·Basic Research·

Role of unc5b in retinal neovascularization in mice with oxygen-induced retinopathy

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Abstract

• AIM: To explore the role of unc5b in retinal neovascularization in murine oxygen-induced retinopathy (OIR).

• METHODS: On postnatal 7 (P7), C57BL/6J mice were exposed to 75%± 2% oxygen for 5 days. On postnatal 12 (P12), the mice were brought back to the room air (21% oxygen) to induce retinal neovascularization. Western blot analysis was performed to examine the temporal expression of unc5b in murine retinas. Double staining for unc5b and isolectin B4 were employed to determine the location of unc5b in murine retinas. The effect of unc5b on retinal neovascularization was evaluated by intravitreal injection of unc5b-FC in mice with OIR. Retinal neovascularization was measured by counting neovascular cell nuclei above the internal limiting membrane and by angiography of flat-mounted retinas perfused with fluorescein dextran.

• RESULTS: Compared to age-matched normal mice, the expression of unc5b was significantly increased in retinas of OIR mice on P17 and P21. Unc5b was apparently expressed in retinal vessels of OIR while being negative in normal retinal vessels. Retinal neovascularization in eyes injected with unc5b-FC was significantly reduced.

• CONCLUSION: Unc5b-FC can effectively inhibit retinal neovascularization induced by OIR. It may serve as a powerful and novel therapy for ischemia-induced retinal disease.

• KEYWORDS: oxygen-induced retinopathy;unc5b;unc5b-FC

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INTRODUCTION

Retinal neovascularization, the abnormal proliferation and migration of new blood vessels from pre-existing vessels in the retinas, occurs in several disease processes such as proliferative diabetic retinopathy, retinopathy of prematurity, and secondary neovascular glaucoma. It is a major cause of blindness. As a conventional therapy, laser photocoagulation reduces reactive neovascularization by destroying part of the peripheral retina to diminish hypoxic tissues. This therapy is only partially effective, and also destructive to the retina, leading to immediate and sometimes significant loss of vision. To find an effective phar- macological treatment to inhibit retinal neovascularization, studies have been conducted to understand the molecular pathogenesis of retinal neovascularization. Several angiogenic factors, such as vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and erythropoietin, have been identified to participate in this process [1]. However, attenuation of any of these angiogenic factors can not completely inhibit retinal neovascularization. It is possible that other factors participate in the angiogenic process.

Unc5b is a receptor of netrin-1, which is one of the major neuronal cues [2]. As a neuronal cue, netrin-1 is bifunctional. It can attract or repel axons [3] when binding to different receptors. Unc5b was identified as a mediator of the repulsive response. Recent studies have proved that netrin-1 was involved in angiogenesis. Unc5b was regarded as the only known netrin-1 receptor with prominent endothelial expression [4]. Unc5b is expressed in developmental angiogenesis and sprouting angiogenesis induced by OIR or matrigel or tumor implantation endothelium. Conflicting results have been reported about the role of unc5b in angiogenesis. Unc5b has been suggested as a pro- or

anti-angioenic receptor. Lu *et al* [5] reported that unc5B functions as a repulsive netrin receptor in endothelial cells controlling morphogenesis of the vascular system, and that activation of unc5b can inhibit developmental and pathological angiogenesis. In contrast, Navankasattusas *et al*[6] and Epting et al's studies [7] suggested that unc5b could function as a pro-angiogenic factor. Umbilical arteries isolated from unc5b-deficient embryos were unable to support vessel outgrowth *in vitro* [6]. Specific deletion of unc5b in the embryonic endothelium of mice led to remarkble reduction of labyrinthine arterioles in the placenta, and knockdown of unc5b in zebrafish disrupted the formation of intersomitic vessels (ISVs), the dorsal longitudinal anastomotic vessels (DLAV), and the parachordal vessels (PAV)[7]. It was reported that the unc5b was only expressed in vessels undergoing active angiogenic sprouting in the retina [8]. However, no study has explored whether unc5b participates in murine retinal neovascularization, and how it affects retinal neovascularization in OIR mice. In the present research, we have proved that unc5b-FC can effectively inhibit neovascularization induced by oxygen-induced retinopathy (OIR).

