Functional analysis of Semaphorin 3A in retinal ganglion cells under hypoxia in vitro

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Purpose*:* Glaucoma is a progressive neurological disease and a main cause of blindness worldwide. Glaucoma causes irreversible damage to retinal ganglion cells (RGCs) and axons, leading to impaired visual function if not properly treated. Currently, there is no cure for chronic glaucoma, and the main therapeutic goal is to maintain visual function by preventing further disease progression. Thus, neuroprotective strategies are necessary to prevent the progression of optic neuropathy in glaucoma. We first analyzed from the viewpoint of hypoxic stimulation because that circulatory failure may occur even in anterior glaucoma before visual field abnormalities appear at recent glaucoma symptoms. Here, we examined the role in RGCs of Semaphorin 3A, which plays an important role in determining axon elongation during development.

Materials and Methods*:* RGC cultures were prepared from the retinas of 4–5-day-old CrIJ Wistar rats and subjected to hypoxia stress (1% O_2) or normoxia (21% O_2). The expression of class 3 Semaphorins was examined by reverse transcription polymerase chain reaction, western blot, and immunofluorescence analyses.

Results*:* Among the secretory class 3 Semaphorins, only the expression of Semaphorin 3A was increased in RGCs under hypoxia conditions; there was no change in the expression of other Semaphorins (3B, 3C, and 3F). Western blot and immunofluorescence analyses revealed down-regulation of the RGC differentiation marker TUJ1 under hypoxia. Hypoxia-induced down-regulation of TUJ1 in RGCs was blocked by siRNA-mediated knockdown of Semaphorin 3A. A multi-array cytokine assay revealed that vascular endothelial growth factor expression was increased in RGCs under hypoxic conditions; its expression was suppressed by siRNA knockdown of Semaphorin 3A.

Conclusions*:* These results indicate that Semaphorin 3A exerts an important neuroprotective function that is closely related to interactions with vascular endothelial growth factor.

Keywords: glaucoma; retinal ganglion cells; hypoxia; neuroprotection; Semaphorin 3A

Introduction

Glaucoma is characterized by the degeneration of optic nerve axons and death of retinal ganglion cells (RGCs). Increased intraocular pressure (IOP) is a major risk factor for glaucoma (AGIS7., 2010; Ernest et al., 2013). While research has provided some insights into the mechanisms underlying glaucoma, the pathogenesis of the disease requires further elucidation. The first-line treatment for glaucoma is the reduction of IOP by pharmacological and surgical intervention. However, even after lowering the IOP, the disease still progresses in some patients (Susanna et al., 2015). This state is pronounced in Asia, where patients manifest glaucoma in the absence of elevated IOP (Iwase et al., 2004). Thus, recent glaucoma research has focused on neuroprotection and the development of neuroprotective drugs for the treatment of glaucoma.

Semaphorins are a family of eight classes of secretory and cell-bound signaling molecules that are defined by a common structure, the 500 amino acid “Sema” domain. Semaphorins are involved in the neural and vascular aspects of visual system development in various species (Luo et al., 1995; Halloran et al., 1999; Campbell et al., 2001; Rosenzweig et al., 2010). Semaphorin 5 (Sema5) and Sema6, which are membrane-bound classes, are involved in retinal lamination (Matsuoka et al., 2011; Matsuoka et al., 2011; Sun et al., 2013) and guidance of RGC axons (Kuwajima et al., 2012). The secreted class 3 Semaphorins (Sema3) are expressed in the developing rat retina (de Winter et al., 2004) and likely influence the developing mammalian visual system. Sema3 proteins were initially described as axon guidance molecules (Kolodkin et al., 1992; Luo et al., 1993); however, they were later shown to be involved in apoptosis, cell migration, immunity, organogenesis, and tumor suppression (Yazdani and Terman, 2006; Neufeld and Kessler, 2008; Suzuki et al., 2008; Mizui et al., 2009;

Pasterkamp and Giger, 2009; Roth et al., 2009). Sema3A antibodies were also shown to rescue RGCs from cell death (Shirvan et al., 2002). Recently, we reported that Sema3F may contribute to the regulation of RGC function and survival in an optic nerve crush system (Ko et al., 2016). Many Sema3A functions in the visual system remain to be elucidated.

