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A Role of Agrin in Maintaining the Stability of Vascular Endothelial Growth Factor Receptor-2 during Tumor Angiogenesis

Highlights
- Extracellular matrix protein Agrin recruits ECs within tumors
- Agrin promotes \textit{in vitro} and \textit{in vivo} tumor angiogenesis
- Agrin and ECM stiffness stabilize VEGFR2 by Lrp4-Integrin $\beta_1$-FAK axis
- Targeting Agrin inhibits tumor angiogenesis by impairing VEGFR2 levels

Authors
Kizito Njah, Sayan Chakraborty, Beiying Qiu, ..., Guillaume Thibault, Xiaomeng Wang, Wanjin Hong

Correspondence
sayanc@imcb.a-star.edu.sg (S.C.), wangxiaomeng@ntu.edu.sg (X.W.)

In Brief
Njah et. al. show that the extracellular matrix protein Agrin recruits blood vessels within tumors. Agrin promotes adherence of endothelial cells (ECs) to tumor cells and enhances tumor angiogenesis. Mechanistically, matrix stiffness and Agrin stabilize VEGFR2 by enhancing interactions with Lrp4-Integrin-$\beta_1$-FAK. Targeting Agrin may inhibit tumor angiogenesis by reducing VEGFR2 levels.
A Role of Agrin in Maintaining the Stability of Vascular Endothelial Growth Factor Receptor-2 during Tumor Angiogenesis

Kizito Njah,1,2,6 Sayan Chakraborty,1,6,7,7 Beiying Qiu,1 Surender Arumugam,1 Anandhkumar Raju,1 Ajaybabu V. Pobbati,1 Manikandan Lakshmanan,1 Vinay Tergaonkar,1 Guillaume Thibault,2 Xiaomeng Wang,1,3,4,5,* and Wanjin Hong1

1Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), 61 Biopolis Drive, Proteos, Singapore 138673, Singapore
2School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore
3Lee Kong Chian School of Medicine, Nanyang Technological University, 59 Nanyang Drive, Singapore 636921, Singapore
4Singapore Eye Research Institute, The Academia, 20 College Road, Discovery Tower Level 6, Singapore 169856, Singapore
5Institute of Ophthalmology, University College London, 11-43 Bath Street, London EC1V 9EL, UK
6These authors contributed equally
7Lead Contact
*Correspondence: sayanc@imcb.a-star.edu.sg (S.C.), wangxiaomeng@ntu.edu.sg (X.W.)
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SUMMARY

Endothelial cell (EC) recruitment is central to the vascularization of tumors. Although several proteoglycans have been implicated in cancer and angiogenesis, their roles in EC recruitment and vascularization during tumorigenesis remain poorly understood. Here, we reveal that Agrin, which is secreted in liver cancer, promotes angiogenesis by recruiting ECs within tumors and metastatic lesions and facilitates adhesion of cancer cells to ECs. In ECs, Agrin-induced angiogenesis and adherence to cancer cells are mediated by Integrin-β1, Lrp4-MuSK pathways involving focal adhesion kinase. Mechanistically, we uncover 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-β1-Lrp4 and additionally stimulates endothelial nitric-oxide synthase (e-NOS) signaling. Therefore, we propose that cross-talk between Agrin-expressing cancer and ECs favor angiogenesis by sustaining the VEGFR2 pathway.

INTRODUCTION

Angiogenesis is central to tumor growth and cancer progression. The imbalance between pro- and anti-angiogenic factors triggers new blood vessel formation that facilitates tumor growth and metastasis (Folkman, 1971, 2002). Various growth factors, cytokines, and mechanical cues are implicated in the induction of angiogenesis within tumors (Carmeliet and Jain, 2011). In this regard, vascular endothelial growth factors (VEGFs) and their cognate receptors (VEGFR1–3) widely expressed in endothelial cells (ECs) are crucial in developmental and tumor angiogenesis. Among them, VEGFR2 has been identified as a critical component of VEGF-mediated angiogenesis (Veikkola et al., 2000). Binding of VEGF to VEGFR2 induces receptor dimerization and subsequent phosphorylation that sustain downstream signaling (Risau, 1997; Veikkola et al., 2000). At the molecular level, activation of VEGFR2 is associated with enhanced EC migration and survival by stimulating Ca2+ immobilization, ERK1/2 and PI3K-Akt activation, and endothelial nitric oxide (e-NO) production (Fulton et al., 1999; Koch and Claesson-Welsh, 2012; Neufeld et al., 1999). Therefore, inhibitors or VEGFR2-blocking therapies that decrease its stability and downstream signaling are of utmost clinical importance (Chung et al., 2010; Ferrara et al., 2004).

Apart from providing structural support for tissues, the extra-cellular matrix (ECM) serves as a mechanotransduction and signaling platform for angiogenesis (Eliceiri and Cheresh, 2001; Ingber, 2002; Stupack and Cheresh, 2002). The contributions of Integrin signaling as communicating receptors between the ECM and tumor cells promote neovascularization and tumor growth (Guo and Giancotti, 2004; Senger et al., 2002). Consistently, ECM-bound VEGF has been shown to prolong VEGFR2 activation and stimulate angiogenesis through Integrin-mediated signaling (Chen et al., 2010), indicating the existence of specific components within the ECM that sustain angiogenesis. We have begun appreciating the role of proteoglycans as key regulators of cell-matrix dynamics in regulating cellular processes such as angiogenesis (Iozzo and Sanderson, 2011). However, their identity and mechanisms mediating the crosstalk with VEGF receptors through Integrin activation during vascularization of tumors remain largely unexplored.

Agrin is a ~210-KDa glycosylated proteoglycan secreted in the basal lamina that clusters acetylcholine receptors (AChRs) in the neuromuscular junctions (NMJs). The C terminus of Agrin binds to low-density lipoprotein receptor-related protein 4 (Lrp4) to activate muscle-specific tyrosine kinase (MuSK), thereby forming a multi-protein complex (Glass et al., 1996; Kim et al., 2008; Ruegg and Bixby, 1998; Zhang et al., 2008). Distinct from its role at NMJs, Agrin is secreted in hepatocellular carcinoma (HCC) and regulates liver tumorigenesis (Chakraborty et al., 2015; Lv et al., 2017). As a potent regulator of cancer-cell-ECM mechanics, Agrin binds to Lrp4, MuSK, and Integrin β1, thereby sustaining...
the mechanoactivity of yes-associated protein (YAP) through maintenance of focal adhesion (FA) integrity (Chakraborty and Hong, 2018; Chakraborty et al., 2017). Although Agrin promotes the survival of hematopoietic stem cells (HSCs) (Mazzon et al., 2011), its impact on ECs and blood vessel formation during tumorogenesis remains unknown. Moreover, as Agrin is expressed in ECs and liver endothelium within HCC lesions (Tátrai et al., 2006), the underlying mechanisms of a tumor-conducive microenvironment caused by Agrin are of prime interest. In pursuit of these precedents, we envisaged a role of Agrin in tumor angiogenesis by recruiting ECs.

