Modulation of Angiopoietin 2 release from endothelial cells and angiogenesis by the synaptic protein Neuroligin 2

Margherita Pergolizzi[*1,2], Laura Bizzozero[*1,2], Elena Riccitelli [1,2], Davide Pascal [1,2], Anna Valeria Samarelli [1,2], Federico Bussolino[#1,2], Marco Arese[# 1,2] *

[1] University of Turin, Department of Oncology, Candiolo 10060, Turin, Italy

[2] Candiolo Cancer Institute – FPO, IRCCS, Candiolo 10060, Turin, Italy

**Corresponding author:* Marco Arese, Candiolo Cancer Institute, Strada Provinciale 142, km 3,95 10060 Candiolo (TO)-Italy. Email: marco.arese@unito.it,

[*]MP, LB equally contributed to this paper

[#]MA and FB equally contributed to this paper

Neuroligin 2 modulates angiogenesis

Keywords: neurovascular parallels, angiopoietin 2, physiological and tumor angiogenesis

Abbreviations: EC: endothelial cells, Ang2: angiopoietin 2, NLGN1: Neuroligin 1, NLGN2: Neuroligin 2, PKC: Protein Kinase C; PKA: Protein Kinase A, CB: collybistin; WPB: Weibel–Palade bodies

Abstract

The synaptic protein Neuroligin 2, similarly to its isoform Neuroligin 1, is produced by endothelial cells, but its activity in the vascular context remains unknown. This study aimed at verifying the hypothesis that Neuroligin 2, in parallel with its extraneuronal involvement in pancreatic beta cells exocytosis, modulated cytokine release from endothelial cells and consequently angiogenesis.

We used in vitro approaches to modulate Neuroligin 2 expression and Neuroligin 2 null mice to test our hypotheses. In vitro, upon VEGF stimulation, Neuroligin 2 silencing strongly reduces Angiopoietin 2 release in the medium and increases the endothelial cell retention of Weibel Palade Bodies, the specialized organelles that store Angiopoietin 2 and various other cytokines. On the contrary, Neuroligin 2 overexpression almost depletes cells of Weibel Palade Bodies, independent of VEGF. In vivo, both the retina and tumor xenografts grown in NLGN2- null mice display an immature vasculature, with lower pericyte coverage and lower Tie2 phosphorylation . At the molecular level NLGN2 colocalizes with its neuronal partner collibystin, a CDC42 guanine nucleotide exchange factor, which is also expressed by endothelial cells and in turn modulates Angiopoietin 2 release.

Neuroligin 2, an inhibitory synaptic protein, modulates a peculiar aspect of vascular function and could represent a novel target of therapy in various fields, from tumor angiogenesis to vascular diseases

Introduction

Neuroligin 1 (NLGN1) is a synaptic protein expressed on endothelial cells (ECs) that modulates angiogenesis and EC adhesion in cooperation with the laminin-binding integrin alpha6 beta1 [1]. Nonetheless, another Neuroligin isoform, Neuroligin 2 (NLGN2), is also highly produced by ECs, but its vascular activities do not overlap with those of NLGN1[1]. Interestingly, outside of the CNS, NLGN2 is involved in insulin release from pancreatic β cells [2].

Angiopoietin 2 (Ang2) is a vascular mediator with diverse activities, ranging from vessel sprouting to *vascular permeability and* inflammation [3] that is stored in secretory organelles of ECs called Weibel–Palade bodies (WPB) [4] along with von Willebrand factor (vWF) and a number of other vascular regulators. Vascular endothelial growth factor (VEGF) is an agonist of Ang2 release from ECs with a prominent role in tumor angiogenesis and vascular remodeling [3]. Here, we report the finding that endothelial NLGN2 modulates the VEGF-induced Ang2 and vWF release from ECs in vitro and in vivo, in cooperation with a neuronal Guanine Exchange Factor (GEF) for the small GTPase CDC42. Moreover, NLGN2 null mice display an altered vascular pattern and reduced pericyte coverage of vessels both in the neonatal retina and in subcutaneous tumors. These phenotypes are compatible with the lack of an agonistic activity of Ang2 towards its receptor Tie2.