MATERIALS AND METHODS

Animal Model of Oxygen –induced Retinopathy C57BL/6J mice(bought from Shanghai Experimental Animal Center of Chinese Academy of Sciences) used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Together with the nursing mothers, seven-day-old C57BL/6J (postnatal 7th day) mice were exposed to 75%±2% oxygen for 5 days. On the 12th day after birth, the mice were moved back to room air (21% oxygen). The mice were exposed to 12-hour cyclical broad spectrum light. The room temperature was maintained at 23-28℃.

Intravitreal Injection On postnatal 12th day, the mice were anesthetized with intraperitoneal injection of pentobarbital sodium. The tip of a 10mm 34-gauge steel needle, mounted on a 5mL Hamilton syringe was entered the eye from the posterior limbus to the vitreous cavity. In six OIR mice, one eye was injected with 1uL of unc5b-FC (1g/L, R&D system, USA), and the other eye was intravitreally injected with phosphate buffer saline (PBS) as a negative control.

Angiography with High–molecular–weight Fluorescein–dextran On postnatal 17th day, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium and perfused through the left ventricle with PBS containing 1mL of 50g/L fluorescein-labeled high-molecular-weight (2,000,000) dextran (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the murine eyes were enucleated and fixed in 40g/L paraformaldehyde for 10 minutes. The retinas were dissected and placed in 40g/L paraformaldehyde for 10 minutes and then flat-mounted with glycerol/PBS (50/50).

Histological Analysis of Neovascularization On postnatal 17th day, the mice were sacrificed with intraperitoneal injection of an overdose of sodium pentobarbital. Their eyes were enucleated, fixed with 40g/L paraformaldehyde in PBS overnight at 4℃ and embedded in paraffin. Serial cross-sections (5mm) of the whole eye were stained with hematoxylin and eosin to visualize cell nuclei. Ten nonserial sections, except sections containing the optic nerve, were analyzed per eye, and the nuclei of new vessels extending from the retina into the vitreous were counted in 60 sections in each group.

Immunohistochemical Localization of Unc5b On postnatal 17th day, the mice were sacrificed and their eyes were enucleated, fixed with 40g/L paraformaldehyde in PBS overnight at 4℃ and then embedded in OCT. Sagittal frozen sections of 12mm thickness were cut and mounted on glass slides. These flat mounts were incubated with 5% bovine serum albumin (BSA) for 2 hours at room temperature to reduced non-specific background staining. Double staining for unc5b and isolectin B4 were employed to determine the location of unc5b in murine retinas. The flat mounts were incubated with primary antibodies diluted in PBS containing 3% BSA overnight at 4℃. The primary antibodies used include goat polyclonal unc5b (1:50, R&D System, USA) and isolectin B4 (1:200 Vector Lab, USA). The sections were rinsed in PBS three times, and then incubated with CY3 (1:200, Invitrogen) or Alexa 488 conjugated antibody (1:400, Invitrogen, USA) for 2 hours at room temperature. In control sections, the primary antibodies were replaced with BSA/PBS mixture. The slides were examined under a confocal microscope.

Western Blot Analysis of Unc5b Protein Retinas were dissected and lysed in RIPA (150mmol/L NaCl, 50mmol/L Tris-HCl, pH 7.4, 2mmol/L EDTA, 1% NP-40). The protein concentration was measured with a BCA Protein Assay Kit (Pierce, USA). Equal amount of cell lysates were loaded onto 10% SDS-polyacrylamide gels. Proteins were blotted onto a polyvinylidene difluoride microporous membrane (Millipore, USA). The membranes were blocked with 5% nonfat milk at 4℃ overnight, and then incubated with goat polyclonal anti-unc5b (1:50, R&D system, USA) or rabbit polyclonal anti-GAPDH antibody (1:3000, Santa Cruz Biotech, USA) for 2 hours at room temperature, followed by incubation with a horseradish peroxidase-conjuncted secondary antibody (1:10000, sigma,USA) for 1 hour at

room temperature. Protein blots were detected using an enhanced chemiluminescence (ECL) kit (Pierce, USA). The experiments were repeated three times. The blot densities were quantified with a gel optical density analysis software. The GAPDH blots were used for standardization. Results were expressed as the relative density of unc5b blot/GAPDH blot.

