldrich	Cat#51298-62-5
Sorafenib (p-Toluene sulfonate salt)	LC Laboratories	Cat#S-8502
Cyclohexamide	Sigma	Cat#C-0934
PF562271	Abcam Biochemicals	Cat#ab141360
Dimethylsulfoxide	Kanto Chemical, co., Inc	Cat#10378-00
Recombinant Rat Agrin	R&D Systems	Cat#550-AG-100
Recombinant Lrp4 (rh-Lrp4)	R&D Systems	Cat#5948-LR
Recombinant VEGF (rh-VEGF)	R&D Systems	Cat#293-VE
C-terminal recombinant Agrin fragment	This paper	N/A
Critical Commercial Assays		
Proteome Profiler Array, Human phosphor-RTK Array Kit	R&D Systems	Cat#ARY001B
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	Promega	Cat#G3580
Cell Tracker Blue CMAC (7-amino-4-chloromethyl coumarin)	Invitrogen	Cat#C2110
DAF-2 DA (5, 6-Diaminofluorescein diacetate)	Sigma-Aldrich	Cat#D225
Human Agrin ELISA kit	MyBiosource	Cat#MBS7217535
Deposited Data		
N/A		
Experimental Models: Cell Lines		
HUVEC - Human Umbilical Vein Endothelial Cells	Lonza	Cat#C2519A

HDMEC – primary Human Dermal Microvascular Endothelial Cells	ATCC	Cat#PCS-110-010
HAEC - Human Aortic Endothelial Cells	Lonza	Cat#CC-2535
HRMEC – Human Retina Microvascular Endothelial Cells	iXCells Biotechnologies	Cat#10HU-079
MHCC-LM3	Dr Kam-Man Hui laboratory (National Cancer Center, Singapore)	N/A
Hep3B 2.1-7	ATCC	Cat# HB-8064
SNU-449	ATCC	Cat# CRL-2234
Experimental Models: Organisms/Strains		
Mouse, CB-17 SCID	InVivos	N/A
Mouse, C57BL/6J	InVivos	N/A
Oligonucleotides		
VEGFR2 (Hs00911700_m1)	Applied Biosystems	Cat# 4331182
18S (Hs03003631_g1)	Applied Biosystems	Cat# 4331182
Agrin (Mm01264855_m1)	Applied Biosystems	Cat# 4331182
Integrin β 1 (Hs00559595_m1)	Applied Biosystems	Cat# 4331182
Lrp4 (Hs00391006_m1)	Applied Biosystems	Cat# 4331182
Lentiviral Agrin shRNA pool Sequence1: 5'- CAGGAGAAUGUCUUCAAGATT-3'	Santa Cruz Biotechnology	Cat# sc-29652-V
Lentiviral Agrin shRNA pool Sequence 2: 5'- CGACGUGUGCUGUGAAGAATT-3'	Santa Cruz Biotechnology	Cat# sc-29652-V
Lentiviral Agrin shRNA pool Sequence 3: 5'- CGACCUCUUCGGAUUCATT-3'	Santa Cruz Biotechnology	Cat# sc-29652-V
scramble shRNA control	Santa Cruz Biotechnology	Cat#sc-108080
Primer for Agrin Z8-insert: Forward Forward: 5' TGGCCAATGAGATCCCCGTC 3'	This paper	N/A
Primer for Agrin Z8-insert: Reverse: 3'CTGGGTCGCTTCGGTGC 5'	This paper	N/A
Software and Algorithms		
Angiogenesis Analyzer; Image J	Carpentier et al., 2012	https://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt
Sprout morphology, Fiji (Image J)	Eglinger et al., 2017	https://github.com/angiogenesis/Sprout_Analysis .

GraphPad Prism 7	GraphPad software	https://www.graphpad.com/scientific-software/prism/
Image J	This paper	https://imagej.nih.gov/ij/

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Mice

All animal experiments performed were done following experimental protocols reviewed by the Biological Resource Center (BRC), Agency for Science Technology and Research (A*STAR) in compliance to the Institutional Animal Care and Use Committee (IACUC) guidelines for the ethical use of animal models in biological research. We used six to eight-week-old male experimental C.B-17 SCID mice purchased from InVivos for Matrigel plug and pulmonary metastasis assays. In the *ex-vivo* mouse metatarsal assay, random ratios of male and female fetal mice pulps were harvested at E16.5 from pregnant female C57BL/6J mice also purchased from InVivos.

Cell Lines

Neonatal-derived Human umbilical vein endothelial cells (HUVEC) and Human aortic endothelial cells (HAEC) were obtained from Lonza (Cat#C2519A and Cat#CC-2535). Human retinal microvascular endothelial cells (HRMEC) were purchased from iXCells Biotechnologies (Cat#10HU-079). Neonatal-derived Human dermal microvascular endothelial cells (HDMEC) were obtained from ATCC (Cat#PCS-110-010). The gender for primary ECs was batch specific. The primary EC's HUVEC, HAEC, and HRMEC were cultured with endothelial growth media (EGM-2) (Lonza, Cat#: CC-3162) supplemented with all relevant growth factors, 2% bovine

serum albumin (FBS) and 1% penicillin-streptomycin (Gibco, Cat#15140148). HDMEC was cultured using microvascular growth media (ATCC, Cat#PCS-100-030) supplemented with all relevant growth factors (ATCC, Cat#PCS-110-040) and 1% penicillin-streptomycin (Gibco, Cat#15140148). The endothelial cells were cultured under standard cell culture conditions. All experiments with primary endothelial cells were performed between 3-7 passages. Hep3B 2.1-7 isolated from an 8-year-old black male and SNU-449 isolated from a 52-year-old Asian male were purchased from ATCC (Cat# HB-8064 and Cat# CRL-2234), while MHCC-LM3 generated from a 39-year-old male was a gift from Dr. Kam-Man Hui laboratory (National Cancer Center, Singapore). The HCC cell lines including MHCC-LM3, Hep3B and SNU-449 were authenticated by ATCC cell line authentication service using short tandem repeat (STR) analysis. All HCC cells were cultured as per standard conditions previously described (Chakraborty et al., 2015).

METHOD DETAIL

Cancer cell and EC co-culture adhesion assay

shControl, shAgrin and shAgrin rescue (expressing rat-Agrin) MHCC-LM3 cell lines were generated as previously described (Chakraborty et al., 2015), pre-labeled with 10 μ M CellTracker blue (7-amino-4-chloromethylcoumarin (CMAC) (Invitrogen) for ~12-24h and incubated at 37°C and 5% CO₂. After incubation, MHCC-LM3 cells were washed with PBS, followed by adhesion buffer (EGM-2 media supplemented with 1mM CaCl₂, 1mM MnCl₂, and 1mM MgCl₂). Fifty thousand labeled cancer cells were reconstituted in adhesion buffer and dispensed into a 24-well plate containing a confluent monolayer of HUVECs; either naïve or pre-transfected for 48h with 150nM scrambled control or Agrin siRNAs. Added cancer cells were imaged immediately (T=0h) by fluorescence microscopy. This was followed by incubation

for 2h to allow cancer/endothelial cell adhesion. Subsequently, the co-culture was washed thrice with PBS to removed non-adherent cells and imaged live (T=2h). The resultant images were analyzed using Image-J software. Data represented as the percentage of adhering MHCC-LM3 at T=2h when compared to initial T=0h.

***In-vivo* Matrigel Plug Assays**

Five million MHCC-LM3 tumor cells were resuspended in 50 μ l of DMEM culture medium and combined with 250 μ l of ice-cold Matrigel (BD Biosciences). The cell-matrigel mix was subcutaneously implanted into the flanks of 7 weeks old Severe Combined Immuno-deficient (SCID) mice with an ice-cold 24G needle. The matrigel mix was allowed to polymerize in the mice to form a solid plug for 14 days, following which the mice were sacrificed and the growing tumor plugs excised. This was fixed overnight with 10% Neutral Buffered Formalin (NBF), embedded in paraffin and sectioned onto slides. The embedded sections were then stained using indicated antibodies by Immunofluorescence (IF) and Immunohistochemistry (IHC), respectively. The stained slides were visualized using Leica Biosystem software (Leica SCN) and quantified using Image J. For Western blotting, tumor plugs from each group were cut equally into small pieces, lysed in RIPA lysis buffer (2.5 μ l/ μ g of tissue), homogenized and sonicated on ice. After which the homogenate was centrifuged at 13000 rpm for 30 min. Subsequently, Bradford reagent was used for total protein estimation and equal protein volumes mixed with 2X sample buffer and resolved using SDS-PAGE. For cell-free matrigel plug assay, 300 μ l of Matrigel was combined with either vehicle control (PBS) or 20 μ g/ml soluble Agrin and injected subcutaneously into the flanks of SCID mice. PBS or soluble Agrin was injected within the Matrigel plug every 4 days and the cell-free plugs were excised 14 days post-inoculation and analyzed as above.

Pulmonary metastasis model

Six-eight-week-old SCID mice were inoculated with 5×10^6 shControl, shAgrin and shAgrin rescue (stably expressing rat-Agrin) MHCC-LM3 cell lines via tail vein injection. After 6 weeks, the mice were euthanized, and their lungs perfused with PBS and 4% paraformaldehyde. The lungs were subsequently fixed in 10% Neutral Buffered Formalin (NBF) and subjected to sectioning and immunohistochemistry. All animal procedures were performed according to the protocols reviewed by the Biological Resource Center, A-STAR.

***In-vitro* angiogenesis assay**

A flat-bottom 96-well plate was pre-cooled on ice and coated with 40 μ l of matrigel (BD Bioscience) and allowed to polymerize by incubating at 37 °C for 15 minutes. Seven-eight thousand viable ECs post-trypsinization was re-suspended in 100 μ l complete EGM-2 medium and plated on the Matrigel and incubated for 6h at 37 °C, 5% CO₂. Representative tube formation was imaged after 6h using phase-contrast microscopy and quantified using Angiogenesis analyzer by Image-J. The total tube length and the total number of master segments were used to determine the tube forming ability of control and treated groups of cells. The master segments consist of pieces of tree delimited by two junctions none exclusively implicated with one branch, called master junctions. Each experiment was performed in triplicates for each condition under investigation.

Recombinant Agrin generation

A ~20kDa C-terminus fragment containing 8 amino acid 'Z' insert and LG3 domain was cloned into a pET28 vector and expressed in *Escherichia coli*. The resultant Agrin protein fragment

was purified in imidazole buffer, gel-filtrated using Superdex75 columns and sterilized by passing through a 45µm filter.

***Ex-vivo* metatarsal sprouting angiogenesis**

The metatarsal sprouting angiogenesis assay was performed as described (Wang et al., 2013). Metatarsal bones isolated from E16.5 C57BL7 mice were treated with either vehicle control (PBS containing 20 µg/mL BSA), VEGF165 (50 ng/ml, PeproTech) or soluble recombinant Agrin (20µg/ml) and cultured in pre-coated plates. Cultured media containing the various treatment conditions was replaced once every two days for a total duration of 10 days. Subsequently, the explants were fixed and stained for CD31 (BD Biosciences, Oxford, UK) and visualized under Nikon TiE inverted fluorescence microscope. CD31-positive sprouting vessels were determined by TRI2 (software developed by the University of Oxford). Each treatment contains at least 8 metatarsals. Statistical significance was determined by One way-ANOVA.

ECM stiffness manipulation

One hundred µl of growth factor reduced Matrigel (BD Biosciences) mixed with rat tail collagen-I (Trevigen, Culterex 3D) having final concentration of either 3.3 mg/ml collagen (hard) or 1.0 mg/ml (soft) was added to 12 well plates and solidified to form gel in 37°C incubator for 30 min (Chakraborty et al., 2017). Ten thousand HUVEC cells were resuspended in 500 µl complete EGM2 media and plated on the hard/soft ECM gel substrate and cultured for 2 days following standard conditions. For VEGFR2 stability assays, HUVEC were seeded on the poly-hydrogel plates of defined stiffness namely: 0.2kPa and 16kPa soft and hard polyhydrogels, respectively (CytoSoft, Advanced Biomatrix, Inc.). Poly-hydrogels of defined stiffness (soft and hard) were prepared according to previously published protocols (Fischer et al., 2012).

Endothelial sprouting assay

Hundred thousand ECs after trypsinizing were resuspended in 2ml of complete EGM-2 media and mixed with 500 μ l of methylcellulose. 20 μ l of the mixture containing ~800 cells were dispensed into each well of a hanging-drop plate (3D Biomatrix, Cat#HDP1096) and incubated overnight at 37°C and 5% CO₂ for spheroid formation. Next, a collagen mix was prepared by adding 126 μ l of rat-tail collagen Type 1 (9.51mg/ml stock) to 414 μ l of cell culture water and 60 μ l of basal medium (M199). 200 μ l of the collagen mix was dispensed into the appropriate wells of a 48-well plate and polymerized by incubating for 30mins at 37°C. The spheroids were gently washed off the hanging-drop plate with double distilled water and centrifuged at 700 rpm using a swing bucket rotor for 5 min and re-suspended in 120 μ l FBS. Another collagen mix prepared as before was mixed with the 60 μ l FBS containing spheroid suspension instead of M199 medium. 250 μ l of the final mixture was dispensed into each well containing bottom collagen matrix and incubated for 2h at 37°C. The appropriate wells were then overlaid with 100 μ l of complete EGM-2 with or without soluble Agrin supplementation. Images of sprouting ECs was captured 24h post-embedding and quantified using Sprout morphology plugin from Fiji (Image J) as described (Eglinger et al., 2017).

Reverse transcription PCR

Total RNA was extracted from HUVEC using Qiagen RNeasy mini kit following the manufacturer's protocol. The total RNA was then reverse-transcribed using a High-Capacity cDNA reverse transcription kit (Applied Biosystems). The generated cDNA was used as a template for the RT-PCR.

Receptor tyrosine kinase (RTK) phosphorylation array

A phospho-RTK screen was performed using the Proteome Profiler Human Phospho-RTK Array Kit (R&D systems #ARY001B) following the manufacturer's protocol. Briefly, the RTK membranes were incubated with Array buffer 1 at room temperature (RT) with mild shaking on a rocking platform. Then, 300µg of whole-cell lysates from siControl or siAgrin HUVECs were diluted in fresh Array buffer 1 to a final volume of 1.5ml and incubated on the membranes at 4°C with mild shaking over-night. Post-incubation, membranes were washed thrice with 1X wash buffer for 10mins each, followed by incubation with phosphor-tyrosine-HRP secondary antibody diluted in Array buffer 2 at a concentration of 1:5000 for 2h at RT. Subsequently, the membranes were washed three times with 1X wash buffer for 10mins each and visualized after overlay with enhanced chemiluminescence (ECL) substrate (Pierce/Bio-Rad).

In-vitro Wound-healing assay

A confluent monolayer of control and Agrin depleted cells in a 6-well plate was subjected to a unidirectional scratch using a 20µl pipette tip. This was followed by washing with 1X PBS at room temperature and incubation in complete EGM-2 culture media at 37 °C and 5% CO₂. Phase-contrast images of the wound area were taken periodically as indicated in the figure legend.

Transwell migration and invasion assays

EC invasion was assessed using the Corning BioCoat Matrigel invasion chamber. The chambers were first hydrated with 250µl of serum-free media. 7000 cells were re-suspended in 200µl of serum-free media and placed into the chamber-inserts. 700µl of complete medium with serum

and/or soluble Agrin was placed in the lower chamber as a chemoattractant and incubated at 37 °C, 5% CO₂ for 24h. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) for 15mins at room temperature (RT) and stained with 1% crystal violet (Sigma-Aldrich) for 20mins. Invading cells were imaged using bright-field microscopy and cell count quantified by Image-J. EC migration assay was performed as above using a migration chamber devoid of Matrigel coating. All other experimental procedures remained unchanged.

EC Proliferation Assay

Cell proliferation was assessed via colorimetry using CellTiter 96® AQueous One Solution Reagent (Promega). Relative cell growth was determined by the formation of colored formazan resulting from a reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by viable cells and the optical density was measured at 490nm wavelength. Briefly, two thousand cells either control or Agrin depleted were resuspended in 100µl complete EGM-2 medium and dispensed into respective wells of a flat bottom 96-well plate and incubated following standard cell culture conditions. Before analysis, 20µl of CellTiter 96® AQueous One Solution Reagent was added to each well-containing cells and incubated for 1h. Relative cell proliferation was determined every 24h for three days by measuring the optical density at 490nm using a microplate reader (Synergy HT).

Immunofluorescence (IF) and confocal microscopy

Control and knockdown cells were cultured on eight-well chamber slides or coverslips overnight. Cells were then washed twice with PBS and fixed for 15min with 4% paraformaldehyde (PFA). Subsequently, the cells were permeabilized for 15min with 0.1% Triton X in Phosphate buffered saline containing 1mM Ca⁺² and 1mM Mg⁺² (PBSCM) at room temperature. The

permeabilized cells were incubated with indicated antibodies in fluorescent dilution buffer (FDB) for 1-2h at RT or overnight at 4°C, followed by 5 washes with PBSCM and incubation with secondary antibody; Alexa Fluor (Thermo Fisher Scientific) for 1h at RT. Slides were again washed five times with PBSCM and mounted with Vectashield medium containing DAPI. The stained cells were then imaged by Olympus or Zeiss confocal microscope. The data were analyzed by either Fluoview or Zen blue software.

Western blot analysis

Indicated cells post-manipulation were washed twice with cold phosphate-buffered saline (PBS) and lysed with cold 1% NP-40 lysis buffer supplemented with 1X protease inhibitor cocktail (Roche Applied Biosciences) for 15mins at 4°C. The cell lysate was centrifuged at 13,000 rpm for 15 min. This was followed by protein estimation using Bradford reagent. Subsequently, 40-50µl of total protein was mixed with an equal volume of 2X Laemmli sample buffer and heated at 95°C for 5 mins. This was followed by resolution with SDS-PAGE gel. The resolved proteins were transferred onto nitrocellulose membrane and blocked in 5% skimmed milk reconstituted in 1X PBS containing 0.1% Tween-20 and probed overnight with the respective primary antibody. The membrane was then washed with 1X PBS supplemented with 0.1% Tween-20; three times at 15 min intervals. This was followed by 1h incubation in conjugated horseradish peroxidase (HRP) secondary antibody (Santa Cruz Biotechnology). Post-incubation, the blot was again washed three times as above and then overlaid with enhanced chemiluminescence (ECL) substrate (Pierce/Bio-Rad) and visualized on X-ray film by image processor or digitally by Chemidoc analyzer (Bio-Rad). The density of the various bands was quantified using the Image-J software.

Dot blot analysis

Indicated conditioned medium or recombinant Agrin protein (10 μ l) was blotted on a nitrocellulose membrane pre-damped in 1X PBS and left to absorb for 15mins at room temperature (RT). The nitrocellulose membrane was then incubated in the ponceau S solution for 15mins at RT. The membrane was then rinsed three times in distilled water with mild shaking for 10mins each time. Afterwards, the membrane was imaged for Ponceau S staining. Subsequently, the membrane was blocked in 5% skimmed milk reconstituted in 1X PBS containing 0.1% Tween-20 and probed overnight with the respective primary antibody. The following day the membrane was developed the same as (western blot analysis) above.

Immunoprecipitation (IP)

Adherent HUVECs were washed twice with PBS (1X) and then lysed with 1% NP-40 lysis buffer on ice for 15mins. The cell lysate was centrifuged at 13000rpm for 30mins and total protein estimation determined using Bradford reagent. 500 μ g of the whole-cell lysate (WCL) was immunoprecipitated using 20 μ l of protein A/G sepharose beads and 2 μ g/ml VEGFR2 antibody (Cell Signaling Technology) on a rocking platform for 2h at 4°C. Subsequently, the immunoprecipitate was spun down at 4000rpm for 2min at 4°C to pellet the beads. The beads were then subsequently washed three times with 500 μ l of 1% NP-40 lysis buffer followed by washing twice with 1X PBS. Subsequently, 20 μ l of 2X Laemmli sample buffer was added to the IP-beads and the bound proteins eluted by heating at 95°C for 5min. The eluted sample was then resolved using SDS-PAGE as described above.