Retinal hypoxia occurs in various ocular disease states, such as central retinal artery occlusion and ischemic central retinal vein thrombosis. Hypoxia has also been associated with the pathogenesis of glaucoma (Flammer, 1994; Tielsch et al., 1995; Chung et al., 1999), diabetic eye disease including retinal and optic nerve head neovascularization (Linsenmeier et al., 1998), and degenerative conditions such as age-related macular degeneration (Cicik et al., 2003; Nowak et al., 2003). Retinal hypoxia is a common cause of visual impairment and blindness (Evangelho et al., 2019). The loss of RGCs occurs in multiple ophthalmic diseases, such as glaucoma and diabetes, with risk factors including hypoxia (Tezel and Yang, 2004; Chen et al., 2007), elevated IOP (Khan et al., 2015), increased glutamate levels (Harada et al., 2007), and aging (Levkovitch-Verbin et al., 2013). Among these risk factors, oxidative stress is thought to be the most important pathway involved in glaucoma progression. Better understanding of the mechanism by which hypoxia leads to RGC damage will help the development of therapies aimed at reducing blindness caused by ischemia and low oxygen conditions in the retina.

Here, we investigated the effects of hypoxia on RGC survival and its effect on the expression of the neural guidance molecule Sema3A. The expression of Sema3A was up-regulated under hypoxic (1% O_2) compared with normoxic (21% O_2) conditions. Furthermore, siRNA-mediated Sema3A knockdown blocked the hypoxia-induced decrease in βIII-tubulin (TUJ1), a neuron-specific marker of RGCs. Our results indicate

that Sema3A may contribute to the survival of RGCs after oxidative stress–induced glaucoma.

Materials and Methods

Ethical approval

This study was performed in accordance with the guidelines of the Committee on Animal Experimentation (Hiroshima University) and the Laboratory Animal Science Research Facility Committee of the Natural Science Center for Basic Research and Development, Hiroshima University (Approval No. A13-169).

Antibodies

Rabbit polyclonal antibodies against Sema3A (ab23393) and GFAP (ab7260) and a mouse monoclonal antibody against TUJ1 (No.80201) (specific for βIII-tubulin) were obtained from Abcam (Minneapolis, MN, USA). A mouse monoclonal antibody against α-tubulin (T9026) was obtained from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibodies against MAP2 (17490-1-AP) and Sema3F (orb11360) were obtained from Proteintech (Rosemont, IL, USA) and Biorbyt (Cambridge, UK), respectively. Horseradish peroxidase–conjugated secondary antibody (mouse, W4021; rabbit, W4011) used in western blotting was obtained from Promega (Madison, WI, USA), and Alexa Fluor 488– (A11001) and 555– (A21429) conjugated secondary antibodies used in immunostaining were obtained from Molecular Probes (Carlsbad, CA, USA).

Purified rat RGC culture

RGC cultures were prepared from the retinas of 4–5-day-old CrIJ Wistar rats using a two-step immune-panning procedure (Uchida et al., 2003; Nakamura et al.,

2011). This culture method is an application of the early RGC primary culture method (Chung et al., 1999; Linsenmeier et al., 1998). In the two-step method, an antibody against Thy-1.1, a ganglion cell surface antibody, is used to selectively collect only RGCs from the retinal cells of rats, followed by primary culture. The direct effects on RGCs not mediated by other cells (glia cells) can be examined. Briefly, retinas were dissociated into a cell suspension using a papain dissociation system (Worthington Biochemical, NJ, USA). The dissociated retinal cells were then incubated in 6 cm cell culture dishes coated with anti-rat macrophage monoclonal antibody (1:50, Chemicon, Temecula, CA, USA) to exclude macrophages. Next, anti-rat Thy1.1 monoclonal antibody (1:300, Chemicon) was added to the dish. The cells were cultured in serum-free basal nerve medium supplemented with 2% B27 supplement, 40 ng/mL brain-derived neurotrophic factor, 40 ng/mL ciliary neurotrophic factor, 10 μM forskolin, 1 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin for 4–5 days in a 37 °C incubator with a humidified atmosphere of 5% CO_2 and 95% air. Cells were then seeded in 6-well plates.

Exposure of RGCs to hypoxia

Purified RGCs were cultured for 4 days. The hypoxia group cultures were transferred to a closed hypoxic chamber with a reduced oxygen atmosphere (1% O_2, 5% CO_2, 94% N_2) and cultured at 37 °C for 3 days.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from cultured RGCs using an RNeasy kit (Qiagen, CA, USA). RT-PCR analysis of 0.1 μg RNA was performed using the one-step RT-PCR kit (Invitrogen, CA, USA). The PCR primer sequences are shown in Table 1. RT was

performed at 50 °C for 30 min; PCR was performed at 94 °C for 2 min, 58 °C for 30 sec, and 72 °C for 1 min for 25 cycles.