Statistical Analysis Data were analyzed by ANOVA followed by the LSD test. $P<0.05$ was considered as statistically significant.

RESULTS

Elevated Expression of Unc5b in Retinas of OIR Mice Western blot analysis demonstrated that the unc5b protein level was increased significantly in retinas of OIR mice compared with normal retinas during the active stages of angiogenesis (P17 and P21), while no significant difference was observed during the pre-angiogenic phase (P12) (Figure 1). Immunostaining showed that unc5b was expressed in the ganglion cell layer, the inner plexiform layer, and the outer plexiform layer, and that the expression of the unc5b was elevated in OIR mice compared with normal mice (Figure 2A, E). There were much more vessels formed in OIR mice compared with the normal mice (Figure 2B, F). Double staining with unc5b and isolectin B4 (the endothelial cell marker) showed that unc5b was apparently expressed in the retinal vessels of OIR mice, while little was expressed in normal mice (Figure 2D, H). These results indicated a critical role of unc5b in promoting retinal neovascularization.

Unc5b –FC Suppressed Retinal Neovascularization in OIR Mice To determine whether unc5b plays a role in retinal neovascularization, we injected unc5b-FC (a fusion of the constant region of human IgG with the ectodomain of unc5b, which is known to bind netrin-1) into the vitreous of the mice. No mice used in this study developed signs of infection and retinal detachment. The patterns of vascular development and neovascularization were evaluated in retinal flat-mounts after fluorescein-dextran perfusion on P17. Retinas from normal mice had both superficial and deep vascular layers that extended from the optic nerve to the periphery. Vessels in the superficial retinal layer formed a fine radial branching pattern. In the deep retinal layer, the vessles formed a polygonal reticular pattern (Figure 3A). Retinas from mice exposed to hyperoxia were characterized by central non-perfused areas and neovascular tufts (Figure 3B). Neovascular tufts were reduced in mice injected with unc5b-FC (Fig. 3D), while no significant reduction of central non-perfused and neovascular tufts were observed in mice injected with PBS (Figure 3C).

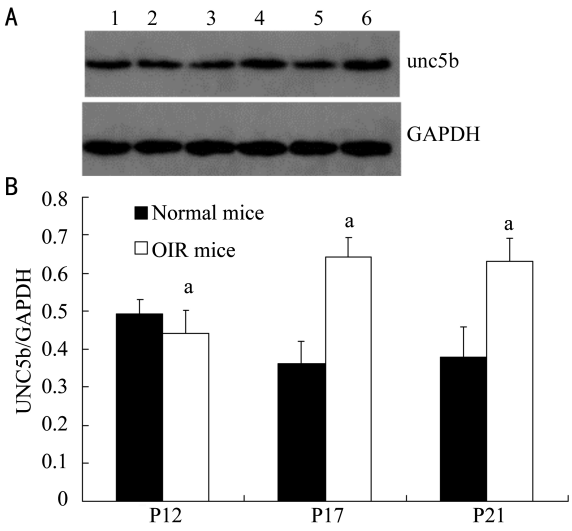

Figure 1 The Unc5b protein level increased significantly in retinas of OIR mice compared with normal retinas on P17 and P21, while no significant difference was observed on P12 A:Representative Western blots of unc5b protein in control and OIR retinas. lane 1: normal, P12; lane 2: OIR, P12; lane 3: normal, P17; lane 4: OIR, P17; lane 5: normal, P21; lane 6: OIR, P21. B: The relative density of unc5b blot/GAPDH blot. On P12, there was no significant difference in unc5b expression in retinas between OIR mice and normal mice ($P>0.05$). However, on P17 and P21, the expression of unc5b in retinas of OIR mice increased significantly compared with normal mice (P17 normal *vs* P17 OIR, $P<0.05$; P21 normal *vs* P21 OIR, $P<0.05$)

To further confirm the inhibitory effect of unc5b-FC on retinal neovascularization, vascular cell nuclei extending beyond the internal limiting membrane were counted. The eyes of control normoxic mice did not contain nuclei anterior to the internal limiting membrane ($n=12$) (Figure 4A), while the average number of neovascular nuclei were 47.8±2.6 per cross-section in non-injected eyes exposed to hyperoxia ($n=10$) (Figure 4B), 46.9±3.2 per cross-section in eyes injected with PBS ($n=10$) (Figure 4C), and 23.4±1.9 per cross-section in eyes injected with unc5b-FC ($n=10$) (Figure 4D). On average, 50% of retinal neovascularization was inhibited by unc5b-FC. The results indicate that local injection of unc5b-FC can significantly decrease retinal neovascularization ($P<0.05$).