Measurement of e-NO production

HUVEC transfected with scrambled control or Agrin siRNA for 48h were sub-cultured in 24-well plates for 24h. Before the experiment, cells were serum-starved for 12-18h using growth factor-free EGM-2 media supplemented only with 1% fetal bovine serum (FBS). Post-starvation, cells were washed thrice with Hank's Balanced Salt Solution (HBSS) (Gibco) and treated with 1µg/ml DAF-2 DA (5, 6-Diaminofluorescein diacetate) diluted in HBSS and incubated for 1h at 37°C and 5% CO₂. Subsequently, the cell monolayer was washed three times with HBSS and incubated for 15-30 min with VEGF (50ng/ml) diluted in HBSS. Relative nitric oxide production was determined by live fluorescent microscopy. Endothelial nitric oxide synthase (eNOS) inhibitor; *N*^ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as a positive control.

Agrin ELISA

Secreted Agrin in the conditioned medium (CM) of MHCC-LM3 and HUVECs was measured using Agrin ELISA kit (MyBiosource, Inc.). Concisely, 50 µl of provided standard solutions and diluted CM was plated into respective strip-wells coated with Agrin antibody. 5µl of balance solution was then added to each well-containing test samples but not in the standards. Subsequently, 100µl of the conjugate solution was added to each well-containing sample excluding the blank control and incubated for 1h at 37 °C. The reaction mixture was then discarded, and the strip-wells washed five times with the wash solution provided. Thereafter, 50µl of substrate A and B was added to each well and incubated for 15 min at 37°C. Subsequently, 50µl of stop solution was added to each well and the respective optical density (O.D.) determined at 450nm using a microplate reader. The OD from the standard strip-wells

was used to generate a standard curve from which the respective Agrin levels in the CM were extrapolated. Each sample was analyzed in triplicates and the data presented as mean +/-SD.

Isolation of Total Membrane Fraction

Control or Agrin depleted HUVECs cultured at ~ 80% confluency were washed twice with ice-cold PBS and lysed for 15min at 4°C with homogenization buffer (20mM HEPES, 1mM EGTA, 250mM sucrose, 1mM EDTA, 10mM KCL) supplemented with 1X protease inhibitor cocktail (Roche). The homogenate was centrifuged at 3000 rpm for 5min. The supernatant was collected and further centrifuged at 8000rpm at for another 5min. The supernatant was again collected and centrifuged at 40,000 rpm for 1h. The homogenate was maintained at 4°C for the duration of the experiment. The pellet was considered a total membrane fraction. Whole-cell lysate pre-fractionation was used as a reference.

Immunohistochemistry

The immunohistochemistry (IHC) on tissue samples was performed as described previously (Chakraborty et al., 2015; Chakraborty et al., 2017). HCC tissue samples were obtained from US Biomax., Inc., and the staining procedure was described as above. Image analysis and quantification were done using Leica image software and IHC tool from Image-J, respectively.

Quantification and Statistical Analysis

All experiments except otherwise stated in the figure legends were performed in three biological replicates. 'N' as stated in the figure legends represents the number of biological replicates. The number of technical repeats per experiment is indicated at the relevant figure legend panels. For *in-vivo* experiments, 'N' refers to the number of animals used. A paired two-tailed student's t-test

or one-way-ANOVA analysis was used to compare groups using GraphPad Prism7 software. Data represent mean $\pm$ standard deviation (S.D.) of the individual experiments of three biological replicates. The data was considered statistically significant when * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, respectively. Data considered insignificant was designated as ns. No prior statistical tests or assumptions were used to determine the sample size of in-vitro and in-vivo experiments. We chose three biological replicates for *in-vitro* experiments as it adheres to the commonly held practice in biomedical research.

CHAPTER 3: RESULTS

Agrin promotes tumor vascularization

In hepatocellular carcinoma (HCC), the overexpression of Agrin is crucial for tumorigenesis and cancer progression (Chakraborty et al., 2015; Chakraborty et al., 2017). Given that tumor progression is angiogenesis-dependent (Folkman, 1971, 2002), we questioned whether Agrin mediates cancer progression by enhancing tumor angiogenesis in HCC. Moreover, the expression of Agrin coincides with the expression of key angiogenic signatures in a multi-cancer panel (**Figure 1.5**), suggesting that Agrin may be required for tumor vascularization or the maintenance of tumor blood vessels.

Considering that the liver parenchyma undergoes significant histopathological changes during HCC development, the expression pattern and/or localization of various oncogenic proteins in this tissue can, therefore, be informative regarding their potential role in disease progression (Tripathy et al., 2018).

To investigate whether Agrin plays a role in tumor vascularization of HCC, we first performed an immunohistochemistry (IHC) analysis of Agrin expression in healthy livers and HCC tissue samples. The intensity of Agrin staining was higher in the HCC tissues when compared to healthy livers (**Figure 3.1**). This observation was consistent with earlier reports showing that Agrin is overexpressed in HCC (Chakraborty et al., 2015). However, we noticed that the pattern of Agrin staining was most prominent in the vicinity of the hepatic endothelium (**Figure 3.1**). Thus, suggesting that Agrin may be relevant for creating a tumor conducive microenvironment that allows for the development or maintenance of tumor blood vessels in hepatocellular carcinoma. Similar conclusions have been made by other researchers regarding growth factors such as VEGF

which exhibits very same expression patterns in the vicinity of the liver endothelium (Chen et al., 2010).

Figure 3.1: *Agrin expression is prominent in the vicinity of tumor microvessels. Bright-field microscopy images of Agrin Immunohistochemistry staining in HCC versus healthy liver samples. Boxes depict enlarged regions. Arrows highlight Agrin clusters in the vicinity of liver microvessels. Scale bar, 100 μ m.*

Many solid tumors including hepatocellular carcinoma begin as avascular lumps of fast-proliferating tumor cells that are usually less than 2-3mm in diameter (Folkman, 2002; Nakashima

et al., 1999). Avascular tumors obtain their metabolic nutrients and oxygen by simple diffusion from nearby capillaries which limit the extent of tumor growth. Without a dedicated blood supply, the growing tumor suffers from nutrient deprivation, hypoxia and tissue necrosis from cytotoxic signals generated by the tumor cells (Ferreira et al., 2002; Folkman, 1975). The transition from avascular to vascular growth is therefore critical for tumor progression.

Consequently, we wondered whether cancer derived Agrin was important for the recruitment of surrounding blood vessels during the early stages of tumor growth. To investigate this, we performed an *in-vivo* Matrigel plug assay, a widely used angiogenic technique to evaluate the formation of new blood vessels by cancer cells or pharmacotherapeutic agents (Akhtar et al., 2002; Lung and Lung, 2012; Malinda, 2009).

Experimentally, control and Agrin depleted MHCC-LM3 liver cancer cells were concocted with Matrigel and inoculated into the flanks of severe combined immunodeficient (SCID) mice. Fourteen days post-inoculation, the tumor-plugs were excised (**Figure 3.2**). The Agrin silenced tumor-plugs were considerably smaller in size when compared to control tumor-plugs. This observation was consistent with our earlier report which showed that Agrin depletion mitigated tumor growth in this xenograft model (Chakraborty et al., 2015). Noteworthy, the qualitative hematological content of the Agrin depleted tumor-plugs was severely lower than that in control tumor-plugs (**Figure 3.2**).

Figure 3.2: In-vivo Matrigel plug assay. Outline of in-vivo Matrigel plug assay using *shControl* and *shAgrin* MHCC-LM3 liver cancer cells which were inoculated into the flanks of SCID mice. Representative tumor-plug images captured 14 days post-inoculation. Scale bar, 1 mm.

Metabolic tissues including tumors require nutrients and oxygen for their activity. These demands are addressed by dedicated capillaries that permeate the tissue peripheries (Hillen and Griffioen, 2007; Warburg et al., 1927). The hematological content of the Matrigel plugs is therefore indicative of the presence of functional tumor capillaries containing red blood cells (RBCs) as such can be used to denote the extent of tumor vascularization (Shchors and Evan, 2007).

However, given that this observation was based on surface analysis that might not be consistent across the tumor mass, we sectioned the respective tumor-plugs and immuno-stained with Agrin antibody to validate the efficiency of Agrin knockdown. As expected, Agrin staining was drastically lower in the Agrin depleted tumor-plugs when compared to control tumors (**Figure 3.3-left panel**). Additionally, we stained for endothelial marker CD-31 to determine the relative density of recruited micro-vessels within these tumor-plugs (**Figure 3.3-right panel**). The Agrin silenced

tumor-plugs revealed decreased CD-31 staining that corresponded to fewer microvessels when compared to control tumor-plugs (Figure 3.3- right panel).

Figure 3.3: Agrin depleted tumor-plugs reveal decreased CD-31 staining and microvessels. Immunofluorescence (IF) verification of Agrin expression in control and Agrin depleted tumor-plug sections (left panel). Immunohistochemistry (IHC) analysis of vessel-associated CD-31 in tumor plug sections (right panel). Arrows depict CD-31 stained microvessels. Image analysis and quantification were done using Leica image software and IHC tool from Image-J, respectively. Data presented as mean $\pm$ SD of five independent tumor plugs per group ($n=5$, *** $P < 0.0001$, student's t -test). Error bars denote standard deviation (SD). Scale bar, 200 μ m.

Consistently, when equal amounts of resected tumor-plugs were analyzed by western blot, Agrin depleted tumor-plugs also revealed decreased CD-31 expression without affecting housekeeping genes; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β -tubulin or β -actin. As the recruited ECs are less in comparison to tumor cells, the overall changes in the levels of endogenous housekeeping proteins contributed by ECs are also negligible. (**Figure 3.4A**). Moreover, given that MHCC-LM3 cells do not express endothelial marker CD-31 and endothelial Agrin knockdown does not affect the expression of CD-31 in HUVEC (**Figure 3.4B**), therefore, we posit that the decreased CD-31 levels in Agrin depleted tumor-plugs resulted from defective recruitment of surrounding endothelial cells during the early stages of tumor growth. Taken together, our data infers that Agrin is important for tumor vascularization during the early stages of localized tumor growth.

Figure 3.4: Agrin depleted tumor-plugs reveal decreased CD-31 levels. (A) Western blot analysis of CD-31 and Agrin expression from equal amounts of excised tumor plugs from (Figure 3.3). Three tumor-plugs were used per group ($n=3$). GAPDH, β -tubulin, and β -actin were used as loading controls. (B) CD31 and Agrin levels in control and Agrin silenced MHCC-LM3 and HUVEC cells respectively. β -actin was used as the loading control.

Agrin facilitates blood vessel recruitment in metastasizing tumor foci

Tumor vascularization is critical for cancer progression and metastatic dissemination of malignant cells to secondary locations. These cells are transported by blood vessels to distal tissues where they rapidly proliferate to form metastatic nodules which also recruit surrounding blood vessels to enhance their progression (Steeg, 2006; Valastyan and Weinberg, 2011). To further ascertain the role of Agrin in tumor vascularization, we utilized a tail vein cancer metastasis model to monitor the effects of Agrin depletion on blood vessel recruitment within metastasizing tumor foci *in-vivo*. Consequently, shControl, shAgrin, and shAgrin cells stably expressing rat Agrin (Agrin rescue) MHCC-LM3 cells were injected into the tail veins of SCID mice and monitored for pulmonary metastasis progression for one month. Agrin silencing robustly mitigated the pulmonary

dissemination of these liver cancer cells when compared to the control cells (**Figure 3.5**). Interestingly, the defective metastasis observed with Agrin depletion was significantly restored when using shAgrin rescue cells (**Figure 3.5**) suggesting that Agrin is important for the metastatic dissemination of these liver cancer cells to the lungs. However, no significant differences were observed in the size of metastatic lesions generated by either control or Agrin depleted liver cancer cells.

Figure 3.5: Silencing Agrin hampered the metastatic dissemination of liver cancer cells to the lungs. Bright-field microscopy images of Hematoxylin and Eosin stained lung sections of mice injected with shControl, shAgrin and shAgrin rescued MHCC-LM3 cells. The dotted lines indicate

tumor lesions. Quantification presented as mean $\pm$ SD of nine lung sections per group ($***P = 0.0002$, $**P = 0.0082$, Student's *t*-test). Error bars denote standard deviation (SD). Scale bar: 100 μ m.

Furthermore, to evaluate the recruitment of blood vessels by these metastatic lesions, we immunostained for endothelial marker CD-31, which revealed notable recruitment of pulmonary blood vessels in the metastatic lesions generated by control cells when compared to Agrin depleted lesions which were grossly deficient in pulmonary blood vessels (**Figure 3.6**). Collectively, this data suggests that cancer derived Agrin promotes blood vessel recruitment and vascularization of localized and metastatic tumor foci during the early stages of tumor growth.

Figure 3.6: Agrin promotes blood vessel recruitment in metastatic tumor foci. Confocal images of CD-31 staining in lung sections of mice injected with shControl, shAgrin, and shAgrin rescued cells. L and T refer to lung and tumor regions, respectively. The dotted lines represent tumor lesions. CD-31 staining within lesions was quantified using Image J. Data presented as mean $\pm$ SD of nine sections from 6 separate lungs analyzed per group (*** $P = 0.0001$ * $P = 0.0178$, Student's t -test). $n=6$ mice per group. Error bars denote standard deviation (SD). Scale bar, $100\mu\text{m}$.

Agrin stimulates endothelial chemotaxis

The angiogenic switch marks the initiation of tumor vascularization and is vital for tumor growth and cancer progression (Bergers and Benjamin, 2003; Hanahan and Folkman, 1996). In adults, angiogenesis is mostly quiescent unless activated by pathophysiological conditions such as wound healing or the menstrual cycle (Carmeliet, 2003). Angiogenesis is tightly regulated by a balance of pro/anti-angiogenic factors. However, pathological conditions such as cancer deregulate this balance in favor of tumor vascularization (Adams and Alitalo, 2007; Ferrara, 2000). During tumor growth, the core of the tumor mass becomes increasingly hypoxic as the tumor size increases. This trigger epigenetic changes that up-regulate pro-angiogenic factors such as VEGF which is secreted into the tumor microenvironment. Thus, creating a concentration gradient that facilitates the recruitment of surrounding endothelial cells by chemotaxis (Pugh and Ratcliffe, 2003; Veikkola and Alitalo, 1999).

Considering that Agrin is highly expressed and secreted in HCC, and the observation that silencing Agrin in these cells led to decreased vascularization of localized and metastatic lesions, we wondered whether secreted Agrin in the tumor microenvironment recruited surrounding

endothelial cells via chemotaxis. Accordingly, we utilized the conditioned medium (CM) of MHCC-LM3 liver cancer cells which expresses and secrete high amounts of Agrin as a chemoattractant to monitor the effect on endothelial chemotaxis via trans-well invasion and migration assays. This technique has been widely used to determine the invasive and migratory properties of cells in response to chemoattractants or pharmacotherapeutic agents (Kramer et al., 2013; Marshall, 2011; Valster et al., 2005). The rationale being that cells are capable of sensing the concentration gradients of growth factors and cytokines in their vicinity and chemotactically respond to it.

Firstly, we evaluated the relative levels of secreted Agrin in the conditioned medium of control and Agrin depleted MHCC-LM3 cells (**Figure 3.7A-upper panel**). As well as total protein expression in the whole-cell lysates (WCL) from the same cells (**Figure 3.7A-lower panel**). As expected, Agrin levels were high in control cells compared to Agrin depleted MHCC-LM3 cells (**Figure 3.7A-upper/lower panels**). Proportionately, shControl CM highly induced the invasion and migration of naïve HUVECs which was robustly decreased when shAgrin CM was used. Interestingly, when 10µg/ml of soluble recombinant Agrin was supplemented in shAgrin CM, that was sufficient to partially rescue HUVEC chemotaxis (**Figure 3.7B-right panel**).

A **B** **HUVEC chemotaxis**

Figure 3.7: Secreted Agrin promotes endothelial chemotaxis. (A) Dot blot analysis detecting secreted Agrin levels in the CM of shControl and shAgrin MHCC-LM3 cells. Ponceau S staining was used as a loading control (upper panel). Whole-cell lysate obtained from the same MHCC-LM3 cells was analyzed by Western blot for Agrin expression. β -actin was used as a loading control (lower panel). (B) Naïve HUVECs were subjected to Boyden chamber trans-well invasion and migration assay using CM from either control, Agrin depleted or Agrin-depleted cells supplemented with 10 μ g/ml of soluble Agrin (sAgrin) in the lower chamber as a chemoattractant. Representative images of invading and migrating cells were acquired via bright-field microscopy and analyzed by Image-J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates. ($n=3$, *** $P < 0.0009$, ** $P = 0.0026$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 10 μ m.

Cancer cells secrete a host of growth factors and cytokines that are important for their proliferation and survival which may also play a role in endothelial invasion and migration (Barderas et al., 2013). As such, we investigated whether the induction of endothelial chemotaxis was specific to secreted Agrin. Appropriately, we immuno-depleted Agrin from shControl CM containing high amounts of secreted Agrin and monitored the effects of this immuno-depletion on endothelial chemotaxis (**Figure 3.8A**). HUVEC invasion and migration were severely mitigated when Agrin was immuno-depleted from shControl CM. As expected, IgG isotype control did not hamper endothelial chemotaxis (**Figure 3.8B**). Our data infers that secreted Agrin is specifically able to induce endothelial chemotaxis.

Figure 3.8: Immuno-depletion of Agrin from MHCC-LM3 conditioned medium hampers HUVEC chemotaxis. (A) Validation of Agrin immuno-depletion from MHCC-LM3 conditioned medium (CM) by dot blot analysis. Ponceau S was used as a loading control. (B) Transwell invasion and migration of HUVEC using IgG or Agrin immuno-depleted MHCC-LM3 CM. Representative images were acquired via bright-field microscopy and quantified by Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, $**P = 0.005$, $*P = 0.0382$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, $10\mu\text{m}$.

Agrin Expression in HCC and endothelial cells

Given that secreted Agrin induces endothelial chemotaxis and tumor vascularization, we sort to understand the sensing mechanism involved in this response. Firstly, we investigated whether endothelial cells express Agrin. To this end, a panel of HCC and endothelial cells were analyzed by real-time polymerase chain reaction (RT-PCR) to determine the relative levels of Agrin mRNA in the respective cells. Though Agrin was expressed in a panel of endothelial cells, Agrin expression was highest in HCC cells (**Figure 3.9A**).

Since Agrin is a secreted proteoglycan, to further ascertain the relative levels in the aforementioned cells, we performed an Agrin enzyme-linked immunosorbent assay (ELISA) using the conditioned medium of MHCC-LM3 cells and HUVEC to measure the relative levels of secreted Agrin. Consistent with the mRNA levels, we observed that MHCC-LM3 cells secrete about 2-fold higher Agrin when compared to equal amounts of HUVEC cells (**Figure 3.9B**). These observations suggest that the difference in Agrin levels between HCC and endothelial cells contributes to the Agrin gradient that is sensed by ECs during chemotaxis.

Figure 3.9: Relative Agrin levels in HCC and endothelial cells. (A) Relative mRNA levels of Agrin in a panel of HCC and endothelial cells. (B) Agrin ELISA depicting the relative levels of secreted Agrin in the CM of the indicated cells. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, $***P < 0.0003$, $**P = 0.0021$, Student's *t*-test). Error bars denote standard deviation (SD).

HCC and endothelial cells express the same variant of Agrin

Given that both ECs and HCC cells express Agrin, we next wondered whether the Agrin variant expressed by these cells is the same. Due to alternative splicing, seven isoforms of Agrin are currently identified as transmembrane or secreted forms which include muscle Agrin lacking the Z-insertion and neural Agrin that bears amino acid insertions at the Z-spliced site (Glass et al., 1996).