Herein, we report that Agrin plays a prominent role in liver cancer cells and EC communication that promotes tumor vascularization. Agrin utilized Lrp4-MuSK and Integrin-β1 signaling to stabilize endothelial VEGFR2 that stimulates angiogenesis by endothelial nitric-oxide synthase (e-NOS) signaling. As inhibition of Agrin destabilized VEGFR2, we propose that targeting Agrin presents an attractive modality for reducing tumor angiogenesis by downregulation of VEGFR2.

RESULTS

Recruitment of ECs within Tumors Mediated by Agrin

Agrin-induced HCC development and its expression in the liver endothelium prompted us to examine its role in angiogenesis (Chakraborty et al., 2015; Tátrai et al., 2006). To this end, we performed a Matrigel plug assay using control and Agrin-depleted MHCC-LM3 liver cancer cells to monitor blood vessel recruitment. The qualitative hematologic content within the Agrin-depleted tumor plugs were considerably lower when compared to control tumor plugs that is consistent with the fact that Agrin depletion severely hampered tumor growth in this xenograft model (Chakraborty et al., 2013) (Figure 1A). The reduction in Agrin immunostaining verified the efficacy of Agrin short hairpin RNA (shRNA) in these tumors (Figure 1B). Agrin depletion reduced the blood vessel marker CD-31 immunostaining and the total number of micro-vessels within growing tumors (Figure 1B). Equal proportions of resected tumor plugs revealed decreased CD-31 levels upon Agrin silencing when compared to those of control tumors, without affecting the levels of housekeeping proteins (glyceraldehyde 3-phosphate dehydrogenase [GAPDH], β-tubulin, and β-actin) (Figure 1C). No substantial decrease in CD-31 levels was observed with Agrin depletion in primary human umbilical vein ECs (HUVECs), suggesting that Agrin does not regulate CD-31 protein levels (Figure S1A). Moreover, as MHCC-LM3 cells do not express CD-31, we posit that lower CD-31 levels in Agrin-depleted tumor plugs is attributed to the diminished recruitment of blood vessels during the early stages of tumor growth (Figures S1A, S1B, and S1C). As the recruited ECs are fewer in comparison to tumor cells, the overall changes in the expression of endogenous housekeeping proteins contributed by ECs are also negligible.

We next evaluated whether secreted Agrin from liver cancer cells recruits ECs through chemotaxis. Accordingly, conditioned medium (CM) from control or Agrin-depleted MHCC-LM3 cells were used as a chemoattractant on naïve HUVECs. The secreted (CM) and cellular levels (WCL) of Agrin were drastically reduced in Agrin-depleted MHCC-LM3 cells, respectively (Figure 1D, left panels). Although the abundance of Agrin in the CM of shControl cells efficiently promoted HUVEC invasion and migration, these effects were significantly diminished by Agrin-deficient CM. The C-terminal Agrin bearing an 8-amino-acid “Z” insert that binds Lrp4 rescued the oncogenic defects observed upon Agrin depletion in HCC (Figure S1B) (Chakraborty et al., 2015; Chakraborty et al., 2017). Therefore, we expressed and purified soluble C-terminal recombinant Agrin (sAgrin) fragment that is recognized by Agrin antibody to rescue the chemotactic defects of Agrin-depleted CM (Figure S1B). Supplementing shAgrin CM with sAgrin significantly increased HUVEC invasion and migration (Figure 1D). In a complementary approach, immuno-depleting Agrin from shControl CM drastically decreased HUVEC chemotaxis (Figure S1C). Comparatively, MHCC-LM3 CM contained ~2-fold higher Agrin than HUVEC CM that may account for an Agrin gradient facilitating HUVEC chemotaxis (Figure S1D).

To determine the impact of HCC-derived Agrin on ECs, we analyzed its relative expression in ECs and HCC. Consistent with secreted Agrin, ECs expressed lower levels of Agrin when compared to aggressive HCC cell lines (Figure S1E). Similar to SK-N-SH neuroblastoma cells, liver cancer cells and ECs express neural Agrin that contains the “ZB” insert, albeit, with the levels being lowest in HUVECs (Figure S1F). Hence, we rationalized whether high levels of secreted Agrin and its involvement in cell-matrix interactions within the tumor environment are important for the adherence of ECs to cancer cells that subsequently...
Figure A: siControl, siAgrin, and siAgrin + sAgrin treatments showed differences in angiogenesis. 

Figure B: siControl, siAgrin, and siAgrin + sAgrin treatments were assessed in HUVEC, HRMEC, HDMEC, and HAEC cell lines. 

Figure C: siControl, siAgrin, and siAgrin + sAgrin treatments were evaluated for cell proliferation. 

Figure D: Different concentrations of anti-Agrin were used to assess its effect on angiogenesis. 

Figure E: siAgrin and β-actin treatments were compared for cell proliferation. 

Figure F: siControl, siAgrin, and siAgrin + sAgrin treatments were analyzed for migration and invasion.
trigger pro-angiogenic events. Accordingly, shControl, shAgrin, or Agrin-depleted liver cancer cells rescued by expressing rat Agrin (Agrin rescue) cells were allowed to adhere to a confluent layer of HUVECs that served as an endothelial substratum (Figure 1E). Owing to the higher Agrin levels in HCC cell lines, the depletion of Agrin in the cancer cells alone significantly decreased their adherence to control ECs, an effect that was partially restored in Agrin rescue cancer cells (Figure 1E, shControl, HUVEC panel). Interestingly, the loss in adherence of cancer cells to ECs was more robust when both cancer and endothelial Agrin were depleted, suggesting that endothelial-Agrin, in part, contribute toward adhesion to cancer cells (Figure 1E, siAgrin HUVEC panel). Under these conditions, Agrin rescued cancer cells partially restored adhesion to ECs, by compensating for adherence defects caused by the loss of endothelial Agrin (Figure 1E, siAgrin HUVEC third panel). Silencing Agrin in SNU-449 and Hep3B cell lines led to similar defects in adhesion to ECs (Figures S2A and S2B, first panels). Even in these cells, the adhesion was maximally inhibited when both endothelial and HCC cell Agrin was silenced and also partially restored when sAgrin was supplemented to the adhesion medium (Figures S2A and S2B, middle and third panels).

