Brief Communications

Wnt3a promotes pro-angiogenic features in macrophages *in vitro*: Implications for stroke pathology

Almudena Fuster-Matanzo[1], Giulia Manferrari[1], Bianca Marchetti[2,3] and Stefano Pluchino[1]

[1]Department of Clinical Neurosciences – Division of Stem Cell Neurobiology, Wellcome Trust-Medical Research Council Stem Cell Institute and NIHR Biomedical Research Centre, University of Cambridge, Cambridge CB2 0HA, UK; [2]Department of Biomedical and Biotechnological Sciences (BIOMETEC), Pharmacology Section, University of Catania Medical School, Catania 95125, Italy; [3]OASI Institute for Research and Care on Mental Retardation and Brain Aging, Neuropharmacology Section, Troina 94018 (EN), Italy
Corresponding authors: Stefano Pluchino. Email: spp24@cam.ac.uk; Almudena Fuster-Matanzo. Email: af541@cam.ac.uk

Impact statement

This work provides a new link between Wnt3a and macrophage-mediated angiogenesis under glucose and oxygen deprivation *in vitro*. Our results reveal how Wnt3a shifts macrophages towards a pro-angiogenic phenotype, which is able–in absence of both glucose and oxygen–of inducing angiogenesis *in vitro*, thus pointing to a synergy between the activation of the pathway and the hypoxia scenario. This work also demonstrates that modulation of cell death is key in order to explain the observed angiogenic effects.

We consider all these findings of significant importance, since no connection between Wnt3a, macrophages, and angiogenesis has been established so far. Furthermore, we do believe that this work provides new and interesting results, with Wnt signaling pathway emerging as an interesting target mediating beneficial outcomes during the inflammatory response undoubtedly linked to stroke pathology, where angiogenesis has been already proposed as a potential mechanism to promote recovery after the injury.

Abstract

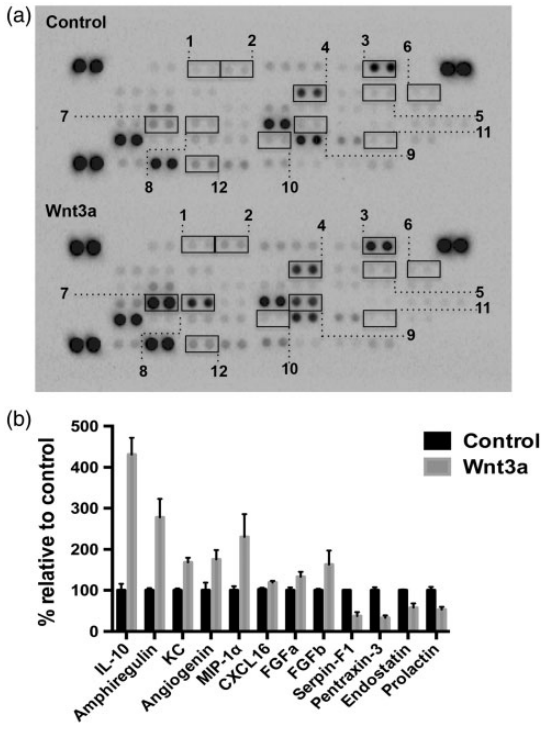
Wnt3a is implicated in several key cellular processes and its expression has been reported in different cell types. Here, we report a novel function for Wnt3a in macrophages, whose exposure to this ligand shifts them towards a pro-angiogenic phenotype capable, under oxygen and glucose deprivation, of inducing *in vitro* tubular pattern structures in endothelial cells resembling capillary-like vasculature. These newly acquired angiogenetic features also include increased proliferation and migration and surprisingly, an increase in cell death. This work provides a new link between Wnt3a and macrophage-mediated angiogenesis under glucose and oxygen deprivation *in vitro*, which are worth further investigation in pathological conditions including stroke, where the stimulation of the angiogenic process might help to recovery after tissue injury

Keywords: Wnt3a, *in vitro* angiogenesis, macrophages, oxygen and glucose deprivation, stroke

***Experimental Biology and Medicine* 2018; 243: 22–28. DOI: 10.1177/1535370217746392**

Introduction

Wnt ligands constitute a family of secreted glycoproteins which are cell- and tissue-specific ligands that orchestrate a wide range of processes in the developing and adult brain.[1] In mammals, a total of 19 proteins have been identified and divided into two classes: the Wnt1 subtype (including Wnt3a), generally triggering the canonical Wnt/β-catenin signaling pathway and the Wnt5a type, which operates via the non-canonical Wnt/planar cell polarity or Wnt/Ca_2^+ pathways.