Identifying the Agrin variant expressed in ECs is imperative in determining the possible mechanism(s) by which ECs sense secreted Agrin in the tumor microenvironment or cancer-cell conditioned medium. Given that even bacteria purified soluble recombinant Agrin bearing the Z8-insert was sufficient to rescue endothelial chemotaxis in Agrin depleted conditioned medium, suggests that these effects on endothelial behavior may not be specific to cancer-derived Agrin but rather a common response to secreted or soluble Agrin regardless of its source. Therefore, we hypothesized that both HCC and endothelial cells express the same variant of Agrin that accounts for EC sensing of the Agrin gradient during chemotaxis and tumor vascularization.

To validate whether ECs and HCC cells express the same variant of Agrin, we utilized PCR primers specific to neural Agrin that flanked the Z8-insert of C-terminal Agrin (**Figure 3.10A**). Since we had earlier identified that HCC cells express the neural variant Agrin (Chakraborty et al.,

2017). Respective complementary DNA (cDNA) sequences from HUVEC, MHCC-LM3 were subjected to PCR amplification using the said primers. As predicted, both MHCC-LM3 and HUVEC expressed the same neural variant of Agrin albeit at lower levels in HUVEC when compared to MHCC-LM3 cells. Neuroblastoma cell line SK-N-SH cells that are known to express neural Agrin, was used as a positive experimental control (**Figure 3.10B**).

Figure 3.10: MHCC-LM3 and HUVECs express neural Agrin with Z8 insert. (A) Model depicting primer design for neural Agrin. The black arrows indicate the respective primers flanking the Z8-insert specific to neural Agrin. **(B)** PCR-amplified neural Agrin resolved by Agarose gel electrophoresis in the indicated cell lines.

Characterization of soluble recombinant Agrin

Having observed that Agrin was important for vascularization of growing lesions and given that soluble recombinant Agrin was sufficient to rescue not only endothelial chemotaxis but also oncogenic defects associated with Agrin depletion in HCC (Chakraborty et al., 2015; Chakraborty et al., 2017), We sort to characterize the recombinant Agrin fragment. Accordingly, plasmid constructs of the C-terminal Agrin fragment that bear the Z8 insert were expressed in bacteria and

purified (**Figure 3.11A**). The ~22kDa fragment obtained was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with instant blue to determine the molecular weight (**Figure 3.11B**). Antibody recognition was achieved by dot blot analysis using Agrin specific antibody with epitope mapping in the C-terminal region (**Figure 3.11C**).

Figure 3.11: Characterization of soluble recombinant Agrin. (A) Cartoon of full-length Agrin highlighting Lrp4 binding site and the recombinant fragment purified for subsequent rescue experiments. (B) ~22kDa recombinant C-terminal Agrin fragment was purified and analyzed by

SDS-PAGE using Instant Blue staining. (C) Antibody detection of recombinant Agrin fragment by dot blot with increasing concentrations of soluble recombinant Agrin as indicated. Ponceau-S staining was used as the loading control.

Agrin facilitates cancer to endothelial cell adhesion

Pro-angiogenic growth factors secreted by growing tumors recruit endothelial cells to the tumor foci by chemotaxis (Carmeliet and Jain, 2000; Kerbel, 2008; Risau, 1997). The subsequent incorporation of these endothelial cells within the tumor mass requires a transient anchorage of ECs by tumor cells. Heterotypic cell adhesion between these cells is therefore vital for tumor vascularization (Kobayashi et al., 2007; Orr et al., 2000; Zetter, 1993).

Cell surface receptors such as Integrins and cadherins in association with extracellular matrix (ECM) components play a critical role in cell adhesion (Albelda and Buck, 1990; Gumbiner, 1996). Recently, we reported that Agrin-induced YAP activation serves as a mechanotransduction signal that attributes ECM stiffness (Chakraborty et al., 2017). Given Agrin's role in cell-matrix dynamics, we next investigated whether Agrin facilitates EC to cancer cell adhesion that may account for Agrin-mediated tumor vascularization. To this end, we performed a heterotypic cell adhesion assay using pre-labeled (cell tracker blue) liver cancer cells (MHCC-LM3) co-cultured on a confluent monolayer of endothelial cells (HUVEC) as outlined (**Figure 3.12A**). Experimentally, Agrin was depleted from both MHCC-LM3 and HUVEC cells by RNA interference. The knockdown efficiency was validated by western blot analysis (**Figure 3.12B**). Fifty thousand pre-labeled shControl, shAgrin or shAgrin cells rescued by expressing rat Agrin (Agrin rescue) MHCC-LM3 cells were allowed to adhere for 2h to a confluent layer of either siControl or siAgrin HUVEC respectively (**Figure 3.12C**). As anticipated, shControl MHCC-LM3

cells revealed robust adhesion to HUVEC when compared to shAgrin cells which showed very little adhesion to ECs after 2 hours (**Figure 3.12C-siControl HUVEC panel**). Interestingly, Agrin rescued cancer cells were able to partially restore adhesion defects associated with Agrin depletion. Notably, the loss in adherence was more robust when both cancer cell and endothelial Agrin were depleted suggesting that endothelial Agrin though to a lesser extent, was required for an effective cancer cell to EC adhesion (**Figure 3.12C-siAgrin HUVEC panel**). Moreover, given that shAgrin rescue cells partially restore adhesion to Agrin silenced ECs, further suggest that cancer-derived Agrin is more critical for this process.

Figure 3.12: Heterotypic cell adhesion assay between Agrin depleted cancer and endothelial cells. (A) Outline of the cancer cell to EC adhesion assay. (B) Western blot analysis of Agrin expression in shControl, shAgrin and shAgrin rescue MHCC-LM3 cells (left panel) and siControl and siAgrin HUVEC (right panel). (C) MHCC-LM3 cells pre-labeled with Cell Tracker blue plated on a confluent monolayer of sicontrol and siAgrin HUVECs for 2h. Representative fluorescent images were taken before adhesion (T=0h) and post-adhesion (T=2h), respectively. Data presented as mean $\pm$ SD of three independent experiments performed in quadruple (n=3, $P < 0.0097$, $*P = 0.0403$, Student's t-test). Error bars denote standard deviation (SD). Scale bar, 10 μ m**

Further, we depleted Agrin in other HCC cell lines; SNU-449 and Hep3B and monitored their adhesion to control and Agrin silenced HUVECs the same as above. Consistently, Agrin knockdown in these cells mitigated cancer to EC adhesion which was also more robust when both cancer and endothelial Agrin were depleted. (**Figure 3.13A-B**). Moreover, exogenous supplementation of soluble recombinant Agrin to the adhesion buffer of the Agrin depleted cells partially rescued the adhesion defects associated with loss of Agrin in both SNU-449 and Hep3B

cells respectively. Collectively, this data proves that Agrin is important for cancer cells to EC adhesion.

Figure 3.13: Agrin mediates cancer to endothelial cells adhesion in SNU-449 and Hep3B. (A-B) Western blot validating Agrin knockdown in HUVEC and SNU-449 (A) and Hep3B (B) cells. β -actin served as the loading control. Fluorescence microscopy images of heterotypic cell adhesion assay of control and Agrin depleted SNU-449 (A) or Hep3B (B) pre-labeled with cell tracker blue was allowed to adhered to control or Agrin depleted HUVEC for 2h. Quantification was done using Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, $***P < 0.0002$, $*P < 0.0356$, Student's t -test) for panel (A); ($n=3$, $***P < 0.0010$, $**P = 0.0068$, $*P < 0.0194$, Student's t -test), for panel (B). Error bars denote standard deviation (SD). Scale bars, $10\mu\text{m}$.

To determine the specificity of Agrin-mediated cancer cells to EC adhesion, we investigated the adhesion of MHCC-LM3 liver cancer cells to non-endothelial cells. To this end, we utilized retinal pigmented epithelial cells (ARPE). When MHCC-LM3 cells pre-labeled as earlier described were plated on a confluent monolayer of ARPE cells, very minimal adherence was observed ($\sim 25\%$). Moreover, these marginal adhesion levels remained unchanged regardless of Agrin expression status in the cancer cells (**Figures 3.14**). Thus, inferring that Agrin-mediated heterotypic cell adhesion is specific for cancer to endothelial cells.

Figure 3.14: Cell Adhesion assay in liver cancer and ARPE cells. Fluorescence microscopy images depicting adhesion of Cell-Tracker blue pre-labeled shControl, shAgrin and shAgrin rescue MHCC-LM3 cells on human retinal pigment epithelial cells (ARPE). Representative images were acquired at the indicated time points before and after adhesion. Quantification was done as in (Figure 3.13). Data denotes mean $\pm$ SD of 3 independent replicates ($n=3$, $***P < 0.0003$, $**P = 0.0043$, Student's t -test). Error bars represent standard deviation (SD). Scale bar, $10\mu\text{m}$.

Cancer-derived Agrin promotes endothelial sprouting

Upon anchorage of migrating endothelial cells to peripheral cancer cells in the tumor microenvironment, tumor vascularization is initiated by the formation of cutaneous capillaries that are subsequently expanded by tumor angiogenesis (Folkman, 1975; Hillen and Griffioen, 2007; Kerbel, 2008). Given that secreted Agrin from cancer cells is important for endothelial chemotaxis and cancer cell to EC adhesion, we next investigated if secreted Agrin was able to stimulate sprouting angiogenesis in endothelial cells. Appropriately, we utilized a spheroid-based sprouting assay. This is a well-established method that has been used extensively to study the effects of

numerous genetic alterations and/or pharmacological compounds on the tube forming ability of endothelial cells. Moreover, this assay offers the advantage of studying sprouting angiogenesis in a three dimensional (3D) environment which is more closely akin to an *in-vivo* setting when compared to other *in-vitro* angiogenesis techniques (Tetzlaff and Fischer, 2018).

Experimentally, endothelial cells were suspended in a hanging drop which allows them to cluster together forming spheroids. The spheroids were then embedded in a 3D collagen matrix and treated with conditioned medium from either shControl or shAgrin MHCC-LM3 cells for 24 hours. Interestingly, endothelial spheres treated with shControl CM revealed numerous endothelial sprouts of considerable tube length. In contrast, spheres treated with shAgrin CM did not reveal any appreciable sprouting (**Figure 3.15**). This suggests that secreted Agrin from cancer cells promotes endothelial sprout formation which in essence contributes towards tumor vascularization.

Figure 3.15: Secreted Agrin from MHCC-LM3 promotes endothelial sprout formation. Phase-contrast microscopy images showing sprouting of HUVEC spheroids treated with shControl or shAgrin MHCC-LM3 conditioned medium (CM) for 24h. Quantification was done using the sprout morphology analyzer on Image J. Data presented as mean $\pm$ SD of three-five spheroids analyzed per group performed in three technical repeats ($n=3$, $**P = 0.0068$, $*P < 0.0100$, student's *t*-test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

As secreted Agrin from liver cancer cells was able to induce EC chemotaxis, cancer cell to EC adhesion and endothelial sprout formation, we then questioned whether Agrin promotes endothelial morphogenesis and capillary formation. Hence, we sort to characterize the functional significance of soluble Agrin (sAgrin) in *in-vitro*, *ex-vivo* and *in-vivo* angiogenesis.

Firstly, since exogenous supplementation of soluble recombinant Agrin (sAgrin) was sufficient to rescue angiogenesis defects associated with Agrin depletion, we evaluated its significance on EC morphogenesis and capillary formation *in-vitro*. To this end, we utilized an *in-vitro* tube formation assay. During angiogenesis, the binding of angiogenic growth factors such as VEGF to its cell surface receptors leads to endothelial activation. Activated endothelial cells secrete proteases that degrade their surrounding basement membrane allowing for endothelial cell migration in response to the angiogenic stimulus. Additionally, the induction of endothelial cells by pro-angiogenic factors also triggers cellular processes that favor endothelial proliferation, survival and cytoskeletal re-organization which allows for the formation of three-dimensional tube-like structures (Arnaoutova and Kleinman, 2010; Folkman and Haudenschild, 1980). This assay has been extensively used to study *in-vitro* angiogenesis based on the fact that when endothelial cells are cultured on a basement membrane gel extract such as Matrigel, they undergo cell

differentiation, and directional migration to form polygonal tube-like networks of blood vessels (Ko and Lung, 2012).

As a result, we pre-treated naïve endothelial cells with 10 μ g/ml sAgrin overnight before subjecting the cells to *in-vitro* tube formation on Matrigel. Notably, when compared to untreated cells, sAgrin treatment on naïve HUVECs enhanced their rates of master segment formation and total tube-lengths during the observed period (**Figure 3.16**).

Figure 3.16: Soluble Agrin promotes the rate of *in-vitro* tube formation. Representative time-lapse phase-contrast images showing rates of *in-vitro* tube formation in untreated and sAgrin (10 μ g/ml) treated HUVEC. Quantification was done using angiogenesis analyzer from Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates (n=3,

*** $P = 0.001$, ** $P < 0.008$, * $P < 0.04$, student's t -test). Error bars denote standard deviation (SD). Scale bar, $100\mu\text{m}$.

Furthermore, Considering that Agrin is a secreted proteoglycan in the ECM, which promotes the recruitment of surrounding endothelial cells to localized and metastatic tumor foci, we envisaged that incorporating Agrin into collagen matrix before the embedding of endothelial spheroids in a 3D sprouting assay might yield enhance sprouting as this mimics an *in-vivo* environment with high amounts of secreted Agrin in the tumor matrix. As predicted, sAgrin incorporated matrices revealed enhanced sprouting in several primary ECs when compared to un-incorporated matrices (Figure 3.17). Thus, suggesting that secreted Agrin in the tumor matrix promotes pro-angiogenic events that contribute to tumor vascularization.

Figure 3.17: Soluble recombinant Agrin stimulates endothelial sprouting in a 3D collagen matrix. Representative images of endothelial sprouting in a 3D collagen matrix for the indicated primary ECs either left untreated or supplemented with 10 μ g/ml sAgrin. Images were captured 24h post-embedding. Quantification was done using a sprout morphology analyzer from Image J. Data presented as mean $\pm$ SD of three spheroids per group performed in three repeats ($n=3$, $**P < 0.0095$, $*P < 0.0240$, student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Soluble Agrin stimulates blood vessel sprouting *ex-vivo*

In-vitro outcomes can often be limited in scope or degree of generalizability when complementary approaches are undertaken *ex-vivo* or *in-vivo*, owing to the lack of proper controls that can effectively mimic the complexities of cellular interactions in a heterotypic environment (Staton et al., 2004). To address this limitation, we further characterized the effect of sAgrin on sprouting angiogenesis in a fetal mouse metatarsal *ex-vivo* model. This technique is increasingly employed to study the angiogenic potential of different growth factors and pharmacological agents by evaluating the effect on endothelial sprouting (Wang et al., 2013). Experimentally, fetal mouse metatarsals were excised on embryonic day 16.5 and cultured for 10 days with or without soluble Agrin supplementation. Subsequently, the explants were fixed and stained for blood vessel marker CD-31. Potent angiogenesis inducer VEGF which is known to stimulate sprouting angiogenesis was used as a positive experimental control. Notably, sAgrin treatment significantly stimulated blood vessel outgrowths which were comparable to VEGF treated metatarsals (**Figure 3.18**).

Figure 3.18: Soluble Agrin stimulates endothelial sprouting in fetal mouse metatarsals. Representative images of sprouting vessels from fetal mouse metatarsals treated with either vehicle (PBS + 20 μ g/ml BSA) (n=11), VEGF (50ng/ml) (n=5) or Agrin (20 μ g/ml) (n=7) for 10 days. Scale bar, 200 μ m. Data presented as mean $\pm$ SD of five-eleven metatarsals per group (***P = 0.0007, 1-way ANOVA). Error bars denote standard deviation (SD).

Soluble Agrin induces vascularization *in-vivo*

Because cancer cells secrete a host of pro-angiogenic growth factors and cytokines that may collectively contribute towards the recruitment of surrounding blood vessels from the tumor environment, we sort to decipher whether these pro-angiogenic events observed were specific to Agrin stimulation. To exclude the influence of other supporting growth factors in Agrin-mediated vascularization, we performed a cell-free *in-vivo* Matrigel plug assay. This is a widely used approach to evaluate the angiogenic potential of growth factors and pharmacological agents (Lung and Lung, 2012). Cell-free assays are imperative to minimize the complexities of multicellular interactions via cell-derived factors which may otherwise conceal the true angiogenic potential of the pharmacotherapeutic agent under investigation (Staton et al., 2009).

To determine if soluble Agrin in isolation from other cell-derived factors can promote angiogenesis *in-vivo*, soluble Agrin or vehicle control was incorporated into growth factor-reduced Matrigel and

injected into the flanks of SCID mice. New doses of vehicle control and soluble Agrin were injected into the cell-free plugs every four days for a total duration of two weeks. Specifically, the Agrin treated plugs revealed significant vascularization compared to control plugs (**Figure 3.19A**). The plugs were subsequently sectioned and stained for blood vessel marker CD-31 which revealed enriched staining in the Agrin treated plugs but not the control plugs (**Figure 3.19B**). This data clearly suggest that Agrin mediates angiogenic cues that promote vascularization and these pro-angiogenic events are isolated from the contributing influences of other tumor-derived factors.

Figure 3.19: Soluble recombinant Agrin promotes vascularization in cell-free matrigel plug assay *in-vivo*. (A) Representative images of subcutaneously implanted cell-free matrigel plug at 14 days post-implantation in SCID mice. Plugs were treated with vehicle control or 20 μ g/ml sAgrin once every 4 days for the indicated duration. Data represents $\pm$ SD of four sponges per group, (n=4, *P = 0.0335, Student's t-test). Scale Bar: 1 mm. (B) Immunohistochemistry staining of vessel-associated CD-31 within matrigel plugs as in panel (A). Four matrigel plug sections per group were quantified by Image J. Data represents $\pm$ SD of four sponges per group (n=4, **P = 0.0017, Student's t-test). Error bars denote standard deviation (SD). Scale Bar: 100 μ m

Agrin silencing hampers *in-vitro* angiogenesis

In hepatocellular carcinoma, secreted Agrin promotes the proliferation, survival and invasive properties of tumor hepatocytes (Chakraborty et al., 2015; Chakraborty et al., 2017). Taking into account that endothelial cells also express and secrete the same neural variant of Agrin that is expressed in HCC cells, we contemplated whether endothelial Agrin is important for EC homeostasis and function. Moreover, the fact that secreted Agrin from liver cancer cells or exogenously supplied stimulated pro-angiogenic phenotype in endothelial cells further warranted an investigation into the potential role of Agrin in endothelial behavior and function. To investigate this, we utilized a ‘loss-of-function’ RNA interference approach that efficiently depleted endothelial Agrin to analyze its functional consequence on primary ECs.

When endothelial cells are cultured on a basement membrane gel extract such as Matrigel, they undergo cell differentiation, and directional migration to form polygonal tube-like networks of blood vessels (Arnaoutova and Kleinman, 2010). To monitor the consequences of Agrin depletion EC function, we subjected to control and Agrin depleted HUVECs to *in-vitro* tube formation assay

on Matrigel. Interestingly, Agrin knockdown significantly decreased the tube forming ability of HUVECs, with a concurrent decrease in the number of master segments and total tube length (**Figure 3.20**). Thus, suggesting that endothelial Agrin is important for EC morphogenesis and cytoskeletal reorganization during *in-vitro* tube formation.

Figure 3.20: Agrin knockdown hampers *in-vitro* tube formation in HUVEC. HUVEC cells transfected with either control or Agrin siRNA were subjected to *in-vitro* tube formation on Matrigel 72h post-transfection. Representative phase-contrast images were acquired 6h after *in-vitro* tube formation assay and analyzed using the angiogenesis analyzer from Image J. Scale bar 100 μ m

Though Agrin knockdown significantly mitigated *in-vitro* tube formation in HUVEC, we sort to validate that this outcome was specific to Agrin depletion and not a sequence-non-specific effect of RNA interference. To determine the specificity of these defects to Agrin silencing, we treated the Agrin silenced HUVEC with increasing concentrations of soluble recombinant Agrin (sAgrin). Noteworthy, supplementing sAgrin in the culture medium of Agrin depleted HUVEC, effectively

rescued the *in-vitro* angiogenesis defects in a dose-responsive manner (Figure 3.21). Thus, validating that the observed defects in *in-vitro* tube formation were specific to Agrin silencing in primary HUVECs.