Materials and Methods

Animals : Mice lacking NLGN2 ($Nlgn2^{tm1}$Bros/J - Jackson Laboratory), previously described [5], were maintained on a mixed C57BL/6 x 129 background (designated B6; 129) in the animal facility of the Institute (IRCC) under standard housing conditions. Mice were sacrificed at P5 and retinas were isolated for analysis. PCR amplification was used for the genotype characterization using the forward common primer (5'GTGAGCTGAATCTTATGGTTAGATGGG3'), the wild-type reverse primer: (5'CCCATCAGTGTACCATTCCCTAAA3'), and the mutant reverse primer (5'GAGCGCGCGCGGCGGAGTTGTTGAC3'). All animal experiments were performed in compliance with guidelines governing the care of laboratory mice of the University of Torino Committee on Animal Research.

Antibodies: Goat polyclonal Human Angiopoietin-2 (AF623), Monoclonal Mouse Human Collybistin/ARHGEF9 (Clone #809309, MAB 7848) and Rabbit Polyclonal Human/Mouse Phospho-Tie-2 (Y992) (AF2720) antibodies were obtained from R&D Systems.

The Rabbit Polyclonal Anti-Human Von Willebrand Factor (A0082) was from Dako.

The mouse anti-HA (sc-7392) was purchased from Santa-Cruz. The Rabbit anti-HA (H6908) and the Isolectin B4 FITC-conjugated from Bandeiraea Simplicifolia were purchased from Sigma.

The Rabbit anti-NG2 Chondroitin Sulfate Proteoglycan Polyclonal antibody was from Millipore.

The Anti-Active Cdc 42 Mouse Monoclonal Antibody (catalog number 26905) was obtained from ewEast Biosciences.

siRNA and cDNA transient transfection: ECs ($2x10^6$) were plated in 10 cm^2 dishes and transfected in opti-MEM® I + GlutaMAX™ (Gibco by Life Technologies) by using the Lipofectamine™ (2 mg/ml) and Plus Reagent (3 mg/ml) (Invitrogen) with 3 μg of DNA. After 3 hours at 37°C the opti-MEM® I was replaced with EGM-2. For transient down-regulation, ECs were seeded in EGM-2, in 6 well plate ($1,2x10^5$ cells/well). ECs were transfected for the next two days with 200 picomoles of each ON-TARGET plus siRNAs (Dharmacon): siRNA NLGN2_B09 (J-008843-09) recognizing the 5'UTR and the ORF sequences of human NLGN2, siRNA NLGN1_A5 recognizing the CDS sequences of human NLGN1 or with Non-targeting siRNA#1 (Scrambled), by using the Oligofectamine (Invitrogen) according to manufacturer's protocol.

Endothelial cells infection: The lentiviruses NLGN2 shRNA, Collybistin shRNA (XM_377014-TRCN0000047617) and CTRL shRNA (RNAi Consortium library SIGMA) were produced as described [6] . One day before the infection, ECs were seeded at concentration of $3,5x10^4$ cells/ml in a 100 mm tissue culture dish. Then the cells were transduced for 36h with the lentiviral particles in the presence of 8 μg/ml Polybrene (Sigma-Aldrich). Before starting every experiment, the efficiency of cell infection was determined by analyzing gene downregulation by qRT-PCR.

In vitro cord formation assay: For *in vitro* cord formation BME was added to each well at a concentration of 8 mg/ml and incubated at 37°C for 30 minutes to allow gel formation. Scramble control and NLGN2 silenced ECs ($2,3 \times 10^4$/well) were plated onto Matrigel in the presence of Recombinant Human $VEGF_{165}$ (50 ng/ml) or VEGF plus angiopoietin 2 (0,2 ug/ml) (R&D systems-catalog number 623-AN) and incubated for 4 hours at 37°C in 5% CO_2 humidified atmosphere. Cells organization was examined (Leica Microsystem, Heerbrugg, Switzerland) and photographed. The lengths of the capillary-like structures were quantified with the imaging software winRHIZO Pro (Regent Instruments Inc.).