(c)

	Angiogenic factors	Statistical significance
1	Amphiregulin	*
2	Angiogenin	*
3	CXCL16	*
4	Endostatin	**
5	FGFa	*
6	FGFb	*
7	IL-10	**
8	KC	**
9	MIP-1 alpha	*
10	Pentraxin-3	***
11	Prolactin	**
12	Serpin-F1	**

Figure 1. Wnt3a induces a pro-angiogenic phenotype in Mφ. (a) Mouse angiogenesis array performed in control Mφ or Mφ^{Wnt3a} (see Material and methods). Numbers represent factors listed on the Table in section c. (b) Graph showing quantification of factors. (c) Table summarizing the changes found in the array analysis. Shadowed factors = anti-angiogenic factors; Light coloured factors = pro-angiogenic factors. $n = 2$ independent arrays were performed, each of one using a four mice-Mφ mix. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *versus* control. Data are expressed as mean ± SEM.

Several evidences point out a role for Wnt signaling in angiogenesis, the formation of new blood vessels from the existing vasculature. Indeed, endothelial cells (ECs) express several Wnt receptors and modulators.[2] On the other hand, it is known that Wnt signaling participates in the induced neuroinflammatory response associated to several neurological disorders including stroke[3] and that specifically Wnt3a induces anti-inflammatory effects in macrophages (Mφ), which in turn acquire regenerative properties.[1]

Mφ constitute a self-renewing tissue-resident population that performs an important immune surveillance function as well as contribute to tissue homeostasis by removing dead cells and toxic materials.[4] A relevant role for Mφ in both pathological and physiological angiogenesis has also been described. Mφ seem to be essential during normal development but they also regulate angiogenesis during tissue injury and repair in the adulthood by secreting trophic factors and contributing to carve out tunnels in the extracellular matrix providing avenues for subsequent capillary infiltration.[5] Finally, Mφ are actively involved in several neurological conditions such as stroke, where they play complex and multifaceted roles.[6] Interestingly, in stroke, overexpression of angiogenic factors is induced after the injury and new capillaries are formed within days after the onset of the ischemia.[7] Thus, understanding the mechanisms regulating angiogenesis might represent an interesting target to look at in post-stroke molecular therapies.

Despite the link Wnt signaling-Mφ-angiogenesis has been suggested in some reports,[5] no connection relative to Wnt3a has been established so far.

Our data demonstrate that Wnt3a and hypoxia act synergistically to promote angiogenesis by regulating essential mechanisms where cell death regulation seems to play a pivotal role.

Material and methods

Reagents

The following reagents were used: Recombinant Wnt3a (R&D Systems, Minneapolis, USA), RNeasy Mini Kit (Qiagen, Hilden, Germany), Proteome Profiler Mouse Angiogenesis Array Kit (ARY015, R&D Systems), Matrigel Matrix Growth Factor Reduced (Corning, NY, USA), LDH cytotoxicity Assay kit (Abcam, Cambridge, UK).

Cell culture and Wnt3a treatment

SVEC4–10 *Mus Musculus* axillary lymph node cells (kindly gift from Prof. Geoffrey L Smith, University of Cambridge, UK) were routinely grown in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/mL penicillin, and100 mg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Mφ were prepared by flushing bone marrow from femurs and tibia of wild-type (WT) C57BL/6 female mice. Cells were cultured in differentiating DMEM medium containing 20% of M-CSF at 37°C in a 5% CO_2 atmosphere for six days. Differentiated Mφ were then treated with 300 ng/ml recombinant Wnt3a using 1% BSA-PBS as vehicle (control) for 6 h. After this time, media were collected for angiogenesis array experiments or replaced by fresh