DISCUSSION

In addition to a role in axon guidance, unc5b has been suggested to be involved in angiogenesis. In the present study, double staining for unc5b and isolectin B4 showed that unc5b was apparently expressed in retinal vessels of OIR mice, but not in normal retinal vessels. This is probably because that unc5b was only expressed in vessels undergoing active angiogenic sprouting during pathological angiogenesis [8]. Western blot analysis showed that the

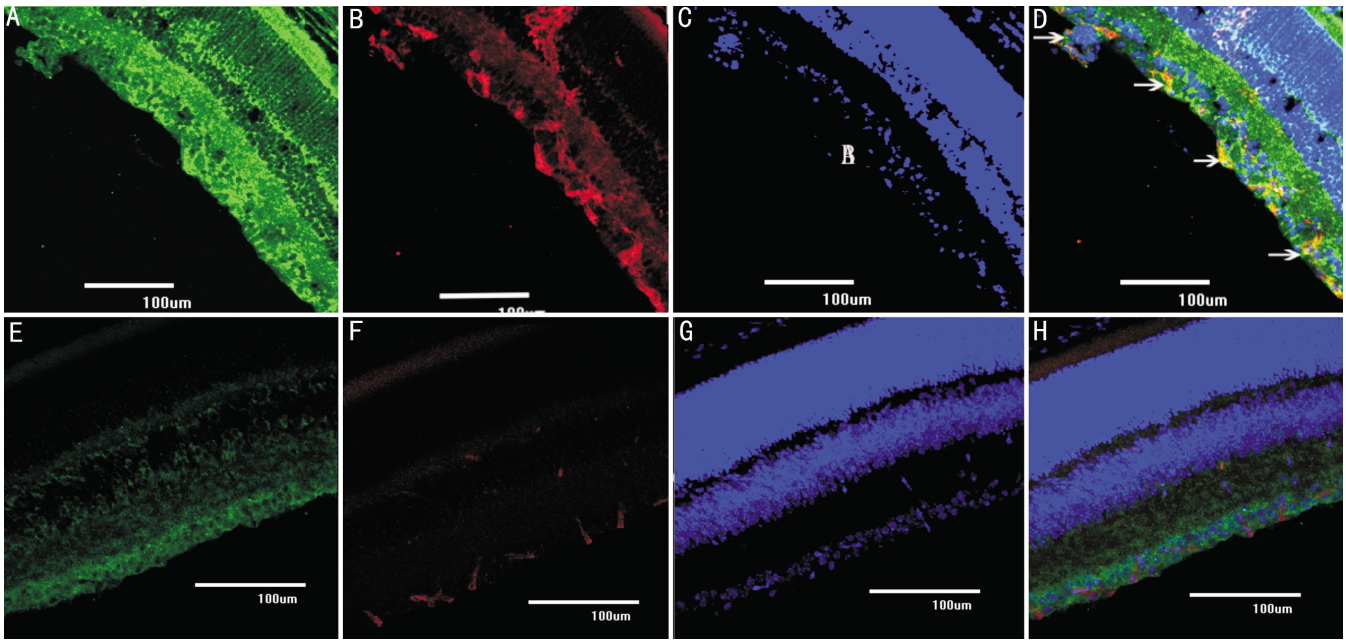

Figure 2 Double immunostaining for unc5b (green) and isolectin B4 (red) on P17 The nuclei were stained blue (DAPI). A-D: OIR mice; E-H: Normal mice. Unc5b was expressed in the ganglion cell layer, the inner plexiform layer, the outer plexiform layer, and retinal vessels of OIR mice. The expression of unc5b was elevated in OIR mice compared with normal mice (Figure 2A, E). There were much more vessels in OIR mice compared with the normal mice (Figure 2B, F). Double staining for unc5b and isolectin B4 (the enothelial cell marker) showed that unc5b was strongly expressed in the retinal vessels of OIR mice, while little was observed in normal mice (Figure 2D, H)