To demonstrate whether Agrin is required for blood vessel recruitment within metastasizing tumor foci in vivo, we inoculated control, Agrin-depleted, and Agrin-rescued MHC-L-M3 cells through tail veins of mice to monitor metastasis progression. First, silencing Agrin significantly inhibited the metastatic dissemination of these liver cancer cells in the lungs, when compared to the control and rescued cells (Figure 1F). Further, metastatic lesions generated by shControl cells efficiently recruited CD-31 enriched pulmonary blood vessels (Figure 1G). Few noticeable lung lesions developed by Agrin-depleted MHC-L-M3 cells were largely devoid of pulmonary blood vessels (Figure 1G). Metastatic tumors caused by Agrin-rescued cells, again, displayed enrichment of pulmonary blood vessels (Figure 1G). Together, these data suggest that cancer cell Agrin supports EC recruitment and adhesion during early stages of localized and metastatic tumor growth.

**Endothelial Agrin Promotes Angiogenesis**

The expression of Agrin overlapped with key angiogenic signatures across multi-cancer panels, further warranting an investigation of its impact on ECs and angiogenesis (Figure S2C). When compared to minimal Agrin expression in normal livers, endothelial linings and surrounding hepatocytes displayed strong Agrin expression in clinical specimens of HCC (Figure S2D), further suggestive of an underlying regulation of cancer-derived Agrin on the endothelium. Given that Agrin was expressed by multiple ECs, we utilized a loss-of-function RNA interference approach that efficiently depleted endothelial Agrin to analyze its functional consequence on ECs (Figures 2A and 2B). As such primary ECs (HUVECs, human retina microvascular EC [HRMECs], human dermal microvascular ECs [HDMECs], and human aortic ECs [HAECs]) were used to measure in vitro angiogenesis (Amautova and Kleinman, 2010). Agrin knockdown significantly decreased their ability to form tube-like structures and branching on Matrigel with an associated reduction in the tube length and master segments (Figures 2A and 2B). This loss of in vitro angiogenesis was restored when sAgrin was supplemented to Agrin-depleted HUVECs in a dose-responsive manner. Similar outcomes were also observed in several ECs (Figures 2A and 2B). Agrin depletion severely inhibited HUVEC and HDMEC spheroid sprouting angiogenesis, which was again rescued by sAgrin (Figure 2C). Additionally, when 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 partially rescued by the supplementation of sAgrin in the culture medium (Figure S3A). In conjunction, a function-blocking antibody against Agrin also inhibited tube formation in a dose-responsive manner (Figure 2D). This inhibition of in vitro angiogenesis by Agrin antibody was obliterated when pre-incubated with soluble Agrin (Figure S3B), thereby suggesting the specificity of sAgrin and its functional abilities on rescuing Agrin-mediated angiogenesis. Together, these gene epistases and function-blocking strategies reveal the relevance of endothelial Agrin to in vitro angiogenesis. Moreover, Agrin silencing also revealed a marked decrease in HVEC proliferation, which was, in part, rescued by sAgrin (Figure 2E). Consistent with its role in cancer cells, Agrin depletion in ECs strikingly inhibited their migratory and invasive rates in trans-well and wound scratch assays (Figures 2F and S3C). Likewise, these defects were partially rescued when soluble Agrin was supplemented as a chemoattractant in the culture medium (Figures 2F and S3C).

Because the majority of the phenotypic defects of Agrin knockdown cells were rescued by supplementing soluble Agrin, we characterized the effects of sAgrin on in vitro, ex vivo, and...

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**Figure 2. Agrin Silencing in ECs Hampers In Vitro Angiogenesis**

(A) Western blot validating Agrin knockdown in HUVECs. In vitro angiogenesis in Control or Agrin-depleted HUVECs alone or treated with indicated concentrations of sAgrin for 12–18 h. Images analyzed by angiogenesis analyzer (ImageJ). Representative data as mean ± SD of triplicates from three biological experiments (n = 3, ***p < 0.0006, **p < 0.0038, *p < 0.0334, Student’s t test). Scale bar, 100 μm.

(B) Western blot validating Agrin knockdown in indicated ECs. In vitro angiogenesis in the indicated ECs transfected as in (A) and rescued with 10 μg/ml sAgrin for 12–18 h. Data presented as mean ± SD of triplicates from three biological repeats (n = 3, ***p < 0.0002, **p < 0.0009, *p = 0.015, Student’s t test). Scale bar, 100 μm.

(C) Sprouting angiogenesis in control or Agrin-depleted ECs alone or supplemented with 10 μg/ml sAgrin. Image analyzed by sprout morphology (ImageJ). Data presented as mean ± SD of three to seven spheroids per group (n = 3, ***p < 0.0072, **p < 0.0034, Student’s t test). Scale bar, 100 μm.

(D) Dose-responsive inhibition of in vitro tube formation in HUVECs pre-treated with immunoglobulin G (IgG) or anti-Agrin (MAb5204) for 24 h. Data shown as mean ± SD of triplicates from three repeats (n = 3, ***p < 0.0006, **p < 0.0064, *p < 0.0340, Student’s t test). Scale bar, 100 μm.

(E) Western blot validation and cell proliferation of siControl, siAgrin, or siAgrin HUVECs supplemented with 10 μg/ml of sAgrin. Data represented as mean ± SD of triplicates from three experiments (n = 3, ***p < 0.0096, **p < 0.0354, Student’s t test).

(F) Invasion and migration of control, Agrin-depleted, and Agrin-silenced ECs supplemented with 10 μg/ml sAgrin for 12–18 h. Data presented as mean ± SD of triplicates from three experiments (***p < 0.0004, **p < 0.0040, *p < 0.0400, Student’s t test). Scale bar, 100 μm.