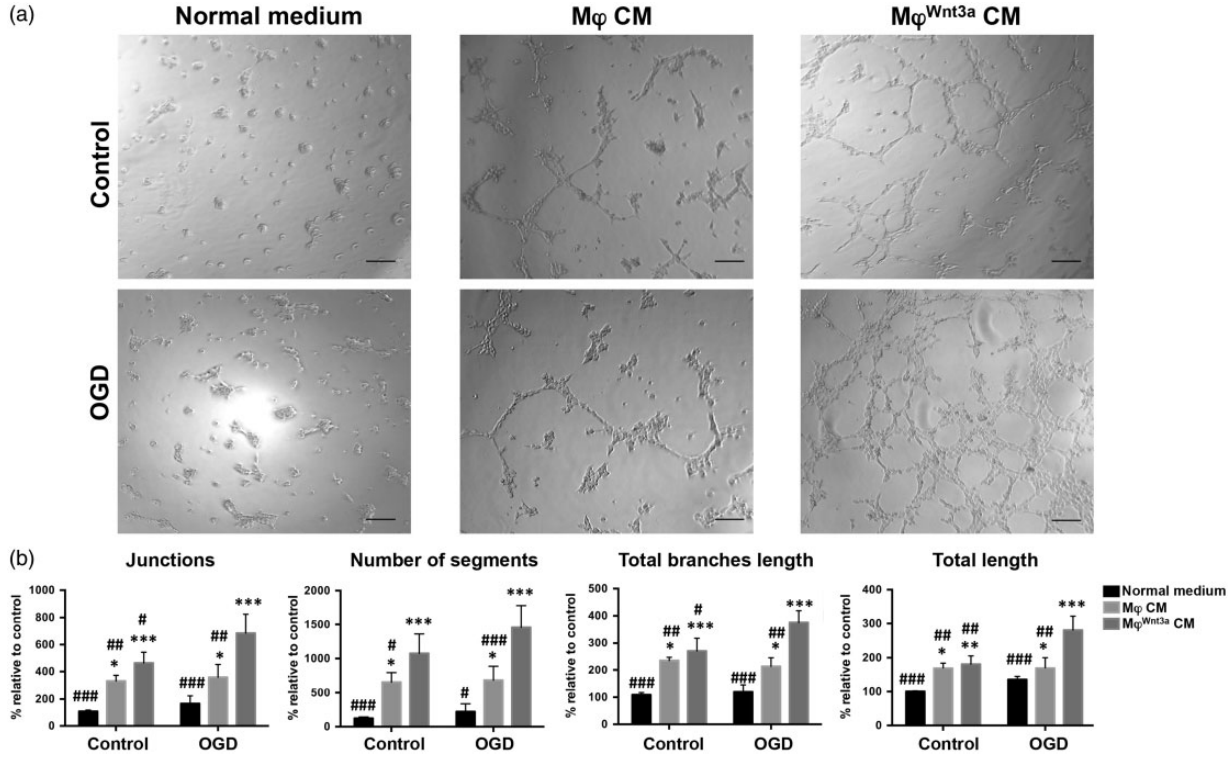


Figure 2. Mφ^{Wnt3a} CM induces *in vitro* angiogenesis in OGD conditions. (a) Tubular formation assay performed in SVEC4–10 cells. (b) Graphs showing parameters obtained with the angiogenesis analyzer plugin (Image J). $n = 5$ independent assays. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* Normal medium. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *versus* OGD Mφ^{Wnt3a} CM. Two-way ANOVA not statistically significant. Scale bar 200 μm. Data are expressed as mean ± SEM.

medium with or without glucose (for oxygen and glucose deprivation (OGD) experiments) for 24 h more to ensure total removal of Wnt3a protein as well as to allow secretion of factors into the medium.

(Additional information is available in Supplementary Material and Methods)

Results

Wnt3a shifts Mφ towards a pro-angiogenic phenotype

Given the ability of Wnt3a to induce the expression of several cytokines,[8] we wonder whether this ligand would be able to provoke any change in the secretion levels of different angiogenic factors in Mφ. Thus, we stimulated these cells with 300 ng/ml of recombinant Wnt3a, a dose able to activate the pathway as we observed in previous experiments (see Figure S1(a)). An angiogenesis proteome profile was then carried out (Figure 1(a) and Figure S1(b)). Wnt3a significantly increased secretion of several factors considered as pro-angiogenic according to the literature.[9] On the other hand, other molecules known to have anti-angiogenic effects were found significantly decreased upon treatment (Figure 1(b) and (c)), suggesting a shift towards an angiogenic phenotype. Additionally, in a reduced number of cases (including fractalkine, interferon gamma-induced protein 10, IP-10 and hepatocyte growth factor, HGF), Wnt3a had an effect opposite of expected (see Figure S1(b)).

Wnt3a treatment and OGD act synergistically to promote angiogenesis *in vitro*

A stroke is a serious life-threatening medical condition characterized by the interruption of blood flow and oxygen and glucose supply, which induce severe tissue damage and where angiogenesis has been proposed as a harmonized target for recovery after the injury.[10] To investigate whether the Wnt3a-induced pro-angiogenic phenotype observed in Mφ might have any effect on angiogenesis under homeostatic conditions and after OGD, which has been widely used as an *in vitro* model for stroke,[11] we performed conditioned medium (CM) experiments in SVEC4–10 ECs in both conditions. To avoid Wnt3a-mediated direct effects, CM was prepared by adding fresh medium to macrophages after Wnt3a treatment 24 h before transferring it to ECs (see Material and methods). As shown in Figure 2 (see also Figure S2), although not statistically significant, medium derived from untreated Mφ was able *per se* to induce an increase in tubular formation in both control and OGD conditions. When we looked at the effects induced by Mφ^{Wnt3a} CM, an increase in different angiogenic parameters was much more evident in OGD samples, pointing to a synergy between both Wnt3a treatment and oxygen and glucose withdrawal.