Immunoblot analysis

RGCs were washed three times with PBS and lysed in 200 μL lysate buffer [containing 150 mM NaCl, 2% SDS, 5 mM EDTA, and 20 mM Tris-HCl (pH 7.5)]. Cell lysates were separated by SDS-PAGE and the proteins were transferred to nitrocellulose membranes. Membranes were then incubated with the indicated antibodies. Immune complexes were detected with enhanced chemiluminescence reagents (GE Healthcare UK, Little Chalfont, UK).

Immunofluorescence analysis

Cultured RGCs were fixed in 100% methanol for 10 min at –20 °C. Cells were then washed with PBS(-) (Ca^{2+}- and Mg^{2+}-free PBS) and blocked in 1% BSA in PBS(-) at room temperature for 1 h. The cells were then incubated with antibodies against TUJ1 (1/1000) and MAP2 (1/2000) for 1 h. After washing with PBS(-), cells were incubated with Alexa Fluor 488– or 555–conjugated secondary antibodies (1/1000) for 1 h. Immunolabeled cells were imaged using a laser confocal microscope (LSM; Carl Zeiss, Jena, Germany).

Rat cytokine array analysis

Culture supernatants or cell lysates were assayed to determine the levels of various cytokines using a Proteome Profiler Rat Cytokine Array Panel A Kit (R&D Systems, Minneapolis, MN, USA).

RNA interference

RGCs were cultured in 6-well plates for 3 days. The culture medium was then removed, and rat-specific small interfering RNA (siRNA) for Sema3A mRNA or non-targeting (control) siRNA (1 μM; Accell Red siRNA) diluted in 1× Accell siRNA delivery medium (Dharmacon, Lafayette, CO, USA) was added. The siRNA-treated RGCs were transferred to 21% O_2 or 1% O_2 environments and cultured for 48 h.

Results

RGC purification and culture under hypoxia

RGC purification was performed as described in previous reports (Chung et al., 1999; Linsenmeier et al., 1998). RGCs were obtained from the retinas of 4–5-day-old Wistar rats following a two-step immunopanning method. Figure 1a shows a representative image of the RGCs after culture for 6 days. RGC neurites were clearly detected by antibodies against neuron-specific TUJ1 (a RGC marker). Several glial cells (detected by GFAP) were also observed; however, most cells were positive for TUJ1 (> 80%). Therefore, rat RGCs were successfully purified by the immunopanning method.

We subsequently compared RGCs cultured under 21% O_2 (control) and 1% O_2 (hypoxic) conditions. As shown in Figure 1b, immunofluorescence analysis indicated that hypoxia stress caused a decrease in the abundance of TUJ1-positive neurites. The hypoxia-induced down-regulation of TUJ1 was confirmed by immunoblot analysis (Figure 1c).

Effects of hypoxia stress on the expression of class 3 Semaphorins

We next evaluated the effects of hypoxic stress on the mRNA expression of class 3 Semaphorins (3A, 3B, 3C, 3F) in cultured RGCs by RT-PCR analysis. The level of Sema3A mRNA in RGCs was significantly increased after 3 days of hypoxic stress

(1% O_2) compared with normoxia (21% O_2) conditions (Figure 2a, b). In contrast, hypoxic stress did not affect the expression levels of the other class 3 Semaphorins. The protein level of Sema3A in RGCs was also increased by hypoxic stress (Figure 2c, d).

Inhibitory effects of Sema3A siRNA knockdown on XXX and dendritic marker expression

To determine the effects of Sema3A during hypoxia stress, we knocked down Sema3A in cultured RGCs using Sema3A siRNA. As shown in Figure 3, the hypoxia-induced depletion of TUJ1 expression was blocked in cells treated with Sema3A siRNA, whereas the negative control siRNA did not block the effect of hypoxia on Sema3A or TUJ1 levels.

Because hypoxia reduced the expression of the axonal marker TUJ1 (Figure 1), we evaluated the effect of hypoxia on the expression of a dendrite marker, MAP2. The results showed that MAP2 levels were not reduced by hypoxia (Figure 4), indicating different effects of hypoxia on axons and dendrites.

Effects of hypoxia on cytokine expression and regulation by Sema3A

We further investigated the effect of hypoxia on the production