Error bars represent SD. See Figures S2 and S3.
Figure 3. Soluble Agrin Promotes Endothelial Sprouting Angiogenesis

(A) Rates of angiogenesis in sAgrin-treated (10 μg/ml) HUVECs. Representative data as mean ± SD of triplicates from three experiments (n = 3, ***p = 0.001, **p < 0.008, *p < 0.04, Student’s t test). Scale bar, 100 μm.

(B) Sprouting angiogenesis of indicated untreated or 10 μg/ml sAgrin supplemented ECs for 24 h. Quantified data (Sprout morphology, ImageJ) presented as mean ± SD of three spheroids per group (n = 3, **p < 0.0095, *p < 0.0240, Student’s t test). Scale bar, 100 μm.

(C) Sprouting from fetal mouse metatarsals treated with vehicle (PBS + 20 μg/ml BSA) (n = 11), VEGF (50 ng/ml) (n = 5), or Agrin (20 μg/ml) (n = 7) for 10 days. Scale bar, 200 μm. Data presented as mean ± SD (**p = 0.0007, 1-way ANOVA).

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in vivo angiogenesis. Of note compared to untreated cells, sAg can treatment on naive HUVECs enhanced their rates of master segment formation and total tube lengths during the observed time period (Figure 3A). Furthermore, in 3D spheroid cultures, soluble Agrin incorporated into a collagen matrix enhanced sprouting angiogenesis among several ECs (Figure 3B). To confirm whether Agrin promotes angiogenesis in an ex vivo metatarsal model, extracted mouse metatarsals at embryonic day 16.5 were treated with either vehicle, soluble Agrin, or VEGF for 10 days. In the absence of other growth factors, Agrin significantly enhanced metatarsal vessel outgrowth, comparable to that induced by VEGF stimulation (Figure 3C). Besides, in a cell-free Matrigel plug inserted subcutaneously in mice, soluble Agrin significantly increased vascularization and promoted the recruitment of ECs, as shown by enhanced CD-31 staining in vivo (Figures 3D and 3E). These studies clearly suggest that Agrin promotes blood vessel outgrowth and angiogenesis.

**Lrp4-Integrin β1-FA Kinase (FAK) Axis Mediate Agrin-Induced Angiogenesis**

The Agrin receptors Lrp4 and MuSK form an oncogenic signal complex with Integrin β1, αv- and β3-dystroglycan, MuSK, and Lrp4 were analyzed in ECs (Figure 4A). Although the knock down of dystroglycan did not hamper HUVEC tube formation, Integrin β1 and Lrp4 depletion robustly inhibited in vitro angiogenesis mimicking that of Agrin-silenced cells (Figures 4B and S4A). MuSK depletion affected tube formation, albeit, to a lesser extent than Integrin-Lrp4 knockdown (Figures 4B and S4B). Interestingly, soluble Agrin supplemented in Lrp4-MuSK and Integrin-β1-depleted HUVECs did not restore in vitro angiogenesis defects, therefore, suggesting that Agrin may utilize Integrin-Lrp4-MuSK receptor(s) axis to mediate tube-formation in vitro (Figures 4C and S4B). Soluble Agrin-mediated rescue of in vitro angiogenesis in Agrin-depleted HUVEC was strikingly abolished when pre-treated with the extracellular domain (ECD) of recombinant Lrp4, suggesting that binding of soluble Agrin to Lrp4 was a key inducer of the pro-angiogenic phenotype (Figure S4C). Furthermore, depletion of Agrin in ECs specifically reduced the mRNA and protein expression of Integrin β1 and Lrp4 that also accounted for reduced FAK activation but did not affect αv/β3-dystroglycan and MuSK levels (Figures 4D, S4D, and S4E). In contrast, sAgrin supplemented in Agrin-depleted HUVEC for 18 h partially restored the protein levels of Integrin β1, Lrp4, and FAK phosphorylation (Figure S4F).

Given that silencing FAK hampered HUVEC tube formation that was not rescued by soluble Agrin (Figure 4C), we hypothesized that FAK activation by Agrin was critical for in vitro angiogenesis. Similarly, pre-treatment of HUVEC with FAK inhibitor (PF562271) abolished the FAK phosphorylation, FAs, and tube formation (Figures 4E and 4F). Even treatment with soluble Agrin failed to rescue tube formation under such circumstances (Figures 4E and 4F). However, removal of the inhibitor allowed FA reorganization. Supplementation of Agrin, but not bovine serum albumin (BSA), strongly promoted tube-forming capabilities of HUVECs under these conditions (Figures 4E and 4F).

**Agrin-Lrp4-Integrin β1-FAK Pathway Adheres Cancer Cells to ECs**

Having demonstrated that cancer and endothelial Agrin was critical for adherence of cancer cells to ECs, we explored whether Integrin-Lrp4 and FAK activation were required for adhesion of cancer cells to ECs. Consistently, we identified that the combined loss of endothelial Integrin β1 (but not Lrp4) and cancer cell Agrin drastically reduced the adhesion of Agrin-depleted MHCC-LM3 cells to HUVECs, and this defect was not rescued when Agrin was restored in liver cancer cells (Figures 4G and S4G). However, when compared to the adhesion of Agrin knockdown HCC cells to control HUVECs, silencing endothelial Lrp4 did not show any synergistic inhibition of adherence when depleted along with cancer cell Agrin (Figures 4G and S4G).

**Agrin Stabilizes Endothelial VEGFR2 for Adhesion to Cancer Cells and Angiogenesis**

Because endothelial receptor tyrosine kinases (RTKs) and their downstream signaling control angiogenesis (Neufeld et al., 1999; Risau, 1997), we sought to understand the underlying...
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mechanisms that may contribute toward decreased angiogenesis upon Agrin depletion. Accordingly, we subjected control and Agrin-depleted HUVECs to an RTK phosphorylation array to screen differentially phosphorylated RTKs that may be associated with reduced angiogenesis (Figure S5A). Several RTKs that are key players in angiogenesis, including VEGFR1, VEGFR2, EphA1, EphB6, PDGFR-β, and Insulin receptor 2, showed a significant loss in their respective phosphorylation status upon Agrin depletion (Figures 5A and S5A). In contrast, phosphorylation of EGF, RYK, EphB2, and MSPR was significantly enhanced upon Agrin depletion (Figures 5B and S5A).