Mφ^{Wnt3a} CM increases ECs migration regardless the oxygen/glucose levels

Migration of ECs, an essential process involved in angiogenesis[12] was then quantified. After 6 h of treatment with

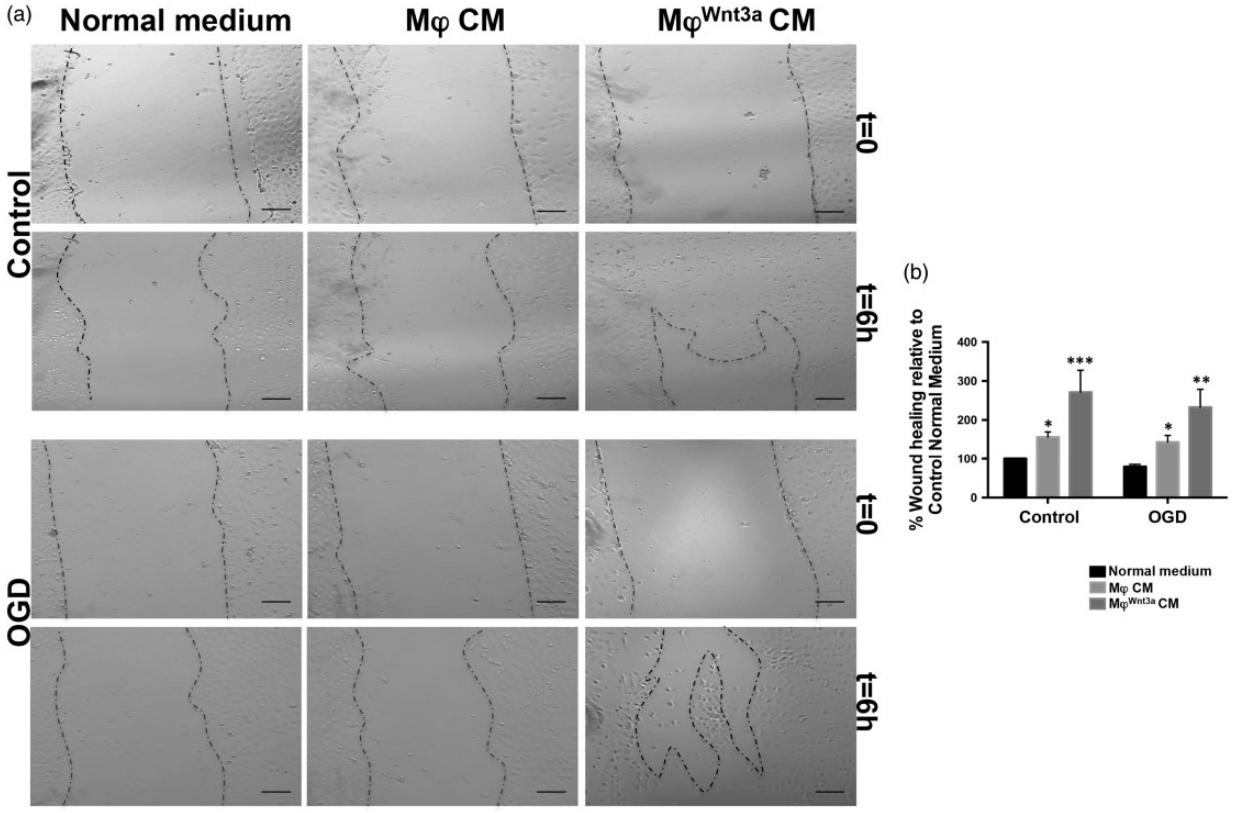


Figure 3. Mφ^{Wnt3a} CM induces cell migration regardless oxygen and glucose levels. (a) Wound healing assay performed in SVEC4–10 cells. (b) Graphs showing percentage of healed area relative to control Normal medium conditions. $n = 5$ independent assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *versus* normal medium. Two-way ANOVA not statistically significant. Scale bar 200 μm. Data are expressed as mean ± SEM.

CM, healing was boosted in those cases in which cells had been treated with Mφ^{Wnt3a} CM regardless oxygen and glucose levels (Figure 3).