Figure 3.21: Soluble Agrin rescues *in-vitro* tube formation in Agrin depleted HUVECs in a dose-dependent manner. (A) Western blot validation of Agrin knockdown in siControl and siAgrin HUVECs. β -actin was used as the loading control. (B) Representative phase-contrast images of *in-vitro* angiogenesis in HUVEC transfected with control or Agrin siRNA for 48h and treated with increasing concentrations of soluble recombinant Agrin (sAgrin) for 12-18h, before subjecting the cells to *in-vitro* tube formation assay. Image analysis and quantification were done using angiogenesis analyzer from Image-J. Data represented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0006$, ** $P < 0.0038$, * $P < 0.0334$, student's *t*-test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Consistently, when Agrin was silenced in a panel of primary ECs including human umbilical vein endothelial cells (HUVEC), human retinal microvascular endothelial cells (HRMEC), human dermal microvascular endothelial cells (HDMEC) and human aortic endothelial cells (HAEC), we observed similar defects in *in-vitro* tube formation that was also rescued when 10 μ g/ml of sAgrin was exogenously supplied to the culture medium of the Agrin depleted ECs for ~18 hours before tube formation. (**Figure 3.22**). Thus, inferring that endothelial Agrin is critical for the cytoskeletal reorganization and directional migration associated with *in-vitro* tube formation in primary ECs.

Figure 3.22: Agrin depletion inhibits in-vitro tube formation in ECs which is rescued by soluble Agrin supplementation. Western blot validation of Agrin knockdown in indicated ECs. β -actin used as a loading control. Phase-contrast images of in-vitro tube formation in the indicated

primary endothelial cells transfected as in (Figure 3.21) and rescued with 10 μ g/ml sAgrin for 12-18h before *in-vitro* tube formation. Quantification performed the same as in Figure 3.21. Data presented as mean $\pm$ SD from three experiments performed in triplicates ($n=3$, *** $P = 0.0002$, ** $P < 0.009$, * $P = 0.015$, Student's *t*-test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Additionally, we utilized an alternative approach to further minimize the effects of RNA interference. As a result, HUVECs were transduced with control or Agrin shRNA bearing lentiviruses to generate a stable pool of Agrin depleted cells, the loss of Agrin decreased *in-vitro* angiogenesis that was also partially rescued by exogenous supplementation of sAgrin in the culture medium (Figure 3.23).

Figure 3.23: Agrin knockdown using lentiviral particles bearing Agrin shRNA impedes *in-vitro* angiogenesis. Western blot showing Agrin knockdown in HUVEC transduced with control or Agrin specific lentiviral shRNA particles. β -actin was used as the loading control. shControl, shAgrin alone and shAgrin supplemented with 10 μ g/ml sAgrin for 24h were subjected to *in-vitro* tube formation. Representative images were acquired by phase-contrast microscopy and quantified by the angiogenesis analyzer from Image-J. Data represent mean $\pm$ SD of three independent experiments done in triplicates ($n=3$, *** $P = 0.0009$, ** $P = 0.0015$, * $P = 0.0179$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Given that Agrin is a secreted proteoglycan that mediates its cellular functions via association with cell-surface receptors, we envisaged that inhibiting endothelial Agrin's connection to these receptors may mimic the same defects in *in-vitro* tube formation as Agrin silencing in primary ECs. Hence, we treated HUVEC with increasing concentrations of Agrin function-blocking antibody that has been used in other studies to decrease Agrin mediated oncogenic events (Chakraborty et al., 2015; Chakraborty et al., 2017). In conjunction with Agrin silencing, blocking Agrin function by antibody treatment also inhibited tube formation in a dose-responsive manner (**Figure 3.24**), which further validates the significance of Agrin to *in-vitro* angiogenesis.

Figure 3.24: Agrin function-blocking antibody hinders in-vitro angiogenesis in a dose-dependent fashion. Dose-responsive inhibition of in-vitro tube formation in HUVEC pretreated with IgG or increasing concentrations of anti-Agrin (MAb5204) for 24h. Representative phase-contrast microscopy images are shown. Image quantification was done using the angiogenesis analyzer from Image J. Data shown as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0006$, ** $P < 0.0064$, * $P < 0.0340$, student's t -test). Error bars denote standard deviation (SD). Scale bar, $200\mu\text{m}$.

Taking into account that the Agrin function-blocking antibody utilized in the aforementioned experiment was able to recognize soluble recombinant Agrin protein in a dot blot analysis, we,

therefore, predicted that pre-incubation of the Agrin function-blocking antibody with soluble recombinant Agrin may neutralize the inhibitory effects on *in-vitro* tube formation. Though anti-Agrin treatment alone was able to hamper *in-vitro* tube formation in HUVEC as previously observed, when pre-incubated with soluble Agrin protein for 4 hours at 4 °C before EC treatment, we did not observe any appreciable decrease in tube formation (**Figure 3.25**), thereby suggesting the specificity of sAgrin and its functional abilities on rescuing Agrin mediated angiogenesis.

Figure 3.25: Soluble Agrin binds Agrin specific antibody. Naïve HUVECs were pretreated overnight with IgG isotype control, Agrin antibody alone or Agrin antibody pre-incubated with sAgrin for 4h at 4°C. The treated cells were then subjected to *in-vitro* tube formation. Representative images were acquired and analyzed as in (Figure 3.24). Data presented as mean $\pm$ SD of three independent experiments done in triplicates ($n=3$, *** $P = 0.0008$, ** $P < 0.0016$, Student's *t*-test). Scale bar, 100 μ m.

Recognizing that *in-vitro* tube formation is mainly a morphogenesis assay, we utilized an alternative approach to study the significance of endothelial Agrin to *in-vitro* sprouting angiogenesis. Hence, Agrin depleted HUVEC and HDMEC was allowed to form spheroids that were subsequently embedded in a 3D-collagen matrix. Agrin depletion severely inhibited HUVEC and HDMEC spheroid sprouting which was again rescued by sAgrin supplementation (**Figure 3.26**). Together, these gene epistases and function-blocking strategies reveal the relevance of endothelial Agrin to *in-vitro* angiogenesis.

Figure 3.26: Agrin knockdown deters endothelial sprouting in a 3D-collagen matrix. Sprouting images in control or Agrin depleted HUVEC spheroids embedded within a 3D collagen matrix alone or supplemented with 10 μ g/ml sAgrin. Quantification was done using the sprout morphology analyzer from Image J. Data presented as mean $\pm$ SD of three-seven spheroids per group (n=3, **P < 0.0072, *P = 0.0034, Student's t-test). (n=3, *** P < 0.0006, **P < 0.0064, *P < 0.0340, student's t-test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

During angiogenesis, the activation of endothelial cells leads to the secretion of matrix-degrading proteases and the activation of signaling pathways that favor endothelial proliferation and directional migration to form tube-like structures (Risau, 1997). Taking into account that Agrin silencing in ECs mitigated their ability to form tube-like structures on Matrigel, we contemplated whether this outcome was linked to an underlying defective proliferation and/or migrational properties that may explicate the observed phenotype. To this end, we investigated the effects of Agrin silencing on endothelial proliferation, invasion, and migration. Appropriately, control and Agrin depleted HUVECs were monitored over three days by MTS proliferation assay. Agrin silencing significantly decreased HUVEC proliferation which was partially rescued by supplementing the Agrin depleted cells with soluble Agrin as indicated (**Figure 3.27**).

Figure 3.27: Agrin is required for endothelial proliferation. (A) Western blot validation of Agrin knockdown in HUVEC with β -actin as a loading control. (B) Cell proliferation assay performed in siControl, siAgrin or siAgrin supplemented with 10 μ g/ml of soluble Agrin for the indicated days, 72h post-transfection with siRNA. Relative cell proliferation was determined by MTS assay and absorbance was measured at 490nm. Data presented as mean proliferation $\pm$ SD of three independent experiments performed in triplicates. ($n=3$, $**P < 0.0096$, $*P < 0.0354$, student's t -test). Error bars denote standard deviation (SD).

Agrin depletion decreased endothelial invasion and migration

In hepatocellular carcinoma, Agrin silencing decreased the invasive and migratory properties of HCC cells (Chakraborty et al., 2015). Moreover, cancer-derived Agrin in the conditioned medium of MHCC-LM3 liver cancer cells sufficiently induced EC invasion and migration. Given the relevance of EC invasion and migration to the angiogenic process, we next evaluated whether endothelial Agrin played a direct role in the invasive and migratory properties of primary ECs. Hence, we depleted Agrin from HUVEC and HDMEC before subjecting the cells to a Boyden chamber invasion and migration assay. Silencing of endothelial Agrin markedly decreased the invasive and migratory properties of these primary ECs which was partially rescued by treating the Agrin depleted cells with soluble Agrin for 18h before the invasion and migration assay (Figure 3.28)

Figure 3.28: Agrin silencing hinders EC invasion and migration. Trans-well invasion and migration assays in control, Agrin depleted and Agrin knockdown ECs supplemented with 10 μ g/ml sAgrin for 12-18h before the experiment. Representative images were acquired via bright-field microscopy and analyzed using Image-J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0004$, ** $P < 0.0040$, * $P < 0.0400$, student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

To independently validate the effect of Agrin silencing on EC migration, we performed a wound (scratch) assay and monitored the rate of wound closure over 12 hours. Compared to control cells which revealed a complete closure of the wound area by the observed 12-hour time point, Agrin depletion decreased the wound-healing abilities of HUVEC over the indicated period. These

defects were partially rescued when exogenous Agrin was supplemented in the culture medium of the Agrin depleted cells (**Figure 3.29**).

Figure 3.29: Agrin knockdown decreases the pace of wound healing in HUVEC. Control and Agrin depleted HUVECs with or without supplementation of 10 μ g/ml sAgrin, were subjected to wound-healing scratch assay. Representative phase-contrast images were acquired at indicated time points. Wound area was quantified by Image J. Data presented as mean wound area $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0002$, ** $P < 0.0088$, * $P = 0.0168$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 10 μ m.

Lrp4-Integrin β 1-FAK axis mediates Agrin induced angiogenesis

At the neuromuscular junction where Agrin's function is best characterized, secreted Agrin via its receptors low-density lipoprotein receptor-related protein 4 (Lrp4) and muscle-specific tyrosine kinase (MuSK), facilitate the aggregation of acetylcholine receptors (AChR) leading to effective neuromuscular transmission (Kim et al., 2008; Ruegg and Bixby, 1998). Moreover, In hepatocellular carcinoma, Agrin also utilizes the same receptor repertoire to form an oncogenic signaling complex in association with Integrin β 1 and FAK that enhances cancer progression (Chakraborty et al., 2015; Chakraborty et al., 2017).

Secreted Agrin binds Lrp4 via the C-terminus. The presence of eight amino acid insertion in the Z-spliced site (Z8) enhances Agrin's affinity to Lrp4. In-vivo, Agrin is susceptible to proteolytic cleavage generating bio-active fragments that mediate Agrins functions. The C-22 Agrin fragment, generated from C-terminal Agrin enhances the formation of dendritic filopodia in the neuronal cells via binding to Agrin receptor Lrp4 (Kim et al., 2008; Zhang et al., 2008; Zong et al., 2012). Because the ~22kDa soluble recombinant Agrin used in rescue experiments is the same C-22 fragment that is naturally generated *in-vivo* by proteolytic cleavage, we envisaged that the same Agrin receptor repertoire observed to mediate Agrin signaling at the neuromuscular junction and in HCC may also be responsible for mediating Agrin's angiogenic cues in ECs. Moreover, the fact that both HCC and endothelial cells express the same neural Agrin variant further supports the hypothesis that these same receptors may be involved in Agrin-mediated angiogenesis.

To this end, we first evaluated the expression of Agrin receptors; Integrin β 1, α - and β -dystroglycan, MuSK, and Lrp4 in HUVEC and HRMEC. Western blot analysis revealed the protein expression of these receptors in both HUVEC and HRMEC respectively (**Figure 3.30**).

Figure 3.30: Agrin receptors are expressed in ECs. Whole-cell lysates of HUVEC and HRMEC were analyzed for expression of indicated proteins by Western blot. β -actin was used as the loading control.

Since Agrin is a secreted proteoglycan in the ECM, the requirement for cell-surface receptors is therefore imperative in mediating intracellular signaling in endothelial cells. If Agrin signals via any of its bonafide receptors; Integrin β 1, α - and β -dystroglycan, Lrp4, and MuSK, then silencing this receptor/s in ECs should reveal similar defects in *in-vitro* angiogenesis comparable to that observed upon Agrin depletion in these cells. Suitably, we silenced each of the aforementioned receptors by RNA interference (**Figure 3.31A**) and subjected the depleted cells to *in-vitro* tube formation on Matrigel (**Figure 3.31B**). The knockdown of the dystroglycan complex did not reveal any significant defects in *in-vitro* angiogenesis, suggesting that Agrin may not require α - or β -dystroglycan for mediating its angiogenic signaling. However, depletion of Integrin β 1, Lrp4 and MuSK similarly affected *in-vitro* tube formation to Agrin depletion albeit to a lesser extent in

MuSK depleted cells (**Figure 3.31B**). Thus, suggesting that Agrin may utilize these receptors to mediate its angiogenic cues.

Figure 3.31: Depletion of Agrin receptors similarly hinders in-vitro tube formation to Agrin silencing. (A) Western blot validating the knockdown of indicated proteins in HUVEC. β -actin as a loading control. (B) HUVECs transfected with siRNAs specific to the indicated proteins were analyzed for in-vitro tube formation on Matrigel. Representative phase-contrast images were acquired after 6h and quantified using the angiogenesis analyzer from Image-J. Data represented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0010$, * $P < 0.0353$, student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Though these receptors may be required for Agrin's signaling in angiogenesis, we questioned whether these were the only receptors capable of mediating Agrin's cues in endothelial cells. Rationally, if Agrin has alternate receptor(s) that mediate its angiogenic functions, under such circumstances, increasing the levels of Agrin in the cell culture environment of Integrin β 1, Lrp4, MuSK, and FAK depleted cells should facilitate the activation of such alternate pathways thus mediating a rescue in *in-vitro* tube formation. Interestingly, supplementing soluble Agrin to these cells did not rescue the associated defects on *in-vitro* tube formation further suggesting

that Agrin utilizes these receptors in mediating its angiogenic cues (**Figure 3.32** and **Figure 3.33**). Moreover, given that these receptors were individually depleted and yet revealed similar defects in *in-vitro* angiogenesis even in the presence of exogenously supplied Agrin, it may suggest a unified pathway where Agrin requires the collective input of the aforementioned receptors in mediating angiogenesis.

Furthermore, if Agrin displayed a signaling preference for any of these receptors, it should, therefore, follow that, the depletion of the alternate receptor in the presence of basal levels of Agrin should not reveal similar defects on *in-vitro* tube formation as the preferred receptor. Nevertheless, even when soluble Agrin was exogenously supplied to augment the basal Agrin levels in these ECs, it failed to rescue the associated defects on *in-vitro* angiogenesis thus inferring that Agrin does not demonstrate a receptor preference for any of these receptors. Thus, our data suggest that Agrin requires the unified input of Integrin $\beta 1$, Lrp4, MuSK, and FAK to mediate angiogenic signaling.

Figure 3.32: Defective in-vitro tube formation associated with Integrin $\beta 1$, Lrp4, and FAK silencing is not rescued by soluble Agrin. Integrin $\beta 1$, Lrp4, and FAK depleted HUVECs were either untreated or supplied with sAgrin (10 μ g/ml) for 18h and subjected to in-vitro tube formation. Presented images were captured and analyzed as in (Figure 3.31). Data presented as mean $\pm$ SD (n=3, ***P < 0.0005, student's t-test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Figure 3.33: Defective in-vitro tube formation associated Lrp4/MuSK depletion is not rescued by soluble Agrin. HUVECs 72h post-transfection with siControl, siMuSK, and combination of siMuSK + siLrp4, were analyzed by in-vitro tube formation with or without supplementation of 10 μ g/ml sAgrin. Representative images were acquired by phase-contrast microscopy and quantified by Image J. Data presented as mean $\pm$ SD of three independent experiments ($n=3$, *** $P = 0.0001$, ** $P < 0.0084$, * $P = 0.0263$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Given that Agrin may require this receptor repertoire for mediating angiogenesis, we were curious to identify the mechanistic underpinnings of this signaling. Reports on Agrin biology show that C-terminal Agrin directly binds to the Lrp4 extracellular domain (ECD). At the

neuromuscular junction, this interaction is crucial for the activation of MuSK and the aggregation of acetylcholine receptors (Kim et al., 2008; Zhang et al., 2008). Moreover, this interaction is also critical in Agrin mediated oncogenic events in HCC. The insertion of amino acids at the z-spliced site by alternative splicing enhances Agrin's affinity for Lrp4 by ~1000 folds (Zong and Jin, 2013).

Recognizing that Agrin does not interact directly with MuSK or Integrin beta 1, but rather with Lrp4, we envisaged that the interaction between Agrin and lrp4 must consequently be critical for the angiogenic process. Therefore, we investigated the significance of this interaction. Rationally, since exogenous supplementation of soluble Agrin rescued angiogenesis in Agrin depleted cells, we pre-treated Agrin silenced HUVECs for 18 hours with either soluble Agrin only, or a pre-incubated mixture of soluble Agrin and recombinant Lrp4 extracellular domain (ECD) (that is known to bind Agrin) before subjecting the treated cells to *in-vitro* tube formation assay on Matrigel. While 10µg/ml of soluble Agrin treatment was sufficient to rescue defects in tube formation, pre-incubating soluble Agrin with recombinant Lrp4 ECD nullified the soluble Agrin mediated rescue of *in-vitro* angiogenesis in Agrin depleted HUVEC (**Figure 3.34**). This data suggests that binding of Agrin to endogenous Lrp4 is a key inducer of the pro-angiogenic phenotype.

Figure 3.34: rh-Lrp4 ECD binds soluble Agrin. Model of Agrin and Lrp4 interaction, showing purified recombinant fragment containing Z8-insert binds to the Lrp4 extracellular domain (ECD). Phase-contrast microscopy images of control and Agrin depleted HUVECs supplemented overnight with 10 μ g/ml sAgrin only or sAgrin (10 μ g/ml) pre-incubated with 20 μ g/ml recombinant human Lrp4 ECD at 4°C for 2h before treatment. Quantification was done the same as in (Figure

3.34). Data presented $\pm$ SD of three independent experimental replicates. ($n=3$, $***P < 0.0008$, $**P = 0.0012$, $*P < 0.0174$, Student's *t*-test). Error bars denote standard deviation (SD). Scale bar, 200 μ m.

Recently, we reported that in HCC, Agrin facilitates a multi-protein complex formation between Integrin β 1-Lrp4-MuSK that is critical for mediating its oncogenic cues and cancer progression (Chakraborty et al., 2015; Chakraborty et al., 2017). Given that our data in this current study points to a unified signaling pathway involving Lrp4, MuSK, and Integrin β 1 that also requires FAK activity, we then asked whether Agrin also facilitated a multi-protein complex formation in ECs that is critical for its angiogenic signaling which may account for the perceived unified approach involving the aforementioned receptors in Agrin-mediated angiogenesis *in-vitro*.

To address this, we first investigated whether silencing Agrin affects the overall protein expression or stability of its receptors Integrin β 1 and Lrp4 in endothelial cells. Interestingly, Agrin depletion decreased the overall expression of Integrin β 1 and Lrp4 that also accounted for a decrease in FAK activation downstream of Integrin β 1 and Lrp4 but did not alter the expression of α/β -dystroglycan or MuSK (Figure 3.35 A-B).

Figure 3.35: Agrin silencing down-regulates the expression of Integrin $\beta 1$, Lrp4, and FAK phosphorylation. (A) Western blot analysis of indicated proteins in control and Agrin depleted HUVEC and HRMEC. β -actin was used as the loading control. (B) Agrin silencing does not affect the expression levels of α/β dystroglycan or MuSK in HUVEC. β -actin was used as the loading control.