VEGFR2 is a highly expressed VEGF receptor in ECs and a master regulator of EC migration, proliferation, and sprouting angiogenesis (Ferrara et al., 2003). Given that the phosphorylation status of VEGFR2 was significantly reduced upon Agrin depletion in ECs, the mechanistic relationship between Agrin and VEGFR2 in the context of tumor angiogenesis was then investigated. Consistent with the RTK screen, phosphorylation at key tyrosine residues (Y951, Y1059, and Y1175) of VEGFR2 implicated in EC migration and sprouting was drastically lower upon Agrin depletion in HUVECs and HRMECs (Figure 5C). However, this effect observed upon Agrin depletion was primarily attributed to a general decrease in the total VEGFR2 protein levels in ECs (Figure 5C). Despite having an interaction with VEGF, Agrin depletion in HUVECs did not alter the expression of VEGF or its other cognate receptors VEGFR1 and VEGFR3, suggesting specificity of Agrin to regulate VEGFR2 (Figures S5B and S5C). VEGF, a potent activator of angiogenesis, was also unable to rescue tube formation defects when exogenously supplied to Agrin-depleted HUVECs, partly inferring that Agrin by the virtue of its binding to VEGF may facilitate VEGFR2-mediated angiogenesis (Figure S5D).

Furthermore, we pursued the following approaches to verify if the decrease in VEGFR2 was consistent with Agrin silencing, thus, ruling out off-target effects of RNA interference. First, time-dependent analysis of VEGFR2 expression upon Agrin small interfering RNA (siRNA) treatment revealed that the levels of VEGFR2 were maximally reduced between days 3–5, coinciding with effective knock down of Agrin (Figure S5E). Second, supplementing soluble Agrin in Agrin-depleted HUVECs rescued the total protein levels of VEGFR2 in a dose-dependent manner (Figure 5D). Third, HUVECs stably transduced by Agrin shRNAs also showed a loss in VEGFR2 protein levels that was restored by supplementing sAgrin (Figure S5F). In addition, dose-dependent treatment with an Agrin-function-blocking antibody also inhibited VEGFR2 levels over a period of 24 h (Figure 5E). Increasing the incubation time for the Agrin antibody treatment revealed a maximal reduction of VEGFR2 levels over a period of 48 h (Figure S5G). Hence, function-blocking Agrin antibodies inhibited VEGFR2 protein levels in a dose- and time-dependent fashion.

Because Agrin depletion rather enhanced VEGFR2 mRNA levels (Figure S5H), we anticipated a role of Agrin in VEGFR2 stability. As such, we deciphered that total VEGFR2 protein was largely de-stabilized within 1 h of blocking protein translation by cycloheximide (CHX) (Figure 5F, top panel). Even though soluble Agrin treatment partially stabilized VEGFR2 within 1–2 h post-CHX treatment (Figure 5F, top panel), we further analyzed its stability within a 1-h time frame, owing to the low half-life of VEGFR2 (Boucher et al., 2017). Pre-treatment with soluble Agrin strongly stabilized VEGFR2 within 15–60 min post-CHX treatment (Figure 5F, bottom panel). Even in MHCC-LM3 cells that express VEGFR2, Agrin depletion reduced its levels that were stabilized in cells rescued with rat-Agrin expression (Figure 5G). Consistent with the in vitro evidence, tumor plugs depleted of Agrin showed reduced VEGFR2 levels (Figures 5H and 5I). In addition, as endothelial Agrin was not perturbed, we posit the reduction of VEGFR2 levels is due to a combination of reduced EC recruitment and loss of VEGFR2 upon Agrin depletion within the tumor plugs. These data cumulatively suggest a role of Agrin in the stabilization of VEGFR2 protein levels.

To decipher whether VEGFR2 is involved in Agrin-mediated adhesion of ECs to cancer cells, we performed MHCC-LM3 and HUVEC adhesion assays in the presence of Sorafenib, a targeted therapy for HCC that blocks VEGFR2 and angiogenesis (Liu et al., 2006). Sorafenib treatment in HUVEC cells partially inhibited the adhesion of control MHCC-LM3 cells (Figure 5J, upper panel). Importantly, this loss of adherence was more robust when using Agrin-depleted MHCC-LM3 cells on HUVECs treated with Sorafenib (Figure 5J, upper panel). Agrin-rescued MHCC-LM3 cells had minimal adherence to Sorafenib-treated HUVECs (Figure 5J, upper panel). Because Sorafenib affects
other RTKs beside VEGFR2, we specifically depleted endothelial VEGFR2 to demonstrate consistent defects in cell adhesion, thus, implying that Agrin requires endothelial VEGFR2 in mediating adhesion of cancer cells to ECs (Figure 5J, lower panel). Notably, soluble Agrin treatment also failed to rescue the in vitro angiogenesis of HUVECs in the presence of Sorafenib or VEGFR2 depletion (Figures 5K and S5). Treatment of ECs with Agrin antibodies reduced VEGFR2 levels and in vitro angiogenesis, which was rescued by exogenous VEGFR2 expression (Figure S5J). Together, these compelling shreds of evidence indicate that Agrin requires endothelial VEGFR2 activity for mediating adhesion to cancer cells and angiogenesis.

**ECM Stiffness Conferring by Agrin Stabilizes VEGFR2**

We have recently reported that Agrin attributed stiffness to the ECM by activating YAP/TAZ (Chakraborty et al., 2017). To probe an ECM-stiffness-based regulation of Agrin on VEGFR2, we cultured HUVECs on soft and hard ECM (Mammoto et al., 2009). Indeed, VEGFR2 and Agrin mRNA and protein levels were enhanced in a stiff ECM (Figure 6A). Treatment of compliant ECM with soluble Agrin that is known to provide stiffness (Chakraborty et al., 2017) stabilized VEGFR2 protein levels in a dose-responsive manner without affecting its mRNA (Figure 6B). On the contrary, knock down of Agrin in HUVECs cultured on stiff ECMs despite enhancing mRNA levels caused a drastic loss of VEGFR2 protein (Figure 6C). The VEGFR2 protein level was dramatically stabilized by soluble Agrin supplemented within the stiff matrix, without any transcriptional alteration (Figure 6C). Moreover, consistent with higher levels of VEGFR2 and Agrin in stiff ECMs, we also found that degradation of VEGFR2 was significantly lower in stiff ECMs (Figure 6D). Likewise, soluble Agrin supplemented in compliant matrices also stabilized VEGFR2 in the presence of CHX treatment, therefore validating ECM-stiffness-based regulation of Agrin on VEGFR2 stability (Figure 6E). Furthermore, compared to Matrigel alone as an ECM substrate, Agrin-supplemented Matrigel supported greater master segments and tube length formation within 1.5–4.5 h (Figure 6F). Accordingly, Agrin supplemented in the Matrigel and culture medium exhibited the fastest rates of in vitro angiogenesis of HUVECs (Figure 6F).