Mφ^{Wnt3a} CM differentially affects cell death and proliferation under OGD conditions

Proliferation and cell death are also essential processes underlying angiogenesis.[12] Upon treatment with Mφ^{Wnt3a} CM, increased proliferation rate in ECs in both normal and OGD conditions was observed with minimum effects in Mφ CM samples (Figure 4(a) and (b); Figure S3(a)). Surprisingly, when cell viability was quantified, a general increase in cell death was observed upon treatment with CM, which was remarkably higher in ECs that had been treated with Mφ^{Wnt3a} CM and subjected to OGD (Figure 4 (c); Figure S3(b)). Although as a whole, gross proliferation percentages (not relativized to Normal medium condition) were higher than the ones corresponding to cell death in all analyzed settings (data not shown), when cell proliferation/cell death ratio was calculated, a clear decrease when Wnt3a treatment and OGD conditions converged was observed, highlighting that cell viability was indeed key to understand the angiogenic effects observed under those conditions (Figure S3(c)).

Discussion

Angiogenesis is regulated by a tight balance between pro- and anti-angiogenic agents and involves a cascade of events of which proliferation, apoptosis, and migration of capillary ECs are essential components.[12] Here, we report how Wnt3a, a member of the canonical Wnt signaling pathway, is able to modulate this balance in Mφ, making them acquire a pro-angiogenic phenotype capable of inducing the formation of new EC tubular structures (capillary-like structures) *in vitro*. We have detected that secretion of eighteen different factors is regulated by exposure to recombinant Wnt3a, being over 65% of them (12/18) in agreement with what expected (8/18 up regulated pro-angiogenic factors [44%] and 4/18 down regulated anti-angiogenic factors [22%]). We believe that this balance is enough to produce remarkable angiogenic effects under OGD. To this regard, we observe how CM derived from both control Mφ and Mφ^{Wnt3a} is able to slightly stimulate tubular formation in control conditions, confirming the previously reported participation of Mφ in the process.[5] However, in OGD, only CM derived from Mφ^{Wnt3a} induced a clear and statistically significant positive effect on *in vitro* angiogenesis, pointing to a synergistic effect between the activation of this pathway and the deprivation of these two essential components in ECs. This synergy might be explained if we take into consideration that, on one hand, some of the factors that