Furthermore, supplementing sAgrin for 18 hours in the culture medium of Agrin depleted HUVEC was sufficient to partially rescue the expression of Integrin $\beta 1$ and Lrp4 with a corresponding increase in FAK activation (**Figure 3.36**). Cumulatively, this infers that endothelial Agrin regulates the expression of its receptors whose levels are collectively required for mediating pro-angiogenic events.

Figure 3.36: Soluble Agrin partially rescues the expression of Integrin $\beta 1$, Lrp4, and FAK phosphorylation. Expression levels of indicated proteins in siControl, siAgrin, and siAgrin treated with 10 μ g/ml sAgrin. β -actin served as the loading control.

Given that silencing Agrin decreased the expression of Lrp4 and Integrin $\beta 1$ that was partially rescued by soluble Agrin, we then questioned the existence of a reciprocal regulation between Lrp4 and Integrin $\beta 1$ that may account for the fact that silencing either receptor revealed similar defects on *in-vitro* tube formation that was not rescued by soluble Agrin supplementation. To this end, we silenced Lrp4 and Integrin $\beta 1$ individually in HUVEC and examined the relative effect on the total expression of the other receptor. Interestingly, silencing either receptor decreased the relative stability of the other (**Figure 3.37**). This finding further supports a unified signaling approach whereby Agrin requires stabilized Lrp4 and Integrin $\beta 1$ to mediate its angiogenic cues.

Figure 3.37: Reciprocal stabilization of Integrin $\beta 1$ and Lrp4 in endothelial cells. Western blot analysis of indicated proteins in Integrin $\beta 1$ and Lrp4 depleted HUVECs. β -actin served as the loading control.

As a downstream mediator of Integrin $\beta 1$ signaling, FAK activation is critical for many cellular processes such as cytoskeletal reorganization and directional migration which are both critical for effective angiogenesis (Giancotti and Ruoslahti, 1999; Guan, 1997). Given that silencing

FAK hampered *in-vitro* tube formation that was not rescued by soluble Agrin, suggests that Agrin-mediated FAK activation was important for its angiogenic signaling.

To further ascertain the role of FAK as a downstream effector of Agrin-mediated angiogenesis, we pre-treated HUVEC cells with solvent control or FAK inhibitor (PF562271) for two hours followed by drug wash-off. The pre-treated cells were either left without further manipulation or exogenously supplied with 10µg/ml sAgrin. Post-manipulation, the cells were monitored for FAK activity and focal adhesion dynamics respectively (**Figure 3.38 A-B**). PF562271 treatment for 2 hours was sufficient to abolish FAK phosphorylation, with a corresponding decrease in focal adhesion assembly which was restored post-drug wash-off. Noteworthy, focal adhesions re-assembly post-drug wash-off was more prominent when soluble Agrin was supplemented to the culture media (**Figure 3.38 C**). Thus, suggesting that Agrin promotes FAK activity and focal adhesion assembly.

Figure 3.38: PF-562271 inhibits FAK phosphorylation and focal adhesion assembly. (A) Outline of PF-562271 experimental set-up in endothelial cells. HUVECs were pretreated with solvent or PF-562271 for 2h followed by drug wash-off. (B) FAK phosphorylation (Y397) was analyzed by Western blot in HUVEC treated with either DMSO (solvent) or FAK inhibitor (PF-562271) for 2h. (C) Similarly, HUVEC were treated for 2h with PF562271, followed by drug-wash off and incubated in complete EGM-2 with or without the addition of 10 μ g/ml sAgrin. Cells were subsequently fixed and stained with pFAK Y397. Representative confocal images are shown. Arrows denote focal adhesion assembly. Scale bar, 10 μ m

Likewise, when HUVEC was similarly pretreated with PF562271 as above and subjected to *in-vitro* angiogenesis assay on Matrigel, this severely hampered tube formation. Supplementing soluble Agrin under these conditions, failed to rescue the defects in tube formation caused by FAK inhibition. Thus, suggesting that Agrin requires FAK activity for mediating its pro-angiogenic cues. Intriguingly, when soluble Agrin was supplemented to the pre-treated cells post-drug wash-off, we observed a significant rescue of *in-vitro* tube formation when compared to the drug-wash-off only. Besides, supplementing bovine serum albumin (BSA) as a protein control instead of sAgrin did not reveal any significant benefits when compared to drug-wash-off only (**Figure 3.39**). Thus, demonstrating that the observed rescue events were Agrin-specific. Moreover, this data validates the significance of FAK activity as a downstream mediator of Agrin's angiogenic signaling.

Figure 3.39: PF-562271 hampers in-vitro angiogenesis which is restored by soluble Agrin post-drug-wash off. HUVEC pre-treated for 2h as in (Figure 3.38) were subjected to in-vitro tube formation on Matrigel using complete EGM-2 either supplemented with 10 μ g/ml sAgrin or 10 μ g/ml BSA in the presence or absence of the inhibitor. Representative images were acquired by phase-contrast microscopy and analyzed by Angiogenesis analyzer from Image-J. Data shown as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0007$, ** $P = 0.0079$, * $P = 0.0228$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Agrin-Lrp4-Integrin β 1-FAK pathway adhere cancer cells to ECs

Taking into account the relevance of endothelial Integrin β 1, Lrp4 and FAK activity to Agrin-mediated angiogenesis, we next questioned whether Agrin requires these receptors to facilitate cancer to endothelial cell adhesion that may account for the recruitment of blood vessels during tumor vascularization. As such, we performed a cell adhesion assay using pre-labeled shControl,

shAgrin and shAgrin rescued MHCC-LM3 liver cancer cells as earlier described. This was allowed to adhere for 2 hours to a confluent monolayer of HUVECs pre-transfected with either siControl, siLrp4 or siIntegrin β 1 respectively. We observed that the combined loss of endothelial Integrin β 1 and cancer cell Agrin drastically reduced the adherence of MHCC-LM3 cells to HUVEC (**Figure 3.40**). However, the combined knockdown of endothelial Lrp4 and cancer cell Agrin did not show any synergistic inhibition of adherence when compared to Agrin depletion only (**Figure 3.40**). Thus, suggesting that endothelial Integrin β 1 but not Lrp4 is critical for Agrin-mediated cancer to endothelial cell adhesion.

Figure 3.40: Silencing of Agrin receptors Integrin $\beta 1$ and Lrp4 hampers cancer to endothelial cell adhesion similar to Agrin depletion. Representative fluorescent images denoting adhesion of pre-labeled shControl, shAgrin, and shAgrin rescued MHCC-LM3 cells to either control, Integrin $\beta 1$ or Lrp4 silenced HUVEC. Images were acquired at the indicated time points and quantified using Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, $*P < 0.0393$, student's t -test). Error bars denote standard deviation (SD). Scale bar, $10\mu m$

To further appreciate the involvement of Agrin and its receptors in the adhesion of cancer to endothelial cells, we utilized a function-blocking approach that eliminates the non-specific effects of RNA interference. Accordingly, we supplemented the adhesion buffer with either IgG isotype control or function-blocking antibodies against Integrin $\beta 1$, Lrp4, and Agrin respectively. Under these conditions, we allowed un-transfected MHCC-LM3 cells to adhere to a confluent monolayer of naïve HUVECs for 2 hours. As expected, IgG control had the maximum adhesion after 2 hours. The presence of function-blocking antibodies against Agrin and Integrin $\beta 1$ greatly hampered cancer cells to EC adhesion. Though function-blocking antibodies against Lrp4 decreased the adherence of MHCC-LM3 cells to HUVEC when compared to IgG control, this defect was more robust in the presence of Integrin $\beta 1$ and Agrin function-blocking antibodies (**Figure 3.41**). This data further supports the significance of cancer-derived Agrin and endothelial Integrin $\beta 1$ but not Lrp4 in heterotypic cell adhesion between cancer cells and ECs.

Figure 3.41: Function-blocking antibody against Agrin or its receptors Integrin $\beta 1$ and Lrp4 hamper cancer cell to EC adhesion. Cell adhesion assay of pre-labeled MHCC-LM3 and HUVEC treated with IgG isotype control or function-blocking antibodies against Integrin $\beta 1$, Lrp4, and Agrin respectively during the adhesion process. Image acquisition and quantification were done as in (Figure 3.40). Data presented as mean $\pm$ SD of three independent replicates. ($n=3$, *** $P < 0.0009$, * $P = 0.0177$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, $10\mu m$.

Considering that the adherence of cancer cells to ECs was minimal when both cancer cell and endothelial Agrin were depleted, we posited that this loss in adhesion was due to decreased Agrin secreted in the conditioned medium by the cancer cells. Therefore, we predicted that supplementing the adhesion buffer with Agrin-rich conditioned medium from control MHCC-LM3 cells, should be sufficient to rescue the adhesion defects associated with Agrin depletion in these cells. Consequently, when shAgrin MHCC-LM3 cells and siAgrin HUVEC were allowed to adhere in the presence of Agrin-rich control MHCC-LM3 conditioned medium, we

observed a significant rescue in cancer to EC adhesion (**Figure 3.42**). Thus, confirming that secreted Agrin in the co-culture environment is vital for heterotypic cell adhesion between cancer cells and ECs.

Furthermore, when function-blocking antibodies against Integrin $\beta 1$, Lrp4 or Agrin was introduced to the adhesion buffer that is supplemented with Agrin-rich conditioned medium same as above, the adhesion promoting effects of Agrin and Integrin $\beta 1$ was nullified by the presence of such function-blocking strategies. However, consistent with our earlier observations, the function-blocking antibodies against Lrp4 did not reveal any significant inhibitory effect on cancer to EC adhesion under similar conditions (**Figure 3.42**). Cumulatively, these experiments reveal that Agrin and Integrin $\beta 1$ are critical mediators of heterotypic cell adhesion between liver cancer cells and ECs. Thus, our findings suggest that though Lrp4 is necessary for *in-vitro* angiogenesis, it is partly dispensable for the cancer cell to EC attachment.

Figure 3.42: *shControl MHCC-LM3 conditioned medium enhances cancer cell to EC attachment which is obliterated by the function-blocking antibodies against Agrin and its receptors Integrin $\beta 1$ and Lrp4. Representative fluorescent microscopy images showing cell adhesion of cell tracker blue labeled shControl and shAgrin MHCC-LM3 cells and either siControl or siAgrin HUVEC, respectively. During the adhesion, the adhesion buffer was augmented with shControl MHCC-LM3 CM and further supplemented with either IgG or function-blocking antibodies against Integrin $\beta 1$, Lrp4, or Agrin, respectively. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates. ($n=3$, *** $P < 0.0001$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, $10\mu\text{m}$.*

Taking into account that FAK activity is vital for Agrin's oncogenic and pro-angiogenic signaling, we next investigated whether FAK activity was also required for Agrin-mediated cancer to EC adherence downstream of Agrin-Integrin $\beta 1$. To this end, pre-labeled cancer cells

same as above were allowed to adhere to HUVEC pretreated with either solvent control or FAK inhibitor PF562271. Inhibition of FAK activity, decreased the adherence of control of cancer cells to PF562271 treated ECs. Notably, this defect on cell adhesion was more robust when Agrin depleted cancer cells were allowed to adhere to PF562271 treated HUVEC. Besides, inhibition of FAK in HUVEC also minimized the adherence of Agrin rescued cancer cells (Figure 3.43). Thus indicating that endothelial FAK activity is crucial for Agrin mediated cancer cell to EC adhesion.

Figure 3.43: FAK inhibition by PF562271 hampers cancer cells to EC adhesion. Control, Agrin knockdown, and Agrin rescued MHCC-LM3 cells prepared as in (Figure 3.42) were allowed to adhere to HUVEC that were pre-treated with either DMSO (solvent) or PF562271 (1 μ M) for 2h. Images of adhering MHCC-LM3 cells were acquired at indicated time points and quantified as in (Figure 3.42). Data represents mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, $**P = 0.0019$, $*P < 0.0149$, student's t -test). Error bars denote standard deviation (SD). Scale Bar: 10 μ m.

Agrin regulates endothelial VEGFR2 for adhesion to cancer cells and angiogenesis

Because Agrin is a secreted proteoglycan in the ECM that requires a signaling axis to mediate extracellular to intracellular communication, we sort to delineate the mechanistic underpinnings of Agrin-mediated angiogenesis. Though we recognize that Agrin signals via its receptors Lrp4 and Integrin β 1 that also requires FAK activity, the identification of the underlying signaling

pathway(s) and the associated effectors recruited in Agrin-mediated angiogenic response remained largely unclear. Therefore, we envisaged that Agrin via its receptors may regulate key angiogenic channels that are consequently disrupted in Agrin silenced ECs.

Receptor tyrosine kinases (RTKs) and their related signaling cascades represent a major extracellular to an intracellular communication channel that regulates key cellular processes such as proliferation and cell survival. Moreover, RTKs also guide cellular responses to both external and internally generated cues that ensure cellular homeostasis (Lemmon and Schlessinger, 2010). Phosphorylation at specific tyrosine residues on these kinases leads to protein activation and corresponding conformational changes that facilitate the binding of associated proteins and signal propagators. The activated kinases ensure desired responses to growth factors and cytokines that are critical for cellular processes including angiogenesis (Hunter, 2009; Schmidt-Arras et al., 2005; Weiss et al., 1997). In endothelial cells, RTKs and their downstream components are vital for mediating the complex activities and cellular dynamics required for effective angiogenesis (Shawver et al., 1997).

Considering that Agrin depletion severely hampered angiogenesis in several ECs, we next examined the effects of Agrin expression on the phosphorylation profiles of forty-nine receptor tyrosine kinases (RTKs) that are commonly expressed on the cell surface membranes of mammalian cells to identify potential pathways implicated in angiogenesis whose activity is dependent on Agrin expression in ECs.

Appropriately, HUVECs transfected with either control or Agrin siRNA were subjected to an RTK phosphorylation array screen to discover differentially phosphorylated RTKs that may be associated with angiogenesis. Noteworthy, we identified several RTKs known to mediate pro-angiogenic responses that revealed decreased phosphorylation profiles upon Agrin silencing

such as VEGFR1, VEGFR2, EphA1, EphB6, PDGFR- β , and Insulin receptor 2 (**Figure 3.44**). Conversely, we also noted other RTKs such as; EGFR, RYK, EphB2, and MSPR whose phosphorylation status was significantly enhanced upon Agrin depletion in HUVEC (**Figure 3.44**).

Figure 3.44: Differential phosphorylation of RTKs in control and Agrin depleted HUVEC.

Western blot validation of Agrin depletion in HUVEC at 72h post-transfection. β -actin was used as a loading control. Control and Agrin silenced HUVEC same as above were subjected to RTK phosphorylation array screen. Lower exposure (left panel) and higher exposure (right panel) blots are shown. Red boxes denote decreased phosphorylation profiles. Blue boxes represent increased phosphorylation status of the respective tyrosine kinases upon Agrin depletion in HUVEC. Quantification of receptor tyrosine kinase (RTK) phosphorylation array in control and Agrin depleted HUVECs was done using dot blot analyzer from Image J.. **(Top panel)** represents RTKs with decreased phosphorylation status upon Agrin knockdown. **(Bottom panel)** shows the RTKs with increased phosphorylation status. Data presented as mean $\pm$ SD of two independent experiments performed in duplicates ($n=2$). Error bars denote standard deviation (SD).

Among the RTKs revealing a decreased phosphorylation status was vascular endothelial growth factor receptor 2 (VEGFR2) a key component of VEGF-mediated angiogenesis. The functional significance of VEGF as a pro-angiogenic factor has been well elucidated in vasculogenesis, developmental and tumor angiogenesis. VEGF signaling via its cognate receptors VEGFR1 and

VEGFR2 regulates endothelial cell proliferation, migration, and sprouting angiogenesis. (Carmeliet, 2005; Gerhardt et al., 2003; Millauer et al., 1993; Shibuya, 2011).

Given that VEGFR2 phosphorylation was significantly reduced upon Agrin silencing in HUVEC, we envisaged that Agrin might be essential for effective VEGF-VEGFR2 signaling that accounts for the decreased angiogenic response upon Agrin silencing in primary ECs. Therefore, we investigated the mechanistic relationship between Agrin and VEGFR2 in the context of tumor angiogenesis.

Firstly, we independently validated the decrease in VEGFR2 activity by analyzing the phosphorylation profiles of key tyrosine residues (Y951, Y1059, and Y1175) in the kinase domain of VEGFR2 that are implicated in EC migration and sprouting angiogenesis. Notably, the observed phosphorylations were drastically reduced upon Agrin silencing in HUVEC and HRMEC respectively (**Figure 3.45**). Nevertheless, we attributed this decrease in VEGFR2 phosphorylation to a general decrease in the total receptor levels that were observed in the Agrin depleted cells (**Figure 3.45**). This data suggest that Agrin is vital for VEGFR2 expression in ECs that account for the decreased phosphorylation profiles upon Agrin knockdown.

Figure 3.45: Agrin depletion down-regulates VEGFR2 expression. Western blot analysis of total and phospho-VEGFR2 at indicated tyrosine residues in control and Agrin knockdown HUVEC and HRMEC. β -actin was used as the loading control.

Since Agrin silencing down-regulated VEGFR2 expression in primary ECs, we next investigated the mechanistic contraptions involved in this regulation. To this end, we performed a real-time polymerase chain reaction (RT-PCR) to determine if this regulation was transcriptionally mediated. However, Agrin silencing in HUVEC did not reveal any significant alterations in the relative mRNA levels of VEGFR2 which conversely was rather upregulated in Agrin depleted ECs (**Figure 3.46**). Thus, eliminating transcriptionally mediated events as the likely mechanism for this regulation.

Figure 3.46: Agrin depletion down-regulates VEGFR2 expression without affecting the mRNA levels. RT-PCR analysis of Agrin and VEGFR2 mRNA levels in HUVEC transfected with control or Agrin specific siRNA. Data were normalized to 18S ribosomal RNA and represented as mean

*+/-SD of three independent experiments performed in triplicates. (n=3, **P = 0.0083, Student's t-test). Error bars denote standard deviation (SD).*

In accordance with the central dogma, that outlines the process of protein synthesis from the genetic code, protein expression can be regulated at several levels along the production chain from transcription, protein translation and post-translational modifications (Crick, 1970; Li and Xie, 2011; Shapiro, 2009). Besides, stability-based mechanisms are also implicated in protein regulation by modulating the rate of protein degradation and bioavailability (Callis, 1995; Hochstrasser, 1996).

Induction of VEGF-VEGFR2 signaling in addition to mediating downstream activation of pro-angiogenic effectors also enhances the expression of VEGFR2 by promoting its stability (Millauer et al., 1993; Shibuya, 2011). Factors that disrupt this ligand-receptor interaction consequently decrease the overall stability of VEGFR2 that also reveals a corresponding decrease in receptor activity (Jopling et al., 2011; Simons et al., 2016).

In the ECM, heparan sulfate proteoglycans (HSPGs) such as Perlecan have been observed to act as co-receptors to secreted growth factors such as VEGF. Perlecan sequesters VEGF within the ECM, preventing its degradation and facilitates VEGF-VEGFR2 interaction (Zoeller et al., 2009). Given that Agrin and Perlecan bear structural similarity and are among the most abundant HSPGs in the basement membrane compartment of the ECM, we hence questioned whether Agrin also functions as a co-receptor for secreted VEGF that might account for defective angiogenesis in Agrin silenced ECs. As a result, we performed and immunoprecipitation assay using whole cell lysate from HUVEC that was pulled down using IgG isotype control or Agrin specific antibodies to determine if Agrin interacts with VEGF.

Notably, we determined that Agrin interacts with VEGF-A 165, though the association was only about ~10% of the total endogenous levels (**Figure 3.47**). Thus, suggesting that the decrease in VEGFR2 expression observed in Agrin depleted ECs, may not result from a defective presentation of VEGF to cell surface VEGFR2 per se.