As clustered Integrin signaling formed a major mechanosensing component in a host of cells, including ECs (Friedland et al., 2009), we further investigated whether soluble Agrin sequestered VEGFR2 in a complex with Integrin β1 and Lrp4 that further account for its stability and functions. Although a minimal basal interaction was observed between VEGFR2 and Integrin β1 and Lrp4 in untreated HUVECs, increased dosage of Agrin robustly enhanced complex formation between VEGFR2-Integrin β1 and Lrp4 (Figure 6G). In fact, soluble Agrin treatment in soft substrates greatly enhanced the formation of VEGFR2-Integrin β1 complex at the leading edges of HUVEC (Figure 6H). However, soluble Agrin-induced VEGFR2-Integrin β1 complex formations were hampered in the presence of Sorafenib in compliant substrates (Figure 6H). Notably, the VEGFR2-Integrin β1 complex was highly evident at the leading edges of HUVECs in stiff substrates, which drastically reduced upon Agrin knockdown due to the loss of VEGFR2 protein stability (Figure 6I). VEGFR2 levels and its complex with Integrin β1 were majorly restored upon soluble Agrin treatment to hard substrates (Figure 6I). Overall, we observed that soluble Agrin stabilized VEGFR2 in both soft and stiff ECMs, whereas Agrin depletion severely affected VEGFR2 stability. Silencing Integrin β1 affected Lrp4 levels and FAK activation, thereby de-stabilizing VEGFR2. (Figure S6A). Likewise, knock down of Lrp4 reduced Integrin levels and FAK activation, accounting for the loss of VEGFR2 stability, while FAK depletion also de-stabilized VEGFR2 without affecting the upstream components Integrin β1 and Lrp4 (Figure S6A). This loss of VEGFR2 was not restored by Agrin treatment (Figure S6A). The reduced VEGFR2 stability in the presence of PF562271 was significantly increased by sAgrin treatment following a drug-wash off in a dose- and time-dependent manner that correlated with FAK activity (Figure S6B). Hence, Agrin utilized its receptor repertoire and FAK to stabilize VEGFR2 in ECs.

**Agrin Controls VEGFR2-e-NOS Signaling in ECs**

Considering Agrin’s role in VEGFR2 stability, we next determined whether Agrin regulates its downstream signaling in ECs. The depletion of Agrin in ECs reduced the steady-state phosphorylation of e-NOS, Akt, and ERK1/2 activations, respectively.
A figure showing various graphs and images related to biological experiments, with legends for further explanation. The figure includes bars, line graphs, and images of cell cultures under different conditions. The experiments appear to involve the use of Agrin and VEGFR2 in soft and hard matrices, with treatments involving CHX and Matrigel. Additional images show the effects of Matrigel only, Matrigel + sAgrin (10 μg/ml), and Matrigel + sAgrin (10 μg/ml sAgrin in culture media) on cell behavior over time. There are also immunofluorescence images with annotations for integrin β1, VEGFR2, and merged images with DAPI. Pearson's coefficients are provided for statistical analysis of the data.
(Figure 7A). To determine whether Agrin affected the functional activation of e-NOS downstream of VEGFR2, we further analyzed the phosphorylation kinetics of e-NOS, Akt, and ERK1/2 upon VEGF stimulation. In control cells, e-NOS phosphorylation was induced as early as 15 min that was sustained until the observed 180 min of VEGF treatment (Figure 7B). In contrast, Agrin depletion abolished the activation of e-NOS under similar temporal stages of VEGF stimulation (Figure 7B). Likewise, the phosphorylations of Akt and ERK1/2 that occurred within 15- to 120-min post-VEGF treatment in control cells were strikingly reduced in Agrin-depleted ones (Figure 7B). Because the activation of e-NOS, Akt, and ERK1/2 maximally occurred within 30-min post-VEGF treatment, we noted that soluble Agrin supplemented along-with VEGF stimulation for 30 min partially restored e-NOS, Akt, and ERK1/2 activity in Agrin-silenced HUVECs (Figure 7C). As such, the loss of Agrin decreases the activation of VEGF-induced VEGFR2-e-NOS-signaling, which is rescued by soluble Agrin supplementation.

e-NOS, majorly localized to the cytoplasm of un-induced serum-starved cells, was shifted to plasma membranes upon VEGF stimulation that is consistent with its functional activation (Figure 7D, panels i and ii) (Govers et al., 2002). This motivated us to investigate whether Agrin depletion affected the plasma membrane localization of e-NOS in ECs. Strikingly, VEGF stimulation failed to recruit e-NOS to the plasma membranes of Agrin-depleted cells (Figure 7D, panels iii and iv). Sub-cellular fractionation also revealed decreased e-NOS in the plasma membrane fractions of Agrin-depleted cells (Figure 7E). No substantial difference in CD-31 levels between control and Agrin-depleted plasma membrane fractions further confirmed that e-NOS was specifically diminished from the plasma membranes of Agrin knockdown cells.

We anticipated a dramatic reduction in e-NO production as an outcome of decreased VEGFR2-e-NOS signaling in the absence of Agrin. Likewise, Agrin depletion severely hampered the production of e-NO, as measured by the formation of triazolo-fluorescein by NO indicator 4,5-diaminofluorescein diacetate (DAF-2-DA) treatment with L-NAME, a known inhibitor for NO production (Boopathy et al., 2017) (Figure 7G). The restoration of tube formation induced by soluble Agrin was blocked by L-NAME treatment in Agrin-depleted HUVECs, suggesting that soluble Agrin required e-NO production to induce in vitro angiogenesis (Figure S6C). Finally, to validate if secreted Agrin from cancer cells enhanced endothelial VEGFR2 stability and e-NOS activity, we incubated control and Agrin-silenced HUVECs with CM from control or Agrin-depleted MHCC-LM3 cells. Treatment of Agrin-depleted HUVECs with the CM from control cells partly rescued VEGFR2 levels, e-NOS activity, and in vitro angiogenesis (Figures 7H and 7I). In contrast, CM from Agrin-depleted cancer cells failed to induce e-NOS phosphorylation or support tube formation in Agrin-depleted HUVECs (Figures 7H and 7I). Consistently, the CM from shControl MHCC-LM3 cells induced greater HUVEC sprouting than Agrin-depleted CM (Figure 7J). Taken together, these data indicate that crosstalk between Agrin-expressing liver cancer cells and ECs regulates e-NOS activity and function.