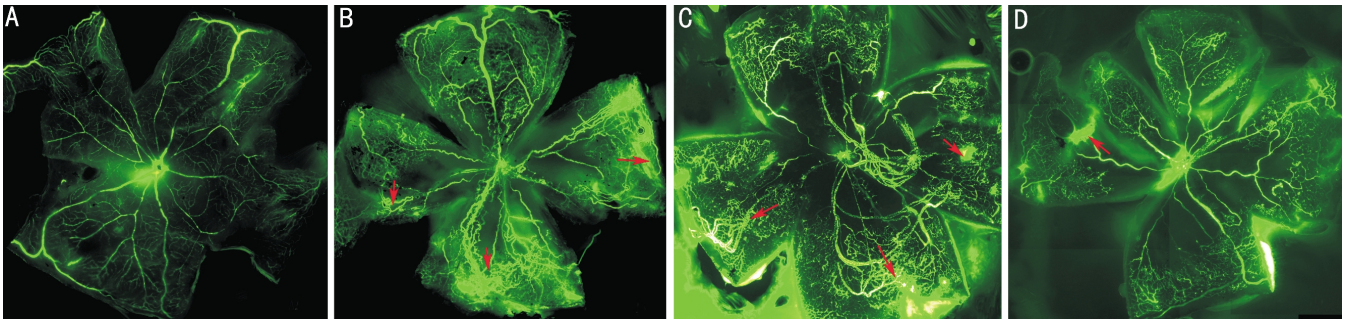

Figure 3 Angiographic assessment of the effects of unc5b–FC on retinal neovascularization. Retinal flat mounts were examined with fluorescein–dextran angiography. The neovascular tufts were indicated by arrows A: Normal age-matched control. The vessels formed a fine radial branching pattern; B: Murine OIR model. Neovascular tufts and central non-perfused area was observed in rentinal flat mounts; C: Mice injected with PBS. Retinal neovascularization and non-perfusion area were not attenuated; D: Mice injected with unc5b-FC. The amount of neovascular tufts was markedly reduced, while the area of non-perfusion was not reduced

expression of unc5b was significantly elevated on P17 and P21 in OIR mice compared to normal age-matched mice. As the expression of unc5b was significantly elevated during the active angiogenic period in OIR mice and localized only in the sprouting vessels, unc5b may play an important role in hypoxia-driven neovascularization.

We injected recombinant unc5b-FC into the vitreous bodies of OIR mice. Unc5b-FC is a fusion of the constant region of human IgG with the ectodomain of unc5b, which is kown to bind netrin-1. Compared with non-injected and PBS-injected eyes, the eyes injected with unc5b-FC showed a marked decrease in neovascularization. This result indicates that unc5b-FC can effectively inhibit the neovascularization induced by OIR, and that unc5b can promote angiogenesis in murine OIR. Although conflicting results have been reported regarding the role of unc5b during angiogenesis, Castets[9,10] has recently reconciled the controversy by showing that binding of netrin-1 to unc5b enhances endothelial cells survival and thus promotes angiogenesis, while unc5b alone enhances apoptosis and inhibits angiogenesis. Our study found that the expression of netrin-1 was elevated significantly in the retinas of OIR mice on P17 and P21, which may be the reason why unc5b acted as a pro-angiogenic factor. The recent study [7] showed that netrin-1 could regulate ELMO1/DOCK180 interaction via Unc5b. We also found that netirn-1 could promote retinal