Figure 3.47: Agrin interacts with VEGF-A. HUVEC lysates were immunoprecipitated with IgG isotype control or Agrin antibody and Western blotted for VEGF-A. Ten percent of the total lysates were used as input control.

Moreover, when Agrin depleted ECs were pretreated overnight with exogenous VEGF and subjected to *in-vitro* tube formation on Matrigel, the supplementation of VEGF was not sufficient to rescue the associated defects in *in-vitro* angiogenesis (**Figure 3.48**). Thus, suggesting that the underlying regulation of VEGFR2 expression by endothelial Agrin is not dependent on VEGF availability but rather a robust destabilization of the receptor that impedes effective VEGF-VEGFR2 signaling in angiogenesis.

Figure 3.48: VEGF-A is unable to rescue in-vitro tube formation in Agrin depleted ECs. *In-vitro tube formation analysis of siControl, siAgrin and siAgrin HUVEC supplemented with 50ng/ml VEGF-A for 12-18h. Representative images were acquired by phase-contrast microscopy and quantified by Angiogenesis analyzer from Image J. Data presented as mean $\pm$ SD of three replicates. ($n=3$, $**P < 0.0032$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.*

Given that Agrin only weakly interacts with VEGF and pre-treatment of Agrin depleted ECs with exogenous VEGF did not rescue defective angiogenesis, we were curious to know whether the associated decrease in VEGFR2 levels upon Agrin silencing was specific to VEGFR2 or a general effect across the different VEGFR's expressed in ECs. Notably, though Agrin interacts with VEGF, silencing Agrin did not alter the expression of VEGF or its other cognate receptors

VEGFR1 and VEGFR3 (**Figure 3.49**). Thus, demonstrating the specificity of Agrin in regulating VEGFR2 expression.

Figure 3.49: Agrin knockdown specifically downregulates VEGFR2 protein levels. Western blot analysis of indicated proteins in control and Agrin depleted HUVEC. β -actin served as a loading control.

Agrin regulates VEGFR2 stability

Taking into account that Agrin silencing did not reduce the mRNA levels of VEGFR2 though drastically decreasing the total protein expression, therefore, we predicted that Agrin might attribute a stability-based regulation of VEGFR2 in primary ECs. In this regard, we first sort to validate that the decrease in VEGFR2 levels observed in Agrin depleted ECs, was consistent with the gradual reduction in Agrin expression post-transfection with Agrin specific siRNA thus, ruling out off-target effects of RNA interference. Accordingly, we performed a time-

dependent analysis of VEGFR2 expression upon Agrin siRNA treatment over a 6-day period. The levels of VEGFR2 were maximally reduced between days 3-5 which also coincided with the effective knockdown of Agrin (**Figure 3.50**). This data confirms the specificity of Agrin-mediated regulation of VEGFR2 expression in primary ECs.

Figure 3.50: Agrin siRNA dynamics and VEGFR2 expression. Cell lysates of HUVEC transfected with control and Agrin siRNA for the indicated days were analyzed by Western blot for Agrin and VEGFR2 expression. β -actin was used as the loading control.

Next, we supplemented Agrin depleted ECs with increasing concentrations for soluble recombinant Agrin (sAgrin) overnight and monitored the effect on VEGFR2 levels. Intriguingly, exogenous addition of soluble Agrin rescued VEGFR2 levels in a dose-responsive manner (**Figure 3.51**). Further demonstrating the specificity of Agrin to regulate the VEGFR2 stability in primary ECs.

Figure 3.51: Soluble Agrin rescues VEGFR2 expression in a dose-responsive manner. Western blot detection of VEGFR2 in control or Agrin depleted HUVECs treated with indicated concentrations of soluble Agrin. β -actin was used as a loading control.

Furthermore, we used an alternative RNA interference, to independently verify Agrin-mediated regulation of VEGFR2 stability. Accordingly, HUVECs and MHCC-LM3 cells were transduced with Agrin shRNA bearing lentiviral particles to generate a stable pool of Agrin silenced cells. Consistently with earlier observations, shAgrin depleted ECs also revealed decreased VEGFR2 levels when compared to the control cells. This was partially rescued when the Agrin depleted cells were supplemented with soluble Agrin (**Figure 3.52 A**). Likewise, when Agrin was silenced in MHCC-LM3 liver cancer cells which also express VEGFR2, Agrin depletion significantly decreased the levels of VEGFR2 in these cells. Moreover, in the shAgrin cells that were rescued by stably expressing rat Agrin, the expression of VEGFR2 was again restored (**Figure 3.52 B**).

Figure 3.52: shRNA mediated knockdown of Agrin decreases VEGFR2 protein levels in HUVEC and MHCC-LM3. (A) HUVEC was transduced with control or Agrin shRNA for 72h. One batch of shAgrin cells was supplemented with 10 μ g/ml of sAgrin for 12-18h and analyzed by Western blot for the indicated proteins. β -tubulin was used as the loading control. **(B)** shControl, shAgrin, and shAgrin rescued cells MHCC-LM3 cells were analyzed by Western blot for Agrin and VEGFR2 expression respectively. β -actin was used as the loading control.

Considering that Agrin function-blocking antibody treatment decreased *in-vitro* angiogenesis and cancer cell to EC adhesion, we then questioned whether exposing ECs to this antibody will also decrease VEGFR2 levels in a similar manner to Agrin silencing. As such, HUVEC were treated with increasing concentrations of Agrin function-blocking antibodies for 24 hours and analyzed by western blot for VEGFR2 expression. Noteworthy, anti-Agrin treatment decreased

the expression of VEGFR2 in a dose-dependent fashion (**Figure 3.53 A**). Moreover, when we increased the incubation time for the Agrin antibody treatment, we observed a maximal reduction of VEGFR2 levels over a period of 48 hours (**Figure 3.53 B**).

Figure 3.53: Anti-Agrin inhibits VEGFR2 protein expression in a dose and time-dependent manner. (A) HUVEC treated with IgG control (10µg/ml) or increasing concentrations of anti-Agrin (MAb5204) for 48h was analyzed by Western blot for Agrin and VEGFR2 detection. β-actin served as the loading control. (B) HUVECs treated with either 10µg/ml IgG or Agrin function-blocking antibody (MAb5204) for the indicated time points were analyzed by Western blot for VEGFR2 and Agrin expression. β-actin served as the loading control.

Similar to the cell-lifecycle, proteins have a limited lifespan that can range from a few minutes to several days at a time. After which these proteins are degraded via proteasomes or lysosomal machinery to their constituent amino acids that serve as building blocks for synthesizing new protein (Goldberg, 2003; Hershko and Ciechanover, 1992). This cycle of protein turnover is

critical for cellular homeostasis as it prevents the accumulation of misfolded, non-functional or potentially toxic proteins which might distort various cellular processes and function (Goldberg and St. John, 1976; Klausner and Sitia, 1990; Wang et al., 2011). Consequently, we sort to determine if Agrin enhances VEGFR2 stability and effectively decreases its intracellular degradation in primary ECs.

Given that Cellular proteins have different half-lives and are constantly recycled by degradation and protein synthesis, it is imperative to inhibit the synthesis of new proteins in order to effectively monitor the stability of those already expressed. As such, we performed cycloheximide (CHX) degradation assay in HUVEC with or without soluble Agrin supplementation to investigate the effect on VEGFR stability. Cycloheximide is a naturally occurring fungicide produced by the bacterium *Streptomyces griseus*, which interferes with the translocation step in protein synthesis thus blocking eukaryotic protein translation (Siegel and Sisler, 1963). This technique has been effectively used in multiple studies to monitor protein degradation over different time points (Ennis and Lubin, 1964; McKeehan and Hardesty, 1969; Schneider-Poetsch et al., 2010).

To this end, HUVECs were either untreated or pre-treated with 10 μ g/ml soluble Agrin overnight before exposure to CHX for 1-3 hours. Interestingly, soluble Agrin treatment increased the stability of VEGFR2 for 1-2 hours post CHX treatment (**Figure 3.54A**). However, because the half-life of VEGFR2 is only ~70 minutes (Boucher et al., 2017), it was difficult to fully appreciate the contributing effects of soluble Agrin on VEGFR2 stability. As such, we further analyzed the stability of VEGFR2 at different time points within a 1-hour duration. Consistently, pre-treatment of HUVEC with soluble Agrin significantly stabilized VEGFR2 levels within 15-60 minutes post-CHX treatment (**Figure 3.54B**). This data strongly suggests

that Agrin enhances the stability of VEGFR2 and decreases the steady-state degradation within the observed time points.

Figure 3.54: Soluble Agrin decreases VEGFR2 degradation. (A-B) HUVEC untreated or pre-treated with soluble Agrin (10 μ g/ml) for 12-18h was subjected to 15 μ g/ml Cycloheximide (CHX) treatment for the indicated time points. Relative VEGFR2 degradation was analyzed by Western blot analysis. The levels of VEGFR2 were normalized to $t=0$ min and quantified using Image J. β -actin was used as the loading control. Data represents mean $\pm$ SD of three independent experiments ($n=3$, $**P < 0.0047$, $*P = 0.0114$, Student's t -test). Error bars denote standard deviation (SD).

Since soluble Agrin treatment conferred increased VEGFR2 stability in HUVEC, we next examined the potential pathway(s) responsible for VEGFR2 degradation in primary ECs. Protein degradation majorly occurs via lysosomal and/or proteasomal pathways which facilitate

the breakdown of polypeptides into their constituent amino acids that are subsequently used for synthesizing new protein.

Consequently, we utilized the peptide-aldehyde proteasomal inhibitor MG132 (carbobenzoxy-Leu-Leu-leucinal) which effectively blocks the proteolytic activity of the 26S proteasome complex (Guo and Peng, 2013; He et al., 2004; Yoshida et al., 2005) and monitored the effect on VEGFR2 degradation after exposure to CHX. Also, we independently inhibited the lysosomal degradation machinery by using Chloroquine a permeable molecular inhibitor that has been extensively shown to block the activity of lysosomal proteins (Gonzalez-Noriega et al., 1980; Wibo and Poole, 1974; Wiesmann et al., 1975).

Experimentally, naïve HUVECs were treated with either MG132, chloroquine (CQ) or sAgrin for ~3 hours before subjugating the cells to CHX treatment in the presence of the respective inhibitors. As earlier observed, soluble Agrin treatment enhanced VEGFR2 stability when compared to Untreated cells. Under similar conditions, inhibiting lysosomal activity using CQ did not promote VEGFR2 stability which in contrast was more de-stabilized in CQ treated cells. Intriguingly, MG132 treatment considerably enhanced VEGFR2 stability in a manner akin to soluble Agrin treatment (**Figure 3.55**). This data suggests that VEGFR2 is mostly degraded by the proteasomal machinery. Thus, informing the proposition that Agrin may promote VEGFR2 expression/stability by decreasing its association with proteasomal structures that are responsible for its degradation.

Figure 3.55. Relative VEGFR2 degradation in MG132 and Chloroquine treated ECs. Western blot analysis of HUVEC cells pretreated with MG132, Chloroquine, and sAgrin for 3 hours prior to CHX exposure for the indicated time points. β -actin was used as the loading control.

Taking into account that Agrin stabilizes VEGFR2 which is vital for tumor angiogenesis, we next queried whether Agrin-mediated stabilization of VEGFR2 was critical for blood vessel recruitment during the early stages of tumor growth. To address this, we first analyzed the relative intensity of VEGFR2 staining in Matrigel tumor-plug sections generated from shControl and shAgrin MHCC-LM3 liver cancer cells respectively. Agrin depleted tumor-plugs revealed decreased VEGFR2 staining when compared to control plugs (**Figure 3.55 A**). Moreover, when equal amounts of the resected tumor-plugs were lysed and western blotted for VEGFR2 expression, Agrin silenced plugs also revealed decreased VEGFR2 levels in comparison to control plugs without affecting the expression of house-keeping genes GAPDH and β -tubulin (**Figure 3.55 B**). However, given that endothelial Agrin was not perturbed, we posit that the observed reduction in VEGFR2 levels is due to a combination of reduced EC recruitment and loss of tumor-derived VEGFR2 upon Agrin silencing. Cumulatively, this data suggests the role of Agrin in the stabilization of VEGFR2 protein levels.

Figure 3.55: Agrin depletion decreases vessel associated VEGFR2 in Matrigel plug assay. (A) Immunohistochemistry (IHC) staining of vessel associated VEGFR2 in control and Agrin depleted Matrigel tumor plugs. Arrows indicate blood vessel associated VEGFR2 (left panel). Data were quantified using the IHC tool from Image J. Data presented as relative VEGFR2 staining in sections (upper panel) or VEGFR2 staining normalized to blood vessel marker CD-31 (lower panel) $n=5$ sections analyzed from 3 different tumor plugs ($***P = 0.0004$, $*P = 0.0105$, Student's t -test). Immunofluorescence detection of Agrin expression in shControl and shAgrin tumor plugs

(right panel). $n=5$ sections from 3 different tumor plugs. Error bars denote standard deviation (SD). Scale bar, 100 μ m. **(B)** Western blot analysis of Agrin and VEGFR2 expression in an equal amount of control and Agrin depleted tumor plugs. GAPDH and β -tubulin were used as loading controls.

VEGFR2 is required for Agrin mediated cancer cell to EC adhesion

Cancer cell to EC adhesion represents a crucial step in tumor vascularization. Earlier we demonstrated that Agrin is vital for cancer to EC attachment. Given the significance of VEGFR2 to tumor angiogenesis, we wondered if stabilized VEGFR2 was critical for Agrin-mediated cancer to EC adhesion.

Appropriately, we performed an adhesion assay using pre-labeled MHCC-LM3 cells and HUVEC in the presence of either solvent control or Sorafenib, a targeted therapy for advanced hepatocellular carcinoma that inhibits VEGFR2 activity and tumor angiogenesis (Llovet et al., 2008). Sorafenib treatment decreased the adhesion of control MHCC-LM3 cells to HUVEC. This defect was more robust when Agrin depleted MHCC-LM3 cells were allowed to adhere to HUVEC in the presence of Sorafenib. Under these conditions even shAgrin rescued cells had minimal adherence to HUVEC (**Figure 3.56 A**). Thus, suggesting that VEGFR2 activity is critical for Agrin-mediated cancer cell to EC adhesion.

Furthermore, since Sorafenib is a multi-tyrosine kinase inhibitor, that also targets other RTKs in combination with VEGFR2, we specifically depleted endothelial VEGFR2 via siRNA mediated knockdown and subjected the cells to another cell adhesion assay same as above. Consistent with Sorafenib treatment, silencing endothelial VEGFR2 also hampered cancer cells to EC adhesion which was also most striking when Agrin depleted cancer cells were allowed to

adhere to VEGFR2 depleted ECs (**Figure 3.56 B**). Further supportive of the role of VEGFR2 in the cancer cell to EC adhesion.

Figure 3.57: VEGFR2 silencing or Sorafenib treatment hampers cancer cells to EC adhesion.

(A) Pre-labeled shControl, shAgrin, and shAgrin rescued MHCC-LM3 cells were allowed to

adhere to HUVEC that were pre-treated for 1h with either solvent (DMSO) or Sorafenib (1 μ M). Representative fluorescent images of adhering MHCC-LM3 cells were captured at 0h and 2h, respectively. Quantification of adherent cells was done using Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates (n=3, $^{**}P < 0.0032$, $^{*}P = 0.0364$, Student's t-test). Scale bar, 10 μ m. (B) Pre-labeled MHCC-LM3 cells same as in (A) were allowed to adhere to control or VEGFR2 depleted HUVEC for 2h. Representative fluorescent images were acquired and quantified as in (A) (n=3, $^{***}P < 0.0007$, $^{**}P = 0.0034$, $^{*}P = 0.0136$, Student's t-test). Error bars denote standard deviation (SD). Scale bar, 10 μ m.

Silencing VEGFR2 in endothelial cells or treatment with Sorafenib has been reported to decrease the tube forming capabilities of ECs (Koolwijk et al., 2001; McGonigle and Shifrin, 2008). Given that Agrin knockdown in ECs decreases VEGFR2 levels with a corresponding decrease in angiogenesis, we then wondered whether maintaining the stability of VEGFR2 was the principal function of Agrin signaling in primary ECs during angiogenesis. To this end, we pre-treated ECs for 1 hour with either solvent control or Sorafenib before subjecting the cells to *in-vitro* tube formation assay. Consistent with other reports, Sorafenib treatment significantly mitigated tube formation in these ECs when compared to solvent control. Besides, soluble Agrin supplementation to these ECs in the presence of Sorafenib did not rescue the defective tube formation (**Figure 3.57 A**). Also, silencing VEGFR2 in HUVEC also hampered *in-vitro* tube formation that was not rescued by soluble Agrin supplementation (**Figure 3.57 B**). These sets of data support the premise that Agrin-mediated stabilization of VEGFR2 is a major target of Agrins' angiogenic signaling in primary ECs.

Figure 3.57: VEGFR2 depletion and Sorafenib treatment hamper in-vitro angiogenesis. (A) HUVEC were pretreated with either DMSO (solvent) or Sorafenib (1 μ M) for 2h and cell lysates were analyzed by western blot for pVEGFR2 (Y1175). Phase-contrast microscopy images of in-vitro tube formation assay in HUVEC pretreated with solvent or Sorafenib (1 μ M) only or

supplemented with 10 μ g/ml soluble Agrin for 2h. Quantification was done using Angiogenesis analyzer from Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P = 0.0006$, * $P = 0.0315$, Student's t -test). Scale bar, 100 μ m.

(B) Western blot validation of VEGFR2 knockdown with β -actin used as a loading control. Control and VEGFR2 depleted HUVECs either untreated or treated with 10 μ g/ml sAgrin for 12-18h were subjected to in-vitro tube formation on Matrigel the same as in (A). Representative images were acquired by phase-contrast microscopy and quantified the same as in (A). ($n=3$, *** $P = 0.0001$, ** $P = 0.0034$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

If the principal role of Agrin's signaling in ECs is to stabilize VEGFR2, then it suffices to presume that the exogenous expression of VEGFR2 in Agrin depleted or functionally inhibited ECs will be sufficient to effectively rescue the angiogenic defects associated with decreased Agrin activity. Consequently, we pre-treated HUVECs with either IgG isotype control or Agrin function-blocking antibody for a total duration of 48 hours to severely de-stabilize VEGFR2. Twenty-four hours into the experiment, the pre-treated cells were transfected with either vector control or VEGFR2-turbo-GFP expression plasmid and incubated for another 24 hours to allow for sufficient VEGFR2 transcription. Post-treatment, we validated the expression of VEGFR2 by western blot analysis (**Figure 3.58 A**). Anti-Agrin treatment drastically de-stabilized VEGFR2 expression when compared to the IgG isotype control. Exogenous expression of VEGFR2 even under conditions of anti-Agrin treatment was sufficient to rescue VEGFR2 levels thus, validating the efficiency of the turbo-GFP-VEGFR2 expression (**Figure 3.58 A**). Intriguingly, the exogenous expression of VEGFR2 under these conditions was also sufficient

to rescue the defective tube formation caused by anti-Agrin treatment (**Figure 3.58 B**). This data infers that Agrin-mediated regulation of VEGFR2 stability is a key component of its angiogenic function.

Figure 3.58: Exogenous expression of VEGFR2 rescues in-vitro tube formation in HUVECs treated with the Agrin function-blocking antibody. (A) HUVEC were treated with either IgG or Agrin antibody for 48h. 24h post-treatment, one batch of anti-Agrin treated cells were transfected with either vector control or VEGFR2-turbo-GFP (VEGFR2-tGFP) expressing plasmid. Cell lysates were analyzed by Western blot for VEGFR2 expression. β -actin served as the loading

control. **(B)** HUVEC treated the same as above were subjected to in-vitro tube formation. Image acquisition and data analysis were done the same as in (Figure 3.57). Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, $***P < 0.0001$, $**P < 0.0021$, student's *t*-test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

Agrin-mediated ECM stiffness stabilizes VEGFR2

Matrix stiffness presents a conducive microenvironment for tumor vascularization and cancer progression (Schedin and Keely, 2011). The rigidity of the extracellular matrix regulates the expression of many functional proteins in endothelial cells including VEGFR2 (LaValley et al., 2017; Mammoto et al., 2009; Santos et al., 2015). Recently, we reported that Agrin-induced YAP activation attributes ECM stiffness that promotes cancer progression in HCC (Chakraborty et al., 2017). Taking into account that Agrin regulates the stability of VEGFR2, we wondered whether Agrin-mediated ECM stiffness contributes towards the underlying stabilization of VEGFR2.