In summary, we propose that secreted Agrin in the tumor micro-environment recruits ECs within localized or metastatic tumor lesions. Agrin binds to its receptors Lrp4 and Integrin β1 and activates FAK on the EC surface, thereby bridging adherence to cancer cells. The Agrin crosstalk and enhanced ECM stiffness, in turn, sequester and stabilize endothelial VEGFR2, triggering downstream e-NOS signaling in ECs that facilitate tumor vascularization (Figure S6D).

**DISCUSSION**

The initial stages of vessel recruitment involve migration and attachment of surrounding ECs to the growing tumor (Hanahan and Folkman, 1996). Comprehensive evidence reveals a role of Agrin in the recruitment of blood vessels and sprouting angiogenesis that contributes to metastatic tumor growth. Several key findings have advanced our understanding of the underlying mechanisms of Agrin-mediated tumorigenesis. First, Agrin is...
important for liver cancer and EC adherence and tumor vascularization. Second, Agrin regulates EC proliferation, invasion, and migration. Although 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. Agrin via Integrin β1-Lrp4 axis serves as a regulator of endothelial FAK to govern EC and liver cancer cell adherence. Third, Agrin promotes angiogenesis by strengthening VEGFR2 stability and downstream e-NOS activation. Together, these findings suggest that targeting Agrin may reduce vascularization within tumors by VEGFR2 downregulation.

To accommodate new vessel expansion and growth, ECs must proliferate, migrate, and invade the basement membrane in response to angiogenic cues (Auspurk and Folkman, 1977; Folkman, 1974). Interestingly, endothelial Agrin serves as an ECM cue to control the angiogenic properties of ECs. Agrin secreted by cancer cells or exogenously supplied is crucial for angiogenesis, which is consistent with other proteoglycans influencing EC’s migratory behavior (Fears and Woods, 2006; Sharma et al., 1998). Agrin isoforms secreted by cancer cell and expressed by ECs are similar. Therefore, both cancer cells and ECs contribute to the total pool of Agrin that acts on ECs through a unified Integrin-Lrp4 receptor axis to stabilize VEGFR2.

Because VEGFR2 is crucial for developmental and tumor angiogenesis (Chung et al., 2010; Klagesbrun and A. D’Amore, 1996; Risau, 1997), the existence of a mechanistic regulation of Agrin and VEGFR2 was uncovered in this study. Soluble Agrin, in particular, has been reported to confer stiffness to its surrounding ECM by activating YAP/TAZ and stiffened matrix further supports tumor vasculature development (Sordeleau et al., 2017; Chakraborty et al., 2017; Frye et al., 2018). We and others have shown that VEGFR2 levels are enhanced by ECM stiffness (Frye et al., 2018; Mammo et al., 2009). Our findings advance the notion that stiff-ECM-associated Agrin stabilizes VEGFR2 in ECs. Owing to the enhanced mechano-signalizing in stiff ECM, a possible explanation for sustained VEGFR2 stability is attributed to the fact that Agrin sequesters VEGF-VEGFR2 in a complex between Integrin β1-Lrp4 in ECs. Secreted Agrin cross-talks between the tumor-ECM and vascular endothelium and contributes toward ECM-stiffness-mediated stabilization of VEGFR2. Agrin localized to the lipid-raft-enriched regions of cancer cells may also sequester VEGF, promoting the interaction between VEGF-VEGFR2 through the assembly of the Integrin-Lrp4-VEGFR2 complex. Whether Agrin- and ECM-stiffness-induced YAP/TAZ play a feed-forward loop in stabilizing the Integrin β1-Lrp4-VEGFR2 complex will also be of future interest.

Phosphorylation of VEGFR2 promotes downstream activation of ERK1/2, Akt, and eNOS, which are central to EC proliferation, invasion, and angiogenesis (Clauss, 2000). Localization of active e-NOS at the cell membranes and NO production are required for vascular permeability and angiogenesis (Govers et al., 2002). Consistent with the loss of VEGFR2 stability, Agrin depletion inhibited e-NOS-Akt-Erk1/2 activation and additionally diminished 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 by cancer cells or when exogenously supplemented may compensate for the loss of e-NOS activity observed in ECs depleted of Agrin. As an outcome of stabilized VEGFR2, these data clearly support the existence of Agrin-mediated crosstalk between cancer and ECs that sustains angiogenesis.

Inhibiting VEGF-VEGFR2 has been shown to decrease EC proliferation and sprouting angiogenesis (Wicki et al., 2012; Ferrara et al., 2005; Jain et al., 2006). Therefore, in combination with existing therapies aimed at the VEGF-VEGFR pathway, we propose that targeting Agrin may present an attractive strategy for reducing tumor angiogenesis by downregulation of VEGFR2.

**STAR METHODS**

**Detailed methods** are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- ○ Mice
- ○ Cell lines

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**Figure 7. Agrin Controls VEGFR2 Downstream Signaling and e-NOS Activation**