Retinal whole mount staining and confocal imaging analysis: Mouse retinas dissection and whole mount immunostaining were performed as previously described [7] with modifications. Eyes were harvested at P5 and fixed in 4% paraformaldehyde (PFA) for 1 hour in ice. Retinas were dissected and washed briefly with DPBS (Sigma) three times and incubated overnight at 4°C in DPBS containing 0.5% Triton X-100 and 0.2% BSA. The retinas were rinsed briefly with DPBS containing 1% Triton X-100 and then incubated overnight at 4 °C in DPBS 1% Triton X-100 with specific antibodies against: Isolectin B4 (40 μg/ml); von Willebrand Factor (vWF 1:200); Phospho-Tie-2 (pTie2 1:50); Neural/glial antigen 2 (NG2 1:50). The retinas were rinsed in DPBS for 2 hours and then incubated for 1 hour at RT in DPBS 1% Triton X-100 containing the appropriate secondary antibody Alexa Fluor conjugate (1:200 Alexa Invitrogen Molecular Probes®) and stained with DAPI (1:5000)

for 45 minutes. Retinas were then washed in DPBS for 1 hour, fixed again with 4% PFA for 30 min at RT and flat-mounted onto a glass coverslip. Images were acquired using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica) and processed using Adobe Photoshop ®.
Retina images were quantified with Image Processing & Analysis in Java software (Image J) as follows: the immunoreactivity of each antigen was calculated as the surface area stained by the corresponding antibody normalized on the total surface vascular area visualized by Isolectin B4. For each quantification at least 5 mice/genotype were analyzed.

Endothelial cells immunostaining and signal quantification: ECs plated on gelatin-coated coverslips were fixed either with 4% PFA or with -20°C Methanol for 10 minutes at room temperature (RT), permeabilized with 0.2% Triton–X in PBS for 8 minutes at RT and saturated with 10% Donkey Serum/1% BSA in PBS for 40 minutes at RT. Slides were incubated with the primary antibodies: mouse anti-HA (1:100), rabbit anti-HA (10µg/ml); rabbit anti-vWF (1:200); goat anti-Angiopoietin 2 (1:50) and mouse anti-Collybistin (1:50) in PBS 10% Donkey Serum/1% BSA for 1 hour at RT and with the appropriate Alexa Fluor secondary antibodies in PBS 5% Donkey Serum for 45 minutes at RT. Finally, cells were stained with DAPI (1:10000) for 10 minutes at room temperature and mounted with immunofluorescence mounting medium (Dako).
Confocal images were acquired using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica). Fluorescent antibody signal was calculated with the Image Processing & Analysis in Java software (ImageJ) measuring the mean pixel intensity of maximum-projected confocal image stacks.

Enzyme-Linked Immunosorbent Assay (ELISA): Cell culture supernatants of NLGN2 overexpressing ECs in basal condition, or NLGN2 silenced ECs treated with vehicle, PMA (50 ng/ml) or VEGF (50 ng/ml) were collected for the analysis of Angiopoietin 2 release. The enzyme-linked immunosorbent assay (ELISA) (Quantikine Human Angiopoietin-2 ELISA kit -R&D Systems Europe, Ltd, Abingdon, UK) was used following the instruction manual. Each sample was measured in triplicate, and the OD values were measured at 450 nm using Synergy HT Microplate Reader. Wavelength correction were made at 540 nm.

Xenografts: WT and NLGN2KO C57BL/6J mice, 5 weeks of age, were subcutaneously inoculated with $2{,}5 \times 10^5$ B16 melanoma cells. Xenograft Tumors were excised when they reached around 1000 mm^3. Immunofluorescence analysis of OCT embedded tumors were performed with Isolectin and Neural/Glial antigen 2 antibodies.
Images were acquired using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica)
and quantified with Image J as follow: the immunoreactivity of each antigen was calculated as the surface area stained by the corresponding antibody normalized on the total surface vascular area visualized by Isolectin B4. For each quantification at least 6 tumors/genotype were analyzed.