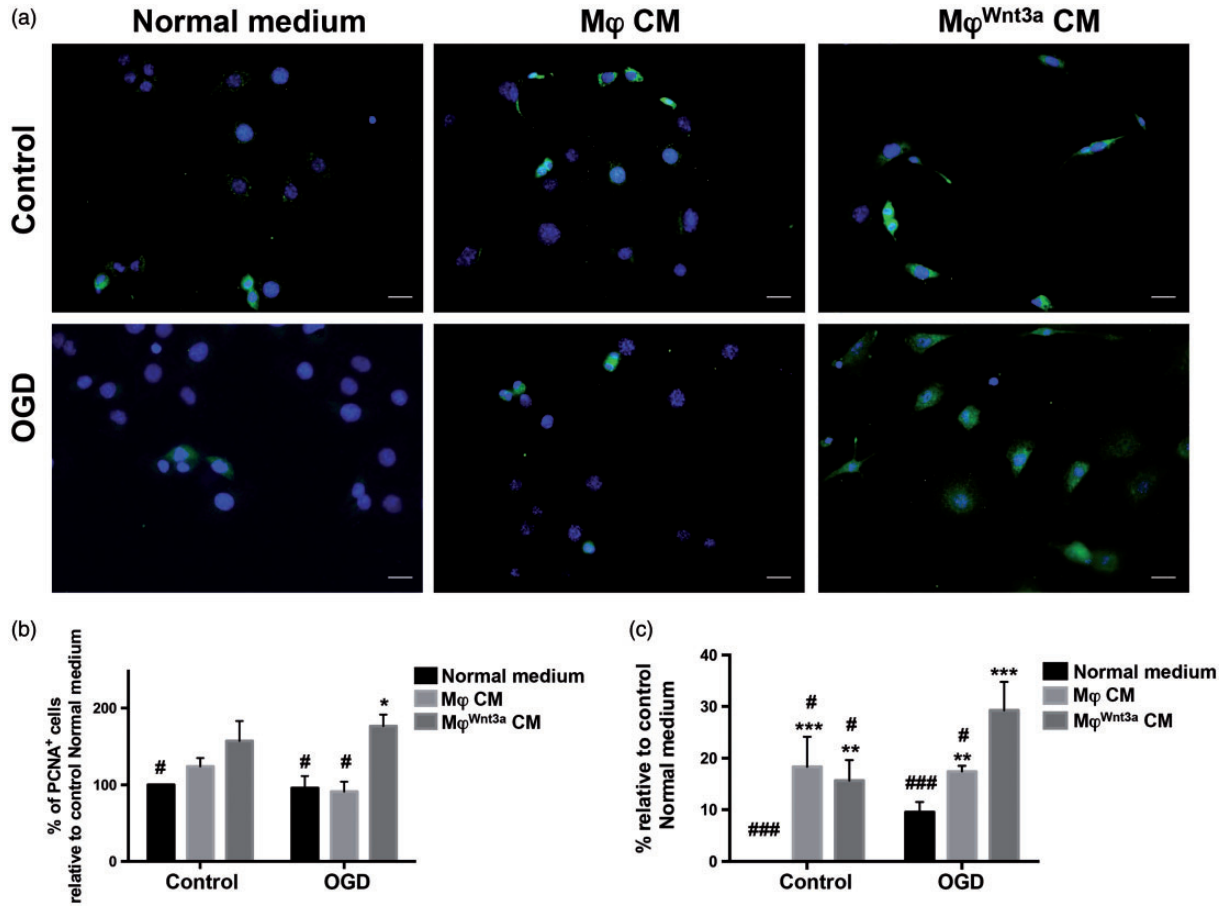


Figure 4. Cell proliferation and viability are differentially affected by Mφ^{Wnt3a} CM and OGD conditions. (a) Fluorescence microscope images showing proliferating PCNA$^+$ cells (green) upon different treatments/conditions. Blue = nuclear staining (DAPI). (b) Quantification of PCNA$^+$ cells. (c) LDH assay results. $n = 5$ independent assays. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* normal medium. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *versus* OGD Mφ^{Wnt3a} CM. Two-way ANOVA not statistically significant. Scale bar 25 μm. Data are expressed as mean ± SEM. (A color version of this figure is available in the online journal.)

are up regulated by Wnt3a treatment (such as Amphiregulin, KC, angiogenin and macrophage inflammatory protein 1-alpha, MIP-1α) are known targets of the hypoxia inducible factor-1 (HIF-1) pathway, the master regulator of oxygen homeostasis.[13–16] On the other hand, other measured factors in our study are known to regulate the expression of HIF-1 transcription factors, such as CXCL16 and basic fibroblast growth factor, FGF (both increased in our model), which up regulates HIF-1α[17,18] or endostatin and prolactin (both decreased in our model), which have been demonstrated to down regulate HIF-1α levels and activity.[19,20] As a result, this would generate a positive loop that would account for the net increase in *in vitro* angiogenesis upon Wnt3a CM treatment and oxygen and glucose withdrawal that we observe. Interestingly, Mφ^{Wnt3a} CM is able to promote EC migration and proliferation in both control and OGD with a similar tendency, but has a differential positive effect on the net cell death rate. Firstly, it is known that hypoxia and glucose deficiency *per se* induce apoptosis.[21] Secondly, some factors released by Mφ upon Wnt3a treatment might have a critical role in the regulation of this process. Thus, some of the mentioned pro-angiogenic factors such as IL-10, amphiregulin, CXCL16, and basic FGF, despite having several anti-apoptotic effects, have been also reported to produce opposite outcomes.[22–25] Interestingly, some factors like fractalkine, IP-10, or HGF, whose regulation in our experimental conditions would not point towards a pro-angiogenic phenotype, might have relevant roles in ECs apoptosis regulation though. The decrease in fractalkine and HGF, with known anti-apoptotic effects[26,27] along with the increase in IP-10, involved in apoptosis induction[28] could create a favorable environment for ECs cell death. Although it seems counterintuitive that EC apoptosis may contribute to vascular morphogenesis, this process might have an important role in deleting wrongly placed EC as nascent vessels enlarge. Indeed, several vascular phenotypes have been described in mice in which regulators of EC apoptosis are disrupted,[29] supporting the idea of this process as a necessary event for proper angiogenesis.

Ischemic stroke is currently the third most frequent cause of mortality in industrialized countries.[30] Consequently, it is presently of outmost importance to shed additional light and provide fundamental information on understanding the basic mechanisms underlying the pathology, which could represent possible steps towards prevention and therapeutic intervention. In this sense,

angiogenesis might be a relevant target process whose up regulation would have several beneficial effects in the hours to weeks after the ischemic injury, such as the promotion of cell survival by growth factors release, the enhancement of neurogenesis by creation of a "vascular niche" or the facilitation of tissue regeneration by allowing Mφ to have access to necrotic brain tissue contributing to its removal.[10] Indeed, a powerful relation between Mφ and microvessel density after cerebral ischemia has been described,[31] thus highlighting the role of mononuclear phagocytes in the post-ischemia angiogenic process.

Although more studies are required to understand the mechanisms triggered by Wnt3a under OGD conditions, our data support the idea of this pathway as an interesting target mediating beneficial outcomes during the inflammatory response in hypoxia; a process that might be worthy to further explore for the development of possible stroke therapies.