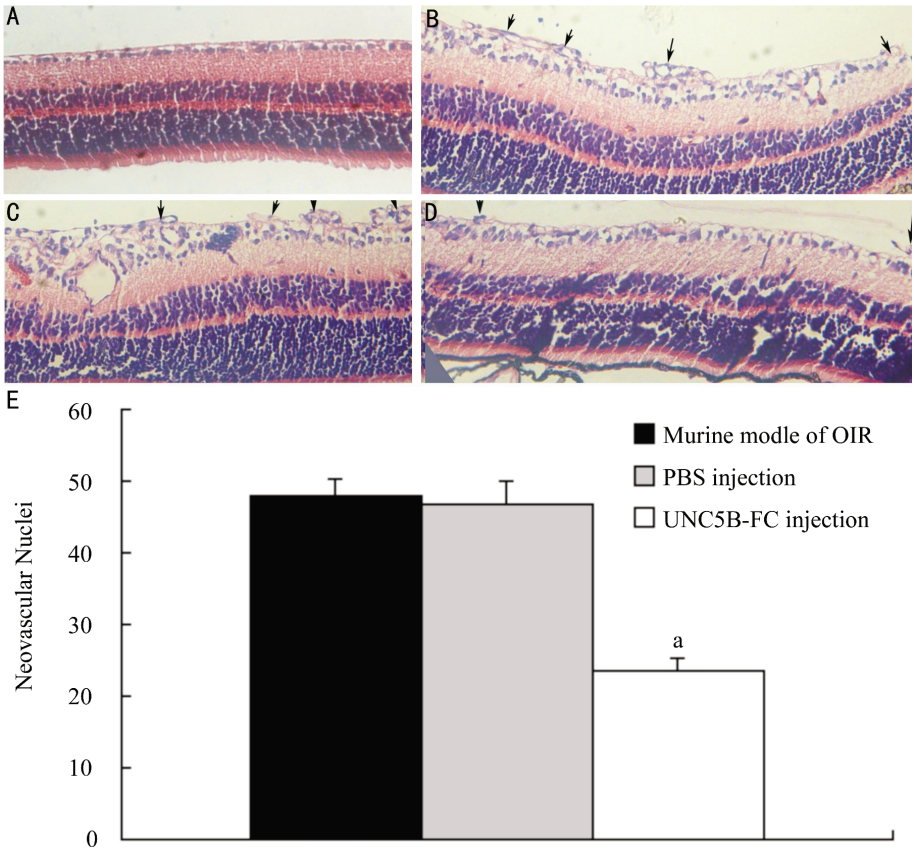

Figure 4 Quantitative analysis of preretinal nuclei in H&E-stained tissue sections The neovascular cell nuclei were indicated by arrows A: Normal mice; B: Murine OIR model; C: Murine OIR model injected with PBS; D: Murine OIR model injected with unc5b-FC;E: Quantification of retinal neovasculari- zation. The mean number of neovascular cell nuclei in eyes injected with unc5b-FC was significantly reduced compared to non-injected and PBS-injected eyes (P<0.05). Differences between non-injected eyes and eyes injected with PBS were not statistically significant (P>0.05)

neovascularization in OIR mice (date not published). As our results demonstrate that unc5b promotes retinal neovascularization, we propose that netrin-1 can promote retinal neovascularization via unc5b.

Wilson *et al* [11] has reported that netrin-1 has pro-angiogenic effects similar to those of VEGF and in our previous studies [12,13] we have proved that VEGF siRNA was effective in inhibiting retinal neovascularization in OIR mice, and about 50%-60% vascular cell nuclei extending beyond the internal limiting membrane were reduced. In our study, we showed 50% of retinal neovascularization was inhibited by unc5b-FC, because netrin-1 was supposed to promote retinal neovascularization in OIR mice via unc5b, so we propose that netrin-1, via its receptor unc5b, has pro-angiogenic effects similar to those of VEGF in OIR mice , this results was coincident with the study of Wilson.

In murine OIR, hypoxia not only leads to retinal vasculopathy, but also neuropathy. Netrin-1 is considered as a inhibitor for axon regeneration in the injured adult spinal cord, and this process is mediated by the receptor unc5b [14,15,16]. In retina, unc5b may regulate the regenerative capacity of adult retinal ganglion cells (RGCs)[17]. Therefore, the increase of unc5b expression in RGCs and the plexiform layers may contribute to regeneration of neurons. Since netrin-1 inhibits the growth of axons when binding to unc5b, inhibition of unc5b by unc5b-FC in the retinas OIR mice may promote the growth of axons. Further studies are needed to prove this hypothesis.

In conclusion, Unc5b-FC can inhibit neovascularization in OIR. It may be a novel therapy for both vasculopathy and neuropathy of OIR. Unc5b is pro-angiogenic in retinal neovascularization and it may be the receptor that mediates the effect of netrin-1 on retinal neovascularization.