Statistical Analysis: Upon verification of normal distribution, the statistical significance of raw data between the groups in each experiment was evaluated using the unpaired Student's *t*-test or ANOVA followed by the Bonferroni post-test. Results are expressed as means ± standard error of the mean (SEM) when derived from averaged experiments, or means ± standard deviation (SD) when derived from several data points of one experiment. *n* represents the number of individual experiments. The asterisks and circles */°, **/°° and ***/°°° in figure panels refer to statistical probabilities (*P*) of <0.05, <0.01 and <0.001, respectively.

Results

NLGN2 and its intracellular partner collybistin modulate Ang2 and vWF release

Our initial analysis showed that Ang2 release in the medium of ECs was sharply reduced by NLGN2 silencing both in basal conditions and upon VEGF stimulation, but not upon stimulation with the protein kinase C (PKC) activating phorbol ester PMA (phorbol 12-myristate 13-acetate[4]) (Figure 1A).

We next focused on the relations between VEGF, NLGN2, and Ang2. NLGN2 downregulation strongly impaired the time-dependent VEGF-induced discharge of WPB from cells, as measured by the amount of Ang2 or vWF within the cells (Figure 1B). Similarly, in vivo, in the postnatal mouse retinal vasculature, the absence of NLGN2 induced an increase in intracellular vWF (Figure 1C). In contrast, NLGN2 overexpression in ECs almost doubled the amount of Ang2 released in the medium (Figure 2A) while strongly reducing both Ang2 and vWF cell content (Figure 2B). This effect was totally independent of VEGF addition (data not shown). Importantly, neither NLGN2 silencing nor overexpression altered the amount of Ang2 mRNA (Figure S1A, B).

We next reasoned on the NLGN2 molecular partners that could potentially be involved in these activities. Two candidates stood out for their properties. One was the family of exchange proteins activated by cAMP (Epac), which are activators of the small G protein RAP [8]. EPAC proteins have been implicated in various exocytotic processes including WPB release [9], and an EPAC isoform (EPAC2/RAPGef4) is known to interact with NLGN2 in neurons[10]. Most of the EPAC-including pathways also involve cAMP signaling through protein kinase A (PKA) [8] and, interestingly, VEGF induced WBP exocytosis is dependent at least in part on cAMP/PKA pathway[11]. Unexpectedly, however, neither NLGN2 overexpression nor silencing was able to modulate RAP1 or PKA activity upon VEGF stimulation (Figure S2A, B, and data not shown). On the other hand, by the independence of PMA-induced release of Ang2 from NLGN2 (Figure 1A), PKC activity was unaffected by NLGN2 expression (Figure S2C).

The second candidate was the neuronal GEF collybistin (CB), a CDC42 activator [12]. In neurons, NLGN2 binds CB and shifts its equilibrium towards an active conformation to regulate the organization of inhibitory postsynaptic densities[13]. Our data showed that CB is expressed by ECs (Figure S3A) and colocalizes with

NLGN2 (Figure S3B), while CB silencing inhibits VEGF activity by causing Ang2 and vWF retention in cells, comparably to NLGN2 downregulation (Figure S3C, D). Moreover, NLGN2 silenced ECs showed a total loss of CB organization (Figure S3E). On the other hand, NLGN2 activity through overexpression, which promotes a strong depletion of Ang2 and vWF from cells even in the absence of VEGF (Figure 2A) was independent of CB expression (Figure S3H).

NLGN2 modulates in vitro and in vivo vascular morphogenesis

Next, we set out to evaluate if NLGN2 affected the angiogenic activities of VEGF on ECs and how this was dependent on Ang2. *In vitro,* NLGN2 downregulation changed the VEGF-induced cord formation of ECs on Matrigel by increasing the total tube length of the vasculature, while the simultaneous addition of exogenous Ang2 restored the normal phenotype (Figure 3A). To demonstrate a role of NLGN2 in physiological angiogenesis we used NLGN2 null mice. In these mice, the total vascular area in the neonatal retina and the density of the meshes were increased, while the typical hierarchical organization of the mature vascular trees was lost (Figure 3B). Interestingly, consistent with an agonist role of Ang2 on Tie2 in this context, Tie2 phosphorylation was reduced in retinas of NLGN2 null mice (Figure 3C). Parallel to the abnormal vessel growth there was a reduced pericyte coverage of retinal vessels (Figure 4A), a phenotype reminding of tumor angiogenesis. To prove this point we grew subcutaneous tumors in NLGN2 null mice, and demonstrated that their vascular bed displayed an additional loss of pericytes when compared with tumors grown in WT mice (Figure 4B).