Authors' contributions: AFM and SP contributed to the conception and design of the study. AFM and GM contributed to the acquisition, analysis and interpretation of data. All authors participated in drafting and revising the manuscript and they all approved the final version of the manuscript for its submission.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

This work was funded by the Italian Multiple Sclerosis Association (AISM, grant 2010/R/31 and grant 2014/PMS/4 to SP), the European Research Council (ERC) under the ERC-2010-StG grant agreement n° 260511-SEM_SEM, the Medical Research Council, the Engineering and Physical Sciences Research Council, and the Biotechnology and Biological Sciences Research Council UK Regenerative Medicine Platform Hub "Acellular Approaches for Therapeutic Delivery" (MR/K026682/1 to SP), the Evelyn Trust (RG 69865 to SP), the Bascule Charitable Trust (RG 75149 to SP) and a core support grant from the Wellcome Trust and Medical Research Council to the Wellcome Trust – MRC Cambridge Stem Cell Institute. AFM was supported by an Intraeuropean Marie Curie Fellowship (IEF) and GM by the Erasmus Fellowship program.

REFERENCES

1. Marchetti B, Pluchino S. Wnt your brain be inflamed? Yes, it Wnt! *Trends Mol Med* 2013;**19**:144–56.
2. Goodwin AM, Sullivan KM, D'amore PA. Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. *Dev Dyn* 2006;**235**:3110–20.
3. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell* 2012;**149**:1192–205.
4. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 2011;**11**:723–37.
5. Newman AC, Hughes CC. Macrophages and angiogenesis: a role for Wnt signaling. *Vasc Cell* 2012;**4**:13.
6. Chen Y, Hallenbeck JM, Ruetzler C, Bol D, Thomas K, Berman NE, Vogel SN. Overexpression of monocyte chemoattractant protein 1 in the brain exacerbates ischemic brain injury and is associated with recruitment of inflammatory cells. *J Cereb Blood Flow Metab* 2003;**23**:748–55.
7. Greenberg DA. Cerebral angiogenesis: a realistic therapy for ischemic disease? *Methods Mol Biol* 2014;**1135**:21–4.
8. Yu CH, Nguyen TT, Irvine KM, Sweet MJ, Frazer IH, Blumenthal A. Recombinant Wnt3a and Wnt5a elicit macrophage cytokine production and tolerization to microbial stimulation via Toll-like receptor 4. *Eur J Immunol* 2014;**44**:1480–90.
9. Yang K-JYaX. (2016) Angiogenesis: a realistic therapy for ischemic stroke. In: Chen J, Zhang JH and Hu X (eds) *Non-neuronal mechanisms of brain damage and repair after stroke.* Berlin: Springer, pp. 93–110
10. Ergul A, Alhusban A, Fagan SC. Angiogenesis: a harmonized target for recovery after stroke. *Stroke* 2012;**43**:2270–4.
11. Tasca CI, Dal-Cim T, Cimarosti H. In vitro oxygen-glucose deprivation to study ischemic cell death. *Methods Mol Biol* 2015;**1254**:197–210.
12. Dimmeler S, Zeiher AM. Endothelial cell apoptosis in angiogenesis and vessel regression. *Circ Res* 2000;**87**:434–9
13. O'Reilly SM, Leonard MO, Kieran N, Comerford KM, Cummins E, Pouliot M, Lee SB, Taylor CT. Hypoxia induces epithelial amphiregulin gene expression in a CREB-dependent manner. *Am J Physiol Cell Physiol* 2006;**290**:C592–600.
14. Fang HY, Hughes R, Murdoch C, Coffelt SB, Biswas SK, Harris AL, Johnson RS, Imityaz HZ, Simon MC, Fredlund E, Greten FR, Rius J, Lewis CE. Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood* 2009;**114**:844–59
15. Lai K, Luo C, Zhang X, Ye P, Zhang Y, He J, Yao K. Regulation of angiogenin expression and epithelial-mesenchymal transition by HIF-1alpha signaling in hypoxic retinal pigment epithelial cells. *Biochim Biophys Acta* 2016;**1862**:1594–607.
16. Storti P, Bolzoni M, Donofrio G, Airoldi I, Guasco D, Toscani D, Martella E, Lazzaretti M, Mancini C, Agnelli L, Patrene K, Maiga S, Franceschi V, Colla S, Anderson J, Neri A, Amiot M, Aversa F, Roodman GD, Giuliani N. Hypoxia-inducible factor (HIF)-1alpha suppression in myeloma cells blocks tumoral growth in vivo inhibiting angiogenesis and bone destruction. *Leukemia* 2013;**27**:1697–706.
17. Yu X, Zhao R, Lin S, Bai X, Zhang L, Yuan S, Sun L. CXCL16 induces angiogenesis in autocrine signaling pathway involving hypoxia-inducible factor 1alpha in human umbilical vein endothelial cells. *Oncol Rep* 2016;**35**:1557–65.
18. Calvani M, Rapisarda A, Uranchimeg B, Shoemaker RH, Melillo G. Hypoxic induction of an HIF-1alpha-dependent bFGF autocrine loop drives angiogenesis in human endothelial cells. *Blood* 2006;**107**:2705–12.
19. Guo L, Chen Y, He T, Qi F, Liu G, Fu Y, Rao C, Wang J, Luo Y. Nuclear-translocated endostatin downregulates hypoxia inducible factor-1alpha activation through interfering with Zn(II) homeostasis. *Mol Med Rep* 2015;**11**:3473–80.
20. Surazynski A, Miltyk W, Wolczynski S, Palka J. The effect of prolactin and estrogen cross-talk on prolidase- dependent signaling in MCF-7 cells. *Neoplasma* 2013;**60**:355–63.
21. Matsushita H, Morishita R, Nata T, Aoki M, Nakagami H, Taniyama Y, Yamamoto K, Higaki J, Yasufumi K, Ogihara T. Hypoxia-induced endothelial apoptosis through nuclear factor-kappaB (NF-kappaB)-mediated bcl-2 suppression: in vivo evidence of the importance of NF-kappaB in endothelial cell regulation. *Circ Res* 2000;**86**:974–81
22. Gimeno MJ, Pascual G, Garcia-Honduvilla N, Prieto A, Alvarez de Mon M, Bellon JM, Bujan J. Modulatory role of IL10 in endothelial cell damage and platelet adhesion. *Histol Histopathol* 2003;**18**:695–702
23. Taira N, Yamaguchi T, Kimura J, Lu ZG, Fukuda S, Higashiyama S, Ono M, Yoshida K. Induction of amphiregulin by p53 promotes apoptosis via control of microRNA biogenesis in response to DNA damage. *Proc Natl Acad Sci U S A* 2014;**111**:717–22.
24. Kee JY, Ito A, Hojo S, Hashimoto I, Igarashi Y, Tsuneyama K, Tsukada K, Irimura T, Shibahara N, Takasaki I, Inujima A, Nakayama T, Yoshie