To address this, we first examined the relative mRNA levels and protein expression of Agrin and VEGFR2 in primary ECs cultured on either soft or hard matrices respectively. Notably, the relative mRNA and total protein expression of VEGFR2 and Agrin were significantly enhanced in ECs cultured on stiff matrix when compared to those cultured on compliant matrices (**Figure 3.59A**). This observation was consistent with an earlier report that also observed an increase in VEGFR2 expression when ECs were cultured on hard substrates (Mammoto et al., 2009).

Given that VEGFR2 levels were majorly de-stabilized in ECs cultured on compliant substrates, we next contemplated whether incorporation of Agrin (known to enhance matrix stiffness) into these compliant matrices will enhance the stability of VEGFR2. As predicted, when ECs were

cultured on soft matrix infused with increasing concentrations of soluble Agrin, we observed a dose-responsive increase in total VEGFR2 expression without any significant alterations to the mRNA levels (**Figure 3.59B**). Thus, validating a stability-based regulation of VEGFR2 by matrix-bound Agrin.

On the contrary, knockdown of Agrin in HUVECs cultured on hard matrices which are known to enhance VEGFR2 expression and stability, revealed a drastic decrease in the total levels of VEGFR2 despite a relative increase in VEGFR2 mRNA levels (**Figure 3.59C**). Hence, suggesting that this decrease in VEGFR2 levels is not due to defective transcriptional machinery but rather a severe deficiency in VEGFR2 stabilization. Besides, when the Agrin depleted ECs were plated on a hard matrix that was supplemented with soluble Agrin, the presence of matrix-bound Agrin was sufficient to rescue the expression of VEGFR2 without any alterations to the relative mRNA levels when compared to that in Agrin depleted HUVECs only (**Figure 3.59C**). This data further validates a stiffness-based regulation of VEGFR2 by matrix-bound Agrin.

Figure 3.59: Agrin stabilizes VEGFR2 in hard and soft matrices. (A) RT-PCR analysis showing the relative mRNA levels of Agrin and VEGFR2 in HUVEC cultured on soft or hard matrices. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates. ($n=3$, $**P = 0.0011$, Student's *t*-test). Western blot analysis of Agrin and VEGFR2 levels in HUVEC cultured on soft or hard matrix. β -actin was used as the loading control. (B) Relative mRNA and protein levels of Agrin and VEGFR2 in HUVECs cultured on soft matrix only or supplemented with increasing concentrations of soluble Agrin for 24h, as analyzed by RT-PCR and Western blot. (C) Control and Agrin depleted HUVECs cultured on hard matrix only or supplemented with 10μg/ml sAgrin for 24h was analyzed by real-time PCR as indicated. Data presented as mean $\pm$ SD of

*three independent experiments performed in triplicates. (n=3, ***P < 0.0001, **P < 0.0048, student's t-test). Western blot analysis of VEGFR2 and Agrin levels in HUVEC cultured as above. Error bars denote standard deviation (SD). β -actin as a loading control.*

Furthermore, we investigated the rate of VEGFR2 degradation in ECs cultured on soft or hard matrices. Suitably, HUVECs culture on hard substrates or compliant matrices were subjected cycloheximide (CHX) treatment and the relative levels of VEGFR2 were monitored at different time points for a total duration of 70 minutes. ECs cultured on hard matrix revealed less degradation over the observe time points when compared to those cultured on compliant matrices (**Figure 3.60 A**). Thus, demonstrating that matrix stiffness in addition to promoting VEGFR2 expression, also enhances its stability.

Considering that the supplementation of soluble Agrin in soft matrix enhanced VEGFR2 stability, without altering the mRNA levels, we then investigated whether matrix-bound Agrin also decreased the rate of VEGFR2 degradation. Accordingly, HUVECs cultured on either soft ECM only or soft ECM supplemented with sAgrin were exposed to CHX for the indicated time points. Consistently, ECs cultured on Agrin-supplemented matrices revealed a slower rate of VEGFR2 degradation when compared to those cultured on soft matrix only (**Figure 3.60 B**). Taken together, this data suggests that Agrin stabilizes VEGFR2 in a stiffness-dependent manner.

Figure 3.60: Soluble Agrin treatment decreases VEGFR2 degradation in soft matrices. (A) HUVEC cultured on soft or hard matrices were treated with 15 μ g/ml cycloheximide (CHX) for the indicated time and analyzed by Western blot for VEGFR2 degradation. Data presented as mean $\pm$ SD of three independent experiments ($n=3$, *** $P = 0.0008$, * $P = 0.0115$, Student's t -test). (B) HUVEC seeded on soft matrix only or supplemented with 10 μ g/ml sAgrin for 12-18h was subjected to 15 μ g/ml CHX treatment for the indicated time points and analyzed by Western blot for VEGFR2 levels. Data presented as mean $\pm$ SD of three independent experiments ($n=3$, * $P < 0.0482$, student's t -test). Error bars denote standard deviation (SD).

Taking into account that Agrin enhances VEGFR2 stability in a stiffness-dependent manner, we next wondered whether incorporating Agrin into Matrigel prior to *in-vitro* tube formation will increase the rate of tube formation owing to enhanced VEGFR2 stability. Consequently,

naïve HUVEC cells were subjected to *in-vitro* tube formation on either Matrigel only, Matrigel infused with 10µg/ml soluble Agrin, or Matrigel incorporated with 10µg/ml soluble Agrin and further supplemented with sAgrin in the cell culture medium. Noteworthy, supplementing Agrin in the Matrigel enhanced the rate of tube formation and supported greater master segments formation and overall tube length within 1.5-4.5 hours (**Figure 3.61**). When Agrin was supplemented in both the matrigel and the culture medium, we observed even faster rates of *in-vitro* angiogenesis (**Figure 3.61**).

Figure 3.61: Soluble Agrin supplementation in Matrigel increases the rate of in-vitro tube formation. HUVEC cultured on Matrigel only or Matrigel containing 10 μ g/ml sAgrin was subjected to in-vitro tube formation with or without supplementation of sAgrin (10 μ g/ml) in the cell culture medium. Representative phase-contrast images were taken at indicated time points and analyzed using Angiogenesis analyzer from Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P = 0.0001$, ** $P < 0.0064$, * $P = 0.0457$, Student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

As clustered Integrin signaling forms a major mechanosensing component in many cell types including ECs, we further investigated whether secreted Agrin sequestered VEGFR2 in a complex with Integrin β 1 and Lrp4 that may further account for its stability and functions. Accordingly, whole-cell lysates from HUVEC cells pre-treated with increasing concentrations of soluble Agrin overnight were immunoprecipitated with either IgG isotype control or VEGFR2 specific antibodies and subsequently probed for Integrin β 1 and Lrp4 interaction. Though a basal interaction was observed in the untreated samples, soluble Agrin treatment significantly enhanced complex formation between VEGFR2-Integrin β 1 and Lrp4 in a dose-responsive manner (**Figure 3.62 A-B**).

Figure 3.62: Soluble Agrin enhances the interaction between Integrin β 1-Lrp4-VEGFR2 (A-B)

HUVEC pretreated with increasing concentrations of soluble Agrin for 12-18h were immunoprecipitated with control or VEGFR2 antibody and analyzed by Western blot for Integrin β 1 (panel A) and Lrp4 (panel B), respectively. Ten percent of the total whole-cell lysate was used as input control. The blots were stripped and re-probed for VEGFR2.

Using an alternate approach, we sort to further characterize the effect of soluble Agrin treatment on VEGFR2-Integrin β 1 complex formation in ECs. To this end, HUVECs pretreated with increasing concentrations of soluble Agrin overnight were immunostaining with VEGFR2 and Integrin β 1 antibodies respectively and analyzed for co-localization. Consistent with the immunoprecipitation data, exogenous addition of soluble Agrin enhanced the co-localization of VEGFR2-Integrin β 1 in a dose-responsive manner (**Figure 3. 63**).

Figure 3.63: Soluble Agrin enhances VEGFR2-Integrin β 1 complex formation. HUVECs were treated overnight with increasing concentrations of sAgrin as indicated. Cells were subsequently fixed and stained with indicated antibodies. Representative confocal images of VEGFR2 and Integrin β 1 co-localization are shown. Scale bar: 10 μ m.

Given that Sorafenib treatment inhibited VEGFR2 activity that also accounted for a decreased *in-vitro* angiogenesis and cancer cell to EC attachment, we next investigated whether Sorafenib treatment mitigated the complex formation between VEGFR2 and Integrin β 1. To this end, HUVEC cells that were cultured on soft polyhydrogels with or without soluble Agrin supplementation were exposed to either solvent control or Sorafenib treatment for 2h prior to immunofluorescence analysis. Soluble Agrin treatment in compliant matrices greatly increased complex formation between VEGFR2 and Integrin β 1 which was especially prominent at the leading edges of HUVEC (**Figure 3.63**). However, soluble Agrin-induced VEGFR2-Integrin β 1 complex formation was greatly hampered in the presence of Sorafenib (**Figure 3.63**). Collectively this suggests that soluble Agrin treatment sequesters VEGFR2 in a complex with Integrin β 1 that contributes to VEGFR2 stability. Also, this data suggests that VEGFR2 activity which is hampered by Sorafenib treatment is required for maintaining complex formation between VEGFR2- Integrin β 1 at the leading edges of ECs.

Figure 3.63: Soluble Agrin enhances VEGFR2-Integrin $\beta 1$ complex formation. HUVEC cultured on soft poly hydrogel were treated with 10 $\mu\text{g/ml}$ sAgrin for 12h before exposing the treated cells to either DMSO (solvent) or 1 μM Sorafenib for 2h. Confocal images of VEGFR2 and Integrin $\beta 1$ are shown. Arrows indicate colocalization. Boxes denote enlarged regions. Data quantified using Pearson's coefficient of colocalization ($n=60-80$ cells per condition, *** $P < 0.0001$, student's t -test). ns denotes in-significant data points. Scale bar: 10 μm .

Furthermore, we investigated the effect of Agrin depletion on VEGFR2-Integrin $\beta 1$ complex formation in HUVECs cultured on hard substrates. Consistent with earlier observations, silencing Agrin in HUVECs cultured on hard collagen matrix did not reveal any co-localization between VEGFR2 and Integrin $\beta 1$ when compared to control HUVECs which showed strong VEGFR2-Integrin $\beta 1$ complex formation that was highly evident at the leading edges. Interestingly, supplementing soluble Agrin to the matrix of the Agrin depleted cells was sufficient to rescued VEGFR2-Integrin $\beta 1$ complex formation at the leading edges of these cells (**Figure 3.64 A**). Moreover, similar observations were also made when stiff polyhydrogels were used instead of a collagen matrix (**Figure 3.64 B**). Put together, this data demonstrates that soluble Agrin promotes VEGFR2-Integrin $\beta 1$ complex formation which contributes to VEGFR2 stability in both soft and stiff matrices.

Figure 3.64: Agrin depletion decreases the co-localization of Integrin $\beta 1$ and VEGFR2. Control and Agrin depleted HUVECs with or without 10 μ g/ml sAgrin was cultured on hard collagen matrix (A) or hard polyhydrogel (B), fixed and stained with Integrin $\beta 1$ and VEGFR2 antibodies respectively. Representative confocal images are shown. Arrows depict co-localization at the leading edges of HUVECs. The nucleus was stained with DAPI. Scale bar: 10 μ m.

In hepatocellular carcinoma, Agrin facilitates complex formation between Lrp4-MuSK-Integrin $\beta 1$ that also requires FAK activity to sustain the mechanoactivity of Yes-associated protein (YAP) leading to cancer progression (Chakraborty and Hong, 2018; Chakraborty et al., 2017). Since we

observe that in ECs, Agrin enhances complex formation between VEGFR2-Integrin β 1-Lrp4 that also requires FAK activity to mediate pro-angiogenic phenotype, we, therefore, investigated the relevance of this multi-protein complex in stabilizing VEGFR2. Suitably, we silenced Integrin β 1, Lrp4, and FAK in HUVEC and examined the effect on VEGFR2 expression (**Figure 3.65 A**). Interestingly, the knockdown of Integrin β 1 decreased Lrp4 expression and FAK phosphorylation which cumulatively led to a decrease in VEGFR2 stability (**Figure 3.65 B, first column**). Similarly, when Lrp4 was silenced in HUVEC, we observed a decrease in Integrin β 1 expression and FAK activation which also revealed a decrease in VEGFR2 stability (**Figure 3.65 B, middle column**). Likewise, knockdown of endothelial FAK though did not alter the expression of Integrin β 1 or Lrp4, revealed a consistent destabilization of VEGFR2 (**Figure 3.65 B, third column**). Collectively, this data suggests that Agrin promotes the formation of a multi-protein complex between VEGFR2-Integrin β 1-Lrp4-FAK that sequesters VEGFR2, enhancing its stability and function. Also, our data suggest that FAK is downstream of Integrin β 1 and Lrp4 in this complex since the depletion of FAK did not alter the expression of Integrin β 1 and Lrp4 but consistently affected VEGFR2 levels.

Figure 3.65: Regulation of VEGFR2 stability and downstream signaling by Agrin receptors. (A) Model outlining the mechanism of Agrin-mediated stabilization of VEGFR2 via the formation of a multi-protein complex with its receptors as indicated. **(B)** HUVECs were transfected with the indicated siRNAs. Two days post-transfection the cells were either left untreated or supplemented with soluble Agrin (10μg/ml) for 18h and Western blotted for VEGFR2 expression. β-actin was used as the loading control.

To further delineate the role of FAK as a mediator of VEGFR2 stability downstream of Agrin-Lrp4-Integrin β1, we pre-treated HUVECs with either solvent control or FAK inhibitor PF562271

for 2 hours to sufficiently block FAK activity. This was followed by drug wash-off and the pre-treated cells supplemented with increasing concentrations of soluble Agrin for another 2 hours. Western blot analysis of these samples revealed a decrease in VEGFR2 stability that corresponded with a decrease in FAK activation. Moreover, when soluble Agrin was exogenously supplied to PF562271 treated cells post-wash-off, we observed a significant rescue of VEGFR2 stability that was consistent with FAK activation in a dose-responsive manner (**Figure 3.66 left panel**).

In an alternate approach, HUVEC cells pre-treated with PF562271 same as above were either left untreated or exposed to 10 μ g/ml soluble Agrin and monitored for 2-4 hours. As expected PF562271 treatment decreased FAK phosphorylation that also accounted for a decrease in VEGFR2 stability. Since PF562271 is a reversible inhibitor, we expected FAK activation to be gradually restored after drug wash-off. In line with this premise, FAK phosphorylation was partially restored by 2 hours post-drug wash off which simultaneously revealed an enhancement in VEGFR2 stability. Interestingly, by the 4-hour time point, soluble Agrin treatment had maximally restored FAK phosphorylation comparable to solvent control. The restoration of FAK activity at this time point also accounted for increased VEGFR2 stability that was more prominent in the soluble Agrin treated cells (**Figure 3.66 right panel**).

Figure 3.66: Soluble Agrin enhances the activation of FAK and VEGFR2 stability. (left panel) HUVECs were pretreated with either solvent control or PF562271 for 2h followed by drug wash-off. Subsequently, cells were supplemented with or without increasing concentrations of soluble Agrin for 2h. **(right panel)** HUVEC treated the same as above, were incubated in complete endothelial growth media (EGM-2) for the indicated time-points post-drug wash-off, in the presence or absence of 10 μ g/ml sAgrin. The respective cell lysates were then analyzed by Western blot for the indicated proteins. β -actin was used as the loading control.

Agrin controls VEGFR2-eNOS signaling in ECs

Considering Agrin's role in VEGFR2 stability, we next examined whether Agrin regulates its downstream signaling in ECs. Though VEGF-VEGFR2 signaling leads to the activation of many intracellular pathways, the major downstream effectors of this signaling as implicated in angiogenesis are; endothelial nitric oxide synthase (eNOS), Protein kinase B (Akt) and Extracellular signal-regulated kinases (ERK 1/2) (Kroll and Waltenberger, 1998). The activation of ERK1/2 and Akt are vital for endothelial homeostasis by promoting EC proliferation and survival. While eNOS induction is critical for endothelial nitric oxide production and vascular permeability (Dellinger and Brekken, 2011; Fulton et al., 1999). Given the relevance of these factors to the angiogenic process, we next examine the effect of Agrin depletion on their respective phosphorylation states. Of note, silencing Agrin in HUVEC and HRMEC revealed an overall decrease in the steady-state phosphorylation of eNOS, Akt, and ERK1/2 respectively (**Figure 3.67**).

Figure 3.67: Agrin silencing decreases the activation of VEGF-VEGFR2 signaling effectors.
Western blot analysis of indicated proteins in control and Agrin depleted HUVEC and HRMEC. β -actin was used as the loading control.

Furthermore, we analyzed the phosphorylation kinetics of eNOS, Akt, and ERK1/2 upon VEGF stimulation in Agrin depleted ECs. Experimentally, Agrin depleted HUVEC were serum-starved for 18 hours before inducing VEGF signaling with 50nmol/ml of VEGF-A 165. This concentration has been shown in various reports to optimally induce VEGF signaling and tube formation in multiple ECs (Boopathy et al., 2017). In the control cells, eNOS phosphorylation was induced as early as 15-minute post-VEGF stimulation and sustained until the observed 180 minutes time point. In contrast, Agrin depletion abolished the activation of eNOS under similar temporal stages of VEGF stimulation. Similarly, the phosphorylation profiles of Akt and ERK1/2 were prominent

within 15-120 minutes in control cells. Consistently, their respective phosphorylation profiles were strikingly reduced in Agrin depleted cells within the same time point (**Figure 3.68**).

Figure 3.68: Induction of VEGF-VEGFR2 signaling in Agrin silenced HUVECs. HUVEC transfected with control or Agrin siRNA were serum-starved for 16-18h before inducing with VEGF (50ng/ml) for the indicated time points and analyzed by Western blot for the respective proteins. β -actin was used as the loading control. The blots were quantified using Image-J.

Next, we investigated whether soluble Agrin supplementation to the Agrin depleted ECs was sufficient to rescue the activation of these downstream effectors of VEGF signaling. Given that the activation of eNOS, Akt and ERK 1/2 maximally occurred within 30 minutes of VEGF stimulation, we supplemented the Agrin depleted HUVECs with soluble Agrin for 18 hours in serum-free media before inducing with 50nmol/ml of VEGF-A for 30 minutes. Notably, when

soluble Agrin was supplemented along with VEGF stimulation for 30 minutes, it partially restored eNOS, Akt and ERK 1/2 activity in the depleted cells (**Figure 3.69**). Taken together, our data suggest that Agrin is required for VEGF-induced eNOS, Akt and ERK1/2 activations.

Figure 3.69: Induction of VEGF signaling in Agrin depleted cells rescued with soluble Agrin. *siControl, siAgrin and siAgrin HUVEC rescued with 10μg/ml soluble Agrin for 12h were serum-starved for another 12h before inducing with VEGF (50ng/ml) for 30 min. The cell lysates were analyzed by Western blot for the indicated proteins. B-actin was used as the loading control.*

Agrin promotes eNOS membrane localization

Membrane localization of eNOS is critical for eNOS activity and vascular permeability. Activated eNOS is primarily associated with the lipid raft subdomains of the plasma membrane. (Church and

Fulton, 2006; Fleming, 2010). Given that silencing Agrin hampered eNOS activation, we consequently wondered whether Agrin regulates eNOS membrane localization that may account for the decreased phosphorylation upon Agrin knockdown. As such, control and Agrin depleted ECs were serum-starved for 18 hours and stimulated with exogenous VEGF for 30 minutes the same as above before immunostaining with the eNOS antibody. Intriguingly, eNOS was majorly localized to the cytoplasm and perinuclear compartments of un-induced serum-starved ECs. However, upon stimulation with VEGF, the eNOS localization was predominantly shifted to the plasma membrane in control ECs; consistent with eNOS activation (**Figure 3.70 panel i-ii**). On the contrary, VEGF stimulation in the Agrin depleted cells did not alter the localization of eNOS which remained mostly in the cytoplasm (**Figure 3.70 panel iii-iv**). Thus, suggesting that Agrin is vital for eNOS membrane localization and activity.