Discussion

This study is a follow-up to other three reports from our laboratory that demonstrate a role for the synaptic proteins Neuroligins in vascular biology [1,6,14]. In particular, we reveal that the major “inhibitory isoform” of the NLGN family helps regulate the secretion of Ang2 and vWF from ECs.

Based on our data we assume that NLGN2 modulates VEGF-induced vWF/Ang2 release without a distinct involvement of the main molecular partners that we considered: the EPAC/RAP, PKA, or PKC pathways [9,11,15]. Besides the complexity of the WPB machinery itself [16] two factors may have impaired our understanding of the VEGF-NLGN2 downstream axis. First, NLGN2 itself modulates exocytosis in pancreatic beta cells through a complex transcellular mechanism that is also context and model dependent [2,17]. Second, VEGF plays a dual role in regulating endothelial exocytosis, triggering pathways that both promote and inhibit this process [18].

Nevertheless, we find that NLGN2 cooperates with yet a novel endothelial mediator, the neuronal GEF CB. Hence, one possibility is that NLGN2 affects exocytosis by modulating the exocyst complex and vesicle docking through a CB-CDC42 axis [19]. Indeed, immunodetection of active CDC42 in ECs shows that NLGN2 silencing

reduces the activity of CDC42 to the same extent as CB downregulation does, at least in basal conditions (Figure S4).

We show that in vitro and in vivo NLGN2 deletion influences vascular morphogenesis. Our data support the idea that Ang2, at least at the concentrations controlled by NLGN2 in vivo, is a Tie2 agonist. While the original view in the vascular field was that Ang2 produced by VEGF-activated endothelium inhibits Tie2 activity, destabilizes blood vessels and induces pericyte loss [20], this antagonist hypothesis has been challenged and the activity of Ang2 has been proposed to be context-dependent. In particular, Ang2 has been reported to function as a Tie2 activator in human tumor xenografts [21]. Accordingly, we show that a lack of NLGN2 in the host induces pericyte loss during the vascular development of the retina and it exacerbates the same phenotype in the already abnormal tumor vasculature. Hence, we hypothesize that NLGN2 silencing in EC causes a loss of an agonist activity of Ang2.

In conclusion, this study along with our previous one[1] reveals that NLGN2, unlike NLGN1, modulates vessel functions not by affecting the extracellular matrix adhesive properties of ECs, but by regulating the release of WPBs, with their large cytokine load and activities ranging from hemostasis to inflammation, to tumor metastasis [3].

Acknowledgements : This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) investigator Grants IG 11503 and 15238 (to M. A.), IG Grant 18652 and 5x1000 12182 (to F.B.) and by Fondazione Umberto Veronesi, Young Investigator Grant 2014 (to M. A.).

References

[1] A.V. Samarelli, E. Riccitelli, L. Bizzozero, T.N. Silveira, G. Seano, M. Pergolizzi, G. Vitagliano, I. Cascone, G. Carpentier, A. Bottos, others, Neuroligin 1 induces blood vessel maturation by cooperating with the α6 integrin, Journal of Biological Chemistry. 289 (2014) 19466–19476.

[2] C. Zhang, A.T. Suckow, S.D. Chessler, Altered Pancreatic Islet Function and Morphology in Mice Lacking the Beta-Cell Surface Protein Neuroligin-2, PloS One. 8 (2013) e65711.

[3] P. Saharinen, L. Eklund, K. Alitalo, Therapeutic targeting of the angiopoietin-TIE pathway, Nature Reviews Drug Discovery. (2017).

[4] U. Fiedler, M. Scharpfenecker, S. Koidl, A. Hegen, V. Grunow, J.M. Schmidt, W. Kriz, G. Thurston, H.G. Augustin, The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies, Blood. 103 (2004) 4150–4156.