O, Sakurai H, Saiki I, Koizumi K. CXCL16 suppresses liver metastasis of colorectal cancer by promoting TNF-alpha-induced apoptosis by tumor-associated macrophages. *BMC Cancer* 2014;**14**:949.
25. Maloof P, Wang Q, Wang H, Stein D, Denny TN, Yahalom J, Fenig E, Wieder R. Overexpression of basic fibroblast growth factor (FGF-2) downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells. *Breast Cancer Res Treat* 1999;**56**:153–67
26. White GE, Tan TC, John AE, Whatling C, McPheat WL, Greaves DR. Fractalkine has anti-apoptotic and proliferative effects on human vascular smooth muscle cells via epidermal growth factor receptor signalling. *Cardiovasc Res* 2010;**85**:825–35.
27. Kakazu A, Chandrasekher G, Bazan HE. HGF protects corneal epithelial cells from apoptosis by the PI-3K/Akt-1/Bad- but not the ERK1/2-mediated signaling pathway. *Invest Ophthalmol Vis Sci* 2004;**45**:3485–92.
28. Liu M, Guo S, Stiles JK. The emerging role of CXCL10 in cancer (Review). *Oncol Lett* 2011;**2**:583–9.
29. Affara M, Dunmore B, Savoie C, Imoto S, Tamada Y, Araki H, Charnock-Jones DS, Miyano S, Print C. Understanding endothelial cell apoptosis: what can the transcriptome, glycome and proteome reveal? *Philos Trans R Soc Lond* 2007;**362**:1469–87.
30. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K, Haase N, Hailpern S, Ho M, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott M, Meigs J, Mozaffarian D, Nichol G, O'donnell C, Roger V, Rosamond W, Sacco R, Sorlie P, Stafford R, Steinberger J, Thom T, Wasserthiel-Smoller S, Wong N, Wylie-Rosett J, Hong Y. Heart disease and stroke statistics – 2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2009;**119**:e21–181.
31. Manoonkitiwongsa PS, Jackson-Friedman C, McMillan PJ, Schultz RL, Lyden PD. Angiogenesis after stroke is correlated with increased numbers of macrophages: the clean-up hypothesis. *J Cereb Blood Flow Metab* 2001;**21**:1223–31.

(Received June 6, 2017, Accepted November 6, 2017)