REFERENCES

1 Pepper MS, Mandriota SJ, Vassalli JD, Orci L, Montesano R. Angiogenesis regulating cytokines: activities and interactions. *Curr Top Microbiol Immunol* 1996;213:31-67

2 Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-Lavigne M. Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. *Nature* 1997;386:833-838

3 Dickson BJ. Molecular mechanisms of axon guidance. *Science* 2002;298: 1959-1964

4 Eichmann A, Makinen T, Alitalo K. Neural guidance molecules regulate vascular remodeling and vessel navigation. *Genes Dev* 2005;19:1013-1021

5 Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Br é ant C, Claes F, De Smet F, Thomas JL, Autiero M, Carmeliet P, Tessier-Lavigne M, Eichmann A. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* 2004;432:179-186

6 Navankasattusas S, Whitehead KJ, Suli A, Sorensen LK, Lim AH, Zhao J, Park KW, Wythe JD, Thomas KR, Chien CB, Li DY. The netrin receptor UNC5B promotes angiogenesis in specific vascular beds. *Development* 2008;135: 659-667

7 Epting D, Wendik B, Bennewitz K, Dietz CT, Driever W, Kroll J. The Rac1 regulator ELMO1 controls vascular morphogenesis in zebrafish. *Circ Res* 2010; 107(1):45-55

8 Larriv é e B, Freitas C, Trombe M, Lv X, Delafarge B, Yuan L, Bouvr é e K, Br é ant C, Del Toro R, Br é chot N, Germain S, Bono F, Dol F, Claes F, Fischer C, Autiero M, Thomas JL, Carmeliet P, Tessier-Lavigne M, Eichmann A. Activation of the UNC5B receptor by netrin-1 inhibits sprouting angiogenesis. *Genes Dev* 2007;21(19):2433-2447

9 Castets M, Coissieux MM, Delloye-Bourgeois C, Bernard L, Delcros JG, Bernet A, Laudet V, Mehlen P. Inhibition of endothelial cell apoptosis by netrin-1 during angiogenesis. *Dev Cell* 2009;16(4):614-620

10 Castets M, Mehlen P. Netrin-1 role in angiogenesis: To be or not to be a pro-angiogenic factor? *Cell Cycle* 2010;9(8):Epub ahead of print

11 Wilson BD, Ii M, Park KW, Suli A, Sorensen LK, Larrieu-Lahargue F, Urness LD, Suh W, Asai J, Kock GA, Thorne T, Silver M, Thomas KR, Chien CB, Losordo DW, Li DY. Netrins promote developmental and therapeutic angiogenesis. *Science* 2006;313:640-644

12 Jiang J, Xia XB, Xu HZ, Xiong Y, Song WT, Xiong SQ, Li Y. Inhibition of retinal neovascularization by gene transfer of small interfering RNA targeting HIF-1alpha and VEGF. *J Cell Physiol* 2009;218:66-74

13 Xia XB, Xiong SQ, Song WT, Luo J, Wang YK, Zhou RR. Inhibition of retinal neovascularization by siRNA targeting VEGF(165). *Mol Vis* 2008;14:1965-1973

14 Löw K, Culbertson M, Bradke F, Tessier-Lavigne M, Tuszynski MH. Netrin-1 is a novel myelin-associated inhibitor to axon growth. *J Neurosci* 2008;28 (5): 1099-1108

15 Manitt C, Thompson KM, Kennedy TE. Developmental shift in expression of netrin receptors in the rat spinal cord: predominance of UNC-5 homologues in adulthood. *J Neurosci Res* 2004; 77(5):690-700

16 Manitt C, Wang D, Kennedy TE, Howland DR. Positioned to inhibit: netrin-1 and netrin receptor expression after spinal cord injury. *J Neurosci Res* 2006;84(8): 1808-1820

17 Ellezam B, Selles-Navarro I, Manitt C, Kennedy TE, McKerracher L. Expression of netrin-1 and its receptors DCC and UNC-5H2 after axotomy and during regeneration of adult rat retinal ganglion cells. *Exp Neurol* 2001;168 (1): 105-115