[5] F. Varoqueaux, G. Aramuni, R.L. Rawson, R. Mohrmann, M. Missler, K. Gottmann, W. Zhang, T.C. Südhof, N. Brose, Neuroligins determine synapse maturation and function, Neuron. 51 (2006) 741–754.

[6] A. Bottos, E. Destro, A. Rissone, S. Graziano, G. Cordara, B. Assenzio, M.R. Cera, L. Mascia, F. Bussolino, M. Arese, The synaptic proteins neurexins and neuroligins are widely expressed in the vascular system and contribute to its functions., Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 20782–7. doi:10.1073/pnas.0809510106.

[7] S. Sawamiphak, M. Ritter, A. Acker-Palmer, Preparation of retinal explant cultures to study ex vivo tip endothelial cell responses, Nature Protocols. 5 (2010) 1659–1665.

[8] J.L. Bos, Epac proteins: multi-purpose cAMP targets, Trends in Biochemical Sciences. 31 (2006) 680–686.

[9] K.W.E.M. van Hooren, E.L. van Agtmaal, M. Fernandez-Borja, J.A. van Mourik, J. Voorberg, R. Bierings, The Epac-Rap1 pathway controls cAMP-mediated exocytosis of Weibel-Palade bodies in endothelial cells, Journal of Biological Chemistry. (2012).

[10] K.M. Woolfrey, D.P. Srivastava, H. Photowala, M. Yamashita, M.V. Barbolina, M.E. Cahill, Z. Xie, K.A. Jones, L.A. Quilliam, M. Prakriya, others, Epac2 induces synapse remodeling and depression and its disease-associated forms alter spines, Nature Neuroscience. 12 (2009) 1275–1284.

[11] Y. Xiong, Y. Huo, C. Chen, H. Zeng, X. Lu, C. Wei, C. Ruan, X. Zhang, Z. Hu, M. Shibuya, others, Vascular endothelial growth factor (VEGF) receptor-2 tyrosine 1175 signaling controls VEGF-induced von Willebrand factor release from endothelial cells via phospholipase C-γ1-and protein kinase A-dependent pathways, Journal of Biological Chemistry. 284 (2009) 23217–23224.

[12] S. Xiang, E.Y. Kim, J.J. Connelly, N. Nassar, J. Kirsch, J. Winking, G. Schwarz, H. Schindelin, The crystal structure of Cdc42 in complex with collybistin II, a gephyrin-interacting guanine nucleotide exchange factor, Journal of Molecular Biology. 359 (2006) 35–46.

[13] A. Poulopoulos, G. Aramuni, G. Meyer, T. Soykan, M. Hoon, T. Papadopoulos, M. Zhang, I. Paarmann, C. Fuchs, K. Harvey, others, Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin, Neuron. 63 (2009) 628–642.

[14] A. Rissone, E. Foglia, L. Sangiorgio, S. Cermenati, S. Nicoli, S. Cimbro, M. Beltrame, F. Bussolino, F. Cotelli, M. Arese, The Synaptic Proteins β-Neurexin and Neuroligin Synergize With Extracellular Matrix-Binding Vascular Endothelial Growth Factor A During Zebrafish Vascular Development, Arteriosclerosis, Thrombosis, and Vascular Biology. 32 (2012) 1563–1572.

[15] O. Lorenzi, M. Frieden, P. Villemin, M. Fournier, M. Foti, U.M. Vischer, Protein kinase C-δ mediates von Willebrand factor secretion from endothelial cells in response to vascular endothelial growth factor (VEGF) but not histamine, Journal of Thrombosis and Haemostasis. 6 (2008) 1962–1969.

[16] T. Nightingale, D. Cutler, The secretion of von Willebrand factor from endothelial cells; an increasingly complicated story, Journal of Thrombosis and Haemostasis. 11 (2013) 192–201.

[17] A.T. Suckow, C. Zhang, S. Egodage, D. Comoletti, P. Taylor, M.T. Miller, I.R. Sweet, S.D. Chessler, Transcellular Neuroligin-2 Interactions Enhance Insulin Secretion and Are Integral to Pancreatic β Cell Function, Journal of Biological Chemistry. 287 (2012) 19816–19826.