(A) Western blot analysis in control and Agrin-depleted ECs.
(B) Serum-starved control or Agrin-depleted HUVECs were induced with VEGF (50 ng/ml) for the indicated time points and analyzed by western blot. Quantified data presented from three experiments.
(C) Serum-starved Control or Agrin-depleted HUVECs or those rescued with 10 μg/ml soluble Agrin for 12 h, induced with VEGF (50 ng/ml) for 30 min, and analyzed by western blot.
(D) Serum-starved control or Agrin-depleted HUVECs induced with 50 ng/ml VEGF for 30 mins were probed for e-NOS staining. Boxed areas represent enlarged panels. Scale bar, 10 μm.
(E) HUVECs treated as in (D) were subjected to sub-cellular fractionation and analyzed by western blot along with respective whole cell lysates, GAPDH and CD31 marked cell lysate and membrane controls, respectively.
(F) Control and Agrin-depleted HUVECs were analyzed for NO production. DMSO and L-NAME treatment served as experimental controls. Data quantified as mean fluorescence intensity ± SD of three repeats. (n = 3, ***p < 0.0004, Student’s t test). Scale bar, 10 μm.
(G) e-NOS phosphorylation in control and Agrin-depleted HUVECs (left panel) or DMSO and L-NAME treated (right panel). β-actin served as loading control.
(H) Control and Agrin-knockdown HUVECs were incubated with CM from shControl or shAgrin MHCC-LM3 cells for 12–18 h and western blotted for indicated proteins. Data quantified from three experiments (n = 3), (**p = 0.0065, *p < 0.0414, Student’s t test).
(I) In vitro angiogenesis in siControl and siAgrin HUVECs either untreated or treated with CM from MHCC-LM3 as in (H). Data presented as mean ± SD of triplicates from three experiments (n = 3, ***p < 0.0010, *p < 0.0245, Student’s t test). Scale bar, 100 μm.
(J) Sprouting angiogenesis in HUVECs treated with shControl or shAgrin MHCC-LM3 CM for 24 h. Data presented as mean ± SD of three spheroids per group from three experiments (*p = 0.0086, *p < 0.0100, Student’s t test). Scale bar, 100 μm. Error bars represent SD. See **Figure S6**.
METHOD DETAILS

- Cancer cell and EC co-culture adhesion assay
- In-vivo Matrigel Plug Assays
- Pulmonary metastasis model
- In-vitro angiogenesis assay
- Recombinant Agrin generation
- Ex-vivo matatarsal sprouting angiogenesis
- ECM stiffness manipulation
- Endothelial sprouting assay
- Reverse transcription PCR
- Receptor tyrosine kinase (RTK) phosphorylation array
- In-vitro Wound-healing assay
- EC Proliferation Assay
- Immunofluorescence (IF) and confocal microscopy
- Western blot analysis
- Immunoprecipitation (IP)
- Measurement of e-NO production
- Agrin ELISA
- Isolation of Total Membrane Fraction
- Immunohistochemistry

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.06.036.

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AUTHOR CONTRIBUTIONS

S.C., K.N., and W.H. conceived the project. S.C. and K.N. designed and performed all the experiments with assistance from B.Q., X.W., A.V.P., and G.T. S.A., A.R., K.N., and S.C. performed the in vivo experiments with inputs from M.L., V.T., and W.H. K.N. and S.C. analyzed the data. S.C. and K.N. wrote the manuscript with inputs from W.H. S.C. and W.H. jointly supervised the project.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

REFERENCES


# STAR METHODS

## KEY RESOURCES TABLE

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<td>Dr Kam-Man Hui laboratory (National Cancer Center, Singapore)</td>
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Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Sayan Chakraborty (sayanc@imcb.a-star.edu.sg).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**

All animal experiments performed were done in accordance with 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 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 retina microvascular endothelial cells (HRMEC) were purchased from ICells Biotechnologies (Cat#10HU-079). Neonatal-derived Human dermal microvascular endothelial cells (HDMEC) was obtained from ATCC (Cat#PCS-110-010). The gender for primary ECs were 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 DETAILS**

**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 10μM 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 μL of DMEM culture medium and combined with 250μl of ice-cold Matrigel (BD Biosciences). The cell-matrigel mix was subcutaneously implanted into the flanks of 7 weeks old Severe Combined Immunodeficient (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 ImageJ. For western blotting, tumor plugs from each group were cut equally into small pieces, lysed in RIPA lysis buffer (2.5 μg/μg 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 μL of Matrigel was combined with either vehicle control (PBS) or 20 μg/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 5X10⁶ 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 Biological Resource Center, A-STAR.

**In-vitro angiogenesis assay**

A flat bottom 96-well plate was pre-cooled on ice and coated with 40μl of matrigel (BD Biosciences) and allowed to polymerize by incubating at 37°C. Seven-eight thousand viable ECs post-trypsinization was re-suspended in 100μl 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. 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 (20 μg/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, Cultrex 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 re-suspended in 2 ml of complete EGM-2 media and mixed with 500 µl of methylcellulose. 20 µl of the mixture containing ~800 cells was 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 µl of rat-tail collagen Type 1 (9.51 mg/ml stock) to 414 µl of cell culture water and 60 µl of basal medium (M199). 200 µl of the collagen mix was dispensed into the appropriate wells of a 48-well plate and polymerized by incubating for 30 mins 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 µl FBS. Another collagen mix prepared as before was mixed with the 60 µL FBS containing spheroid suspension instead of M199 medium. 250 µl 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 µl 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 (ImageJ) 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 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, 300 µg of whole cell lysates from siControl or siAgrin HUVECs was diluted in fresh Array buffer 1 to a final volume of 1.5 ml and incubated on the membranes at 4 °C with mild shaking over-night. Post-incubation, membranes were washed thrice with 1X wash buffer for 10 mins each, followed by incubation with phospho-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 10 mins 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 20 µl 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 Corning BioCoat Matrigel invasion chamber. The chambers were first hydrated with 250 µl of serum-free media. 7000 cells were re-suspended in 200 µl of serum-free media and placed into the chamber-inserts. 700 µl 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. Similarly, ECs were plated in the lower chamber as a chemoattractant and incubated at 37 °C, 5% CO₂ for 24h. Subsequently, cells were fixed with 4% parafomaldehyde (PFA) for 15 mins at room temperature (RT) and stained with 1% crys-tal 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® AQsous One Solution Reagent (Promega). Relative cell growth was determined by the formation of colored formazan resulting from 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 re-suspended 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. Prior to analysis, 20 µl of CellTiter 96® AQsous One Solution Reagent was added to each well containing cells and incubated for 1h. Relative cell proliferation was determined every 24h for a three day period 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 15 mins with 4% parafomaldehyde (PFA). Subsequently, the cells were permeabilized for 15min with 0.1% Triton X in Phosphate buffered saline containing 1mM Ca²⁺ and 1mM Mg²⁺ (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 softwares.

**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.

**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. 500μg of whole cell lysate (WCL) was immunoprecipitated using 20μl of protein A/G Sepharose beads and 2 μg/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 500μl of 1% NP-40 lysis buffer followed by washing twice with 1X PBS. Subsequently, 20μl 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. Prior to 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 Balance 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 live by fluorescent microscopy. Endothelial nitric oxide synthase (eNOS) inhibitor; N”-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as 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 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 wash solution provided. Thereafter, 50μl of substrate A and B was added to each 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 were performed as described previously (1,2). 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 ± standard deviation (SD) 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.