Figure 3.70: Silencing of Agrin in ECs hampers eNOS membrane localization. Serum-starved control or Agrin depleted HUVEC were either untreated or induced with 50ng/ml VEGF for 30 mins and subsequently probed with an e-NOS antibody. The nucleus was stained with DAPI (blue). Representative confocal images are shown. Boxed areas represent enlarged panels. Scale bar: 10 μ m.

In an alternate approach, control and Agrin depleted HUVECs treated the same as above, were subjected to subcellular fractionation assay. The total plasma membrane fractions were isolated and analyzed by western blot to determine the relative levels of eNOS at the membrane. Consistent with the immunofluorescence data, subcellular fractionation also revealed decreased eNOS levels in the plasma membrane fractions of Agrin depleted HUVECs when compared to control fractions (Figure 3.71). No substantial differences were observed in the plasma membrane levels of endothelial marker CD-31 between control and Agrin depleted fractions, which further confirms that eNOS was specifically diminished from the plasma membrane fractions of the Agrin knockdown cells.

Figure 3.71: Loss of eNOS membrane fractions in Agrin knockdown HUVECs. HUVEC treated the same as in (Figure 3.70) was subjected to subcellular fractionation to isolate total membrane fractions. The resultant samples were analyzed by Western blot for eNOS and Agrin levels. Respective whole cell lysates were also analyzed along-with membrane fractions. GAPDH and CD-31 were used as loading controls for WCL and membrane fractions, respectively.

Given that eNOS activity was severely affected by Agrin depletion in HUVEC, we anticipated a corresponding decrease in endothelial nitric oxide production as an outcome of decreased VEGFR2-eNOS signaling. To verify this, we utilized a cell-permeable nitric oxide indicator 4,5-diaminofluorescein diacetate (DAF2-DA) which has been used in other studies to measure endothelial nitric oxide production (Boopathy et al., 2017). As expected, Agrin depletion severely hampered eNOS activation and endothelial nitric oxide production (**Figure 3.72 A-C**). Which was comparable to that observed when HUVEC cells were treated with eNOS specific inhibitor; N-nitro-L-arginine-methyl ester hydrochloride (L-NAME), a well-known suppressor of nitric oxide production (**Figure 3.72 A-C**).

Figure 3.73: Agrin silencing leads to decreased endothelial nitric oxide production. (A) Western blot analysis of e-NOS phosphorylation in HUVEC treated with either solvent control or L-NAME. (B) Control and Agrin depleted ECs analyzed for the indicated proteins the same as in (A). β -actin was used as the loading control. (C) Control and Agrin depleted HUVECs were analyzed for nitric oxide (NO) production using DAF-2DA nitric oxide indicator. DMSO and L-NAME treated cells were used as negative and positive controls respectively. Representative live-cell fluorescence and phase-contrast images are shown. Data quantified as mean fluorescence intensity $\pm$ SD of three independent experiments performed in triplicates. ($n=3$, *** $P < 0.0004$, student's t -test). Error bars denote standard deviation (SD). Scale bar, 10 μ m.

Taking into account that blood vessels are lined by a single layer of endothelial cells interconnected by tight junctions (Dejana, 2004). Vascular permeability mediated by eNOS is therefore critical for the formation of new blood vessels by angiogenesis. During neovascularization of solid tumors, endothelial stimulation by angiogenic growth factors leads to a temporal disruption in the tight junctions of adjoining endothelial cells thus, allowing for endothelial migration and sprouting angiogenesis (Connolly et al., 1989; Dvorak et al., 1995; Fukumura et al., 2001). Given the significance of endothelial permeability to VEGF-mediated angiogenesis, we, therefore, sort to validate the relevance of eNOS activity on *in-vitro* angiogenesis. Accordingly, we pre-treated ECs with either solvent control or eNOS inhibitor L-NAME for 1 hour before subjecting the cells to *in-vitro* tube formation on Matrigel. Noteworthy, L-NAME treated cells revealed significant defects in *in-vitro* tube formation which was not rescued by soluble Agrin supplementation (**Figures 3.73**). Thus, suggesting that Agrin requires active eNOS to mediate its angiogenic cues.

Figure 3.73: L-NAME treatment causes defective *in-vitro* tube formation which was not rescued by soluble Agrin supplementation. Representative phase-contrast images of ECs treated with DMSO or L-NAME only or supplemented with 10 μ g/ml sAgrin. Quantification was done using the angiogenesis analyzer tool from Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates

Earlier, we showed that silencing Agrin severely hampered *in-vitro* angiogenesis that was effectively rescued when soluble Agrin was supplemented in the culture medium of the Agrin depleted ECs. Given that soluble Agrin did not rescue *in-vitro* tube formation in L-NAME treated HUVECs, we then questioned whether soluble Agrin-mediated eNOS activation was critical for the rescue of *in-vitro* tube formation in Agrin silenced ECs. To verify this, Agrin depleted HUVECS were either left untreated or rescued with soluble Agrin in the presence of either solvent control or L-NAME and analyzed by *in-vitro* tube formation. As expected, silencing Agrin decreased *in-vitro* tube formation which was effectively rescue by soluble Agrin in the presence of solvent control but not in the presence of eNOS inhibitor L-NAME (**Figure 3.74**). Thus validating the premise that Agrin requires eNOS activity for mediating pro-angiogenic events.

Figure 3.74: Soluble Agrin mediated rescue of in-vitro tube formation requires eNOS activity. HUVEC was transfected with control or Agrin siRNAs. 48h post-transfection, one batch of Agrin depleted cells were supplemented with 10 μ g/ml sAgrin for 18h. The Agrin depleted cells supplemented with sAgrin were further treated with either solvent control or L-NAME for 1h

before subjecting the cells to in-vitro tube formation on Matrigel. Representative images were acquired by phase-contrast microscopy and analyzed by the angiogenesis analyzer from Image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0001$, ** $P < 0.0032$, * $P < 0.0226$, student's t -test). Error bars denote standard deviation (SD). Scale bar, $100\mu\text{m}$.

Earlier, we showed that secreted Agrin from MHCC-LM3 liver cancer cells was able to stimulate endothelial chemotaxis and sprouting angiogenesis. Given that vascular permeability is critical for tumor vascularization, we wondered whether secreted Agrin by HCC cells was able to rescue VEGFR2 stability and eNOS activity in Agrin depleted ECs. To this end, control and Agrin depleted HUVECs were treated with the conditioned medium from shControl or shAgrin MHCC-LM3 liver cancer cells for 18 hours. Interestingly, Agrin depleted HUVECs treated with shControl conditioned medium, partially rescued VEGFR2 expression and eNOS activity which remained drastically low in cells treated with shAgrin C.M (Figure 3. 75)

Figure 3.75: Secreted Agrin in the conditioned medium of MHCC-LM3 cells can rescue VEGFR2 expression and eNOS activity in Agrin silenced ECs. Control and Agrin knockdown HUVECs were incubated with CM from either shControl or shAgrin MHCC-LM3 cells for 12-18h and analyzed by Western blot for the indicated proteins. Data quantified from three experiments ($n=3$), (** $P = 0.0065$, * $P < 0.0414$, Student's t -test). β -actin was used as the loading control. Error bars denote standard deviation (SD).

Next, we queried whether this rescue of VEGFR2 stability and eNOS activation was sufficient to rescue the defects in *in-vitro* angiogenesis associated with Agrin depletion in primary ECs. Accordingly, Agrin depleted HUVECs were pre-treated with MHCC-LM3 conditioned medium same as above and subjected to *in-vitro* tube formation on Matrigel. Consistently, shControl conditioned medium but not shAgrin was able to partially rescue defective *in-vitro* tube formation in Agrin depleted HUVEC (**Figure 3.76**). Taken together, these data indicate that crosstalk between Agrin expressing liver cancer cells and ECs regulate eNOS activity and pro-angiogenic phenotype.

Figure 3.76: Secreted Agrin in the conditioned medium of MHCC-LM3 cells can partially rescue in-vitro tube formation in Agrin silenced ECs. Representative phase-contrast microscopy images of siControl and siAgrin HUVEC were subjected to in-vitro tube formation after pre-treated with C.M. from shControl or shAgrin MHCC-LM3 cells for 12-18h. Quantification was done using the angiogenesis analyzer tool from image J. Data presented as mean $\pm$ SD of three independent experiments performed in triplicates ($n=3$, *** $P < 0.0010$, * $P < 0.0245$, student's t -test). Error bars denote standard deviation (SD). Scale bar, 100 μ m.

In summary, we propose that secreted Agrin in the tumor microenvironment recruits endothelial cells within localized and metastatic tumor lesions. Agrin binds to its receptors Lrp4 and Integrin β 1 which leads to the activation of endothelial FAK thereby bridging the adherence of cancer cells to endothelial cells. The crosstalk and enhanced ECM stiffness mediated by Agrin, in-turn, stabilizes VEGFR2 by sequestering it in a multi-protein complex with its receptors Lrp4, Integrin β 1, and FAK. Thus sustaining VEGFR2 signaling and eNOS activity that promotes tumor vascularization (**Figure 3.77**)

Figure 3.77: Model of Agrin in angiogenesis and tumor vascularization. Model depicting Agrin's role in angiogenesis and tumor vascularization.

CHAPTER 4: DISCUSSION

Angiogenesis is critical for tumor growth and cancer progression (Folkman, 1971, 2002). Analysis of Agrin expression in a multi-cancer panel revealed an overlap with key angiogenic signatures that have been implicated in the vascularization of solid tumors. Moreover, immunostaining of Agrin in HCC tissue sections revealed a distinct pattern of Agrin staining that was prominent in the vicinity of the liver endothelium (Njah et al., 2019). Agrin expression in tumor hepatocytes is associated with enhanced oncogenic activity (Chakraborty et al., 2015). Given the connection between tumor angiogenesis and cancer progression, we uncovered that Agrin promotes tumorigenesis and cancer progression by enhancing tumor vascularization during the early stages of localized and metastatic tumor growth (Njah et al., 2019). This discovery reiterates the premise that tumor progression is angiogenesis dependent.

During HCC development, the liver parenchyma undergoes significant histopathological changes that are critical for tumor growth. Similar to other secreted proteoglycans and growth factors, Agrin is secreted into the tumor matrix and promotes a conducive microenvironment for blood vessel recruitment and the maintenance of tumor capillaries (Tripathy et al., 2018). Tumors below 2-3mm in diameter are usually avascular since they can readily obtain their metabolic requirements by simple diffusion from nearby capillaries. However, tumor vascularization is critical for cancer progression. Our comprehensive analyses reveal a role of Agrin in tumor vascularization and metastatic dissemination of liver cancer cells. Several key findings have advanced our understanding of the underlying mechanisms of Agrin mediated tumorigenesis. Firstly, we uncovered that secreted Agrin in the tumor microenvironment promotes the recruitment of surrounding endothelial cells to the tumor foci by stimulating endothelial chemotaxis and sprouting angiogenesis (Hanahan and Folkman, 1996). Moreover, secreted Agrin is also critical

for cancer cells to EC adhesion which is vital for the anchorage of sprouting endothelial cells during tumor angiogenesis and the metastatic dissemination of extravasating tumor cells.

Similar to HCC cells whereby Agrin promotes their viability and invasive properties, endothelial Agrin facilitates EC proliferation, invasion, and migration which are all vital for the angiogenic process. Also, Agrin promotes focal adhesion (FA) assembly which is critical for tumor motility and metastatic dissemination of tumor cells. Additionally, Agrin also enhances FA-mediated cytoskeletal reorganization and directional migration of endothelial cells during sprouting angiogenesis. Though endothelial FAK is known to mediate cancer cell homing during primary tumorigenesis and metastasis (Hiratsuka et al., 2011; Tavora et al., 2010), the up-stream ECM regulators governing this process are largely unknown. Here we show that Agrin via Integrin β 1-Lrp4 serves as a regulator of endothelial FAK activity and FA assembly that facilitates angiogenesis and enhances cancer cells to EC adhesion during tumor vascularization. On a molecular level, Agrin promotes angiogenesis by strengthening VEGFR2 stability and downstream e-NOS activation. Together, these findings suggest that targeting Agrin may reduce vascularization within tumors via VEGFR2 down-regulation. Moreover, Agrin-mediated stabilization of VEGFR2 is also critical for cancer cells to EC adhesion during tumor angiogenesis. To accommodate capillary expansion, ECs must proliferate, invade and migrate through their surrounding basement membrane in response to angiogenic cues (Ausprunk and Folkman, 1977; Folkman, 1974). Interestingly, secreted Agrin serves as an ECM cue to control the angiogenic properties of ECs. Moreover, primary endothelial cells, mouse metatarsals or matrigel plugs treated with soluble Agrin displayed enhanced rates of sprouting angiogenesis and vascularization respectively. Increasingly, the role of secreted proteoglycans as mediators of extracellular to intracellular communication via cell surface receptors is being appreciated. The evidence that

Agrin secreted from cancer cells or when exogenously supplied is crucial for angiogenesis is consistent with other proteoglycans influencing EC's migratory behavior (Fears and Woods, 2006; Sharma et al., 1998). Due to alternative splicing, Agrin is expressed as transmembrane or secreted variants which include neural and muscle Agrin. The fact that both liver cancer cells and ECs express the neural variant of Agrin shows that both cancer cells and ECs contribute to the total pool of Agrin within the tumor microenvironment that acts on ECs through a unified Integrin-Lrp4 receptor axis to stabilize VEGFR2. Since VEGFR2 is crucial for developmental and tumor angiogenesis (Chung et al., 2010; Klagsbrun and A. D'Amore, 1996; Risau, 1997), the existence of a mechanistic regulation of Agrin and VEGFR2 uncovered in this study is of prime interest in curbing tumor vascularization and cancer progression. Soluble Agrin, in particular, has been reported to confer stiffness to its surrounding ECM by activating YAP/TAZ and attributing matrix stiffness that presents a conducive environment for the development of tumor vasculature (Frye et al., 2018; Mammoto et al., 2009). Besides, our findings advance the notion that stiff ECM associated with Agrin stabilizes VEGFR2 in ECs. Besides the enhanced mechanosignaling in stiff ECM, another possible explanation for sustained VEGFR2 stability is attributed to the fact that Agrin sequesters VEGF-VEGFR2 in a complex between Integrin β 1-Lrp4 in ECs thereby decreasing its degradation and sustaining its angiogenic signaling. Extracellular to intracellular communication is vital for cellular homeostasis and the activation of signaling pathways that promotes cell function. Here we uncovered that Agrin-mediated cross-talks between the tumor-ECM and its receptors Integrin β 1 and Lrp4 on the endothelial surface favors heterotypic cell adhesion between liver cancer cells and ECs that is important during the recruitment of surrounding endothelial cells to the tumor foci. Besides, Agrin-mediated complex formation between these receptors also sustains FAK activity that is collectively required for mediating

angiogenic signaling and VEGFR2 stabilization. Agrin localized to the lipid raft enriched regions of cancer cells may sequester VEGF promoting the interaction between VEGF-VEGFR2 through the assembly of Integrin-Lrp4-VEGFR2 complex. Whether Agrin and ECM stiffness induced YAP/TAZ play a feed-forward loop in stabilizing the Integrin β 1-Lrp4-VEGFR2 complex will also be of future interest. The phosphorylation of VEGFR2 promotes downstream activation of ERK1/2, Akt, and eNOS, which are central to EC proliferation, invasion, and angiogenesis (reference). Consistent with the loss of VEGFR2 stability, Agrin depletion inhibits e-NOS-Akt-ERK1/2 activation and additionally diminishes e-NOS from cell membranes. This implies a gross defect of e-NOS activation in the cell membranes of ECs in the absence of Agrin. Agrin secreted from cancer cells or when exogenously supplemented compensates for the loss of e-NOS activity observed in Agrin depleted ECs. As an outcome of stabilized VEGFR2, secreted Agrin promotes sprouting angiogenesis and tumor vascularization. Thus, our findings support the existence of Agrin-mediated crosstalk between cancer and ECs that sustains tumor angiogenesis. Inhibiting VEGF-VEGFR2 has been shown to decrease EC proliferation and sprouting angiogenesis (Ferrara et al., 2005; Jain et al., 2006). Therefore, in combination with existing therapies targeting the VEGF-VEGFR pathway, we propose that targeting Agrin may present a new modality for reducing tumor angiogenesis via the downregulation of VEGFR2.

FUTURE PERSPECTIVES

Hepatocellular carcinoma is a highly vascularized tumor. The overexpression of Agrin in HCC has been shown to mediate tumorigenesis and cancer progression. Agrin silencing in HCC xenograft models revealed considerable mitigation in tumor growth and vascularization. Targeting Agrin in HCC might yield substantial clinical benefits in reducing the tumor burden and decreasing the

associated vascularization of these tumors. In this study, we demonstrated the significance of Agrin in tumor vascularization during the early stages of tumor growth. In hepatocellular carcinoma (HCC), Agrin is reported to be overexpressed and secreted in the extracellular matrix (ECM) of the tumor cells. We revealed that secreted Agrin in the tumor microenvironment creates an Agrin gradient that is sensed by surrounding endothelial cells (ECs) and they respond by chemotaxis towards the tumor foci in both localized and metastatic lesions. Furthermore, we showed that secreted Agrin is crucial for the adhesion of cancer cells to ECs which is a vital step in the vascularization of solid tumors from recruited ECs in the tumor vicinity. However, the precise mechanism by which Agrin mediates cancer cell to EC attachment needs to be elucidated. Moreover, whether Agrin independently activates vascular permeability by disrupting endothelial tight junctions remains unclear. Also, we observed that Agrin binds VEGF-A. However, the exact mechanisms of this association and the structural domains involved remain to be elucidated. In the ECM, secreted heparan sulfate proteoglycans (HSPGs) such as Perlecan which bears structural similarity to Agrin, has also been identified to bind secreted VEGF. Perlecan sequesters VEGF and prevents its degradation within the ECM. Moreover, Perlecan facilitates the presentation of VEGF to membrane-bound VEGFR2 thereby sustaining VEGF signaling in endothelial cells. Whether Agrin also serves as a co-receptor to secreted VEGF within the ECM remains to be clarified. Additionally, we reveal that Agrin regulates the stability of VEGFR2, a key mediator of VEGF-VEGFR2 signaling in developmental and tumor angiogenesis. We propose that Agrin which is none to confer matrix stiffness stabilizes VEGFR2 in a stiffness dependent manner. We showed that VEGFR2 is rapidly degraded in the absence of Agrin. The precise mechanism underlying this degradation needs to be further elucidated. In our investigations, we observed that inhibiting lysosomal degradation pathways by treating the ECs with chloroquine did not confer

any benefits in VEGFR2 stability. But inhibition of the proteasomal degradation complex using MG132 substantially decreased the degradation of VEGFR2. The underlying trafficking mechanisms that may be activated in the absence of Agrin remains unclear. Endothelial nitric oxide synthase (eNOS) activity is important for vascular permeability a vital process during tumor vascularization. In this study, we reveal that Agrin is important for eNOS membrane localization and activity. The membrane localization of eNOS is critical for eNOS activation. At the cell surface, eNOS is associated with the lipid raft subdomains of the plasma membrane. Agrin has also been linked to the lipid raft subdomains. Whether Agrin by its proximity to lipid raft-associated eNOS directly facilitates eNOS activation independent of VEGF-VEGFR2 signaling remains to be expounded.

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