[18] K. Matsushita, M. Yamakuchi, C.N. Morrell, M. Ozaki, B. O'Rourke, K. Irani, C.J. Lowenstein, Vascular endothelial growth factor regulation of Weibel-Palade-body exocytosis, Blood. 105 (2005) 207–214.

[19] N. Porat-Shliom, O. Milberg, A. Masedunskas, R. Weigert, Multiple roles for the actin cytoskeleton during regulated exocytosis, Cellular and Molecular Life Sciences. 70 (2013) 2099–2121.

[20] D. Keskin, J. Kim, V.G. Cooke, C.-C. Wu, H. Sugimoto, C. Gu, M. De Palma, R. Kalluri, V.S. LeBleu, Targeting vascular pericytes in hypoxic tumors increases lung metastasis via angiopoietin-2, Cell Reports. 10 (2015) 1066–1081.

[21] C. Daly, G.D. nd Yancopoulos, others, Angiopoietin-2 functions as a Tie2 agonist in tumor models, where it limits the effects of VEGF inhibition, Cancer Research. 73 (2013) 108–118.

Figure legends

FIGURE 1 Neuroligin2 regulates VEGF-induced WPBs release in vitro and in vivo

A) Ang2 release assays were performed by Elisa on ECs supernatants, transfected with control (si RNA SCRL) or NLGN2 siRNA (siRNA NLGN2) treated with vehicle (PBS), PMA (50ng/ml) or VEGF (50ng/ml) for 40 minutes (A). NLGN2 silenced cells displayed a strong decrease in VEGF-induced Ang2 secretion; NLGN2 downregulation does not affect PMA ability to induce Ang2 release; The graphs shows the percentage of Ang2 release in culture media with respect to siRNA SCRL. Values are expressed as mean ± S.E.M. (n=3 independent experiments). Asterisks and circles indicate statistical significance *versus* cells treated with vehicle and VEGF, respectively; one-way ANOVA with Bonferroni test (n=3): $^{**}p<0.01$,$^{***,\circ\circ\circ}p<0.001$..

B) Confocal microscopy analysis performed on ECs, transfected with a short hairpin control (shRNA CTRL) and a shRNA sequence specific for NLGN2 (shRNA NLGN2), treated with VEGF (50ng/ml) for 0, 5, 10, 20 and 40 minutes. Cells were immunostained with anti-Ang2 (red, upper panels and graph) and anti-vWF (green, lower panels and graph) antibodies. In NLGN2 downregulated cells VEGF is unable to induce WPB/Ang2 release, which then accumulate intracellularly. The images are representative of 1 out of 3 reproducible experiments (scale bar: 10 μm). The graphs show the fluorescent area immunodecorated with each antibody. Values are expressed as mean ± S.E.M. Asterisks indicate statistical significance *versus* cells infected with shRNA CTRL; unpaired Student's t test, two-tailed (n = 3, total cells analyzed n = 30): $^{*}p<0.05$; $^{***}p<0.001$.

C) P5 postnatal mouse retina of WT and NLGN2 KO (Nlgn2−/−) mice were immunostained with isolectin B4 to visualize the retinal vascular vessels (green) and with the anti-vWF antibody (red). NLGN2 KO retinas showed a clear intracellular accumulation of WPBs with respect to WT; this accumulation is localized predominantly in areas of active proliferation at the growing front of the vascular plexus. Scale bar: 100 μm. The graph shows the fluorescent area of vWF staining normalized on the total vascular surface area. Values are expressed as mean ± S.E.M. (n = 5 retinas for each genotype), unpaired Student's t test, two-tailed ($^{*}p = 0.025$).

FIGURE 2: **Neuroligin2 overexpression induces Ang2 and WPBs release from cells**

A) Ang2 release assays were performed by Elisa on ECs supernatants overexpressing the retroviral vector pBABE, either EMPTY or containing NLGN2 cDNA (pBABE NLGN2). The overexpression of NLGN2 in basal condition doubled the presence of Ang2 in ECs supernatants. The graph shows the percentage of Ang2 release in culture media with respect to pBABE empty. Values are expressed as mean ± S.E.M. (n=3 independent experiments). Asterisks indicate statistical significance *versus* cells transfected with pBABE empty; unpaired Student's t test, two-tailed (n=2): p=0.0012

B) Confocal microscopy analysis performed on ECs overexpressing the retroviral vector pBABE, either EMPTY, or containing NLGN2 cDNA (pBABE NLGN2). Cells were immunostained with anti-HA (red), anti-vWF (green), anti-Ang2 (magenta) antibodies and DAPI (blue). NLGN2 overexpressed cells displayed a significant reduction in WPBs and Ang2 intracellular fluorescence with respect to control cells. The images are representative of 1 out of 3 reproducible experiments (scale bar: 10 μm). The graph shows the fluorescent area immunodecorated with each antibody. Values are expressed as mean ± S.E.M.; unpaired Student's t test, two-tailed (n = 3, total cells analyzed n = 21): ***p<0.001.

FIGURE 3

Phenotipic effects of Neuroligin2 silencing in vitro and in vivo.

A) ECs transfected with control (shRNA CTRL) and one shRNA sequence specific for NLGN2 (shRNA NLGN2) were seeded on 48-well plates coated with BME (8 mg/ml) and treated 4 hours with VEGF (50ng/ml) in presence or absence of human recombinant Ang2 (rhAng2, 0.2 μg/ml). Tube length was quantified through WinRHIZO Pro and is represented in the histograms. Images and graphs shown are representative of 1 out of 3 reproducible experiments, each in triplicate. Data are expressed as mean ± S.D. One-way ANOVA with Bonferroni test: °°, $p < 0.01$ for shCTRL + VEGF versus shNLGN2 + VEGF and **, $p < 0.01$ for shNLGN2 + VEGF versus shNLGN2 + VEGF + Ang2. NLGN2 downregulation increases the length of the total tube vasculature formed upon VEGF treatment and this phenotype is reversed by exogenous Ang2.

B,C) Post-natal angiogenesis (B), Tie2 activation (C) in p5 mouse retina of WT and NLGN2 KO (Nlgn2−/−) mice immunostained with isolectin B4 to visualize the retinal vascular vessels (green, B,C), anti-pTie2 (red, C). The absence of NLGN2 results in increased total vascular area and density of the vascular network in the neonatal retina and decreased activation of the Ang2 receptor Tie2. The graphs in B show the total surface vascular area, in C the pTie2 fluorescent area normalized on the total isolectin area. Values in B,C are expressed as mean ± S.E.M.. Unpaired Student's t test, two-tailed: B (n=9 retinas for each genotype) *, p = 0.0015); C (n=5 retinas for each genotype) **, p =0.0032

FIGURE 4: Neuroligin2 silencing reduces pericyte coverage of vessels in physiological and tumoral angiogenesis

A) Vascular pericyte coverage in p5 mouse retina of WT and NLGN2 KO (Nlgn2−/−). Retinas of mice were immunostained with isolectin B4 to visualize the retinal vascular vessels (green), and anti-NG2 to visualize pericytes (red). The absence of NLGN2 results in decreased pericyte coverage . The graphs shows the pericyte coverage (measured as the colocalization between NG2 and isolectin, normalized on the total isolectin area. Values are expressed as mean ± S.E.M.. Unpaired Student's t test, two-tailed (n=4 retinas for each genotype) *, p =0.0492.

B) NG2 (red) and Isolectin (green) staining and quantification in B16 -derived tumors from WT and NLGN2 KO mice (six animals per group). Tumors grown in NLGN2 KO mice displayed a reduction in pericyte coverage

compared to WT. Images are representative of 1 out of 6 tumors per group (scale bar, 100 μm). Graphs show the pericyte coverage (measured as the colocalization between NG2 and isolectin, normalized on the total isolectin area). Values are expressed as mean ± S.E.M. (n = 48 images), unpaired Student's t test, two-tailed (**, p = 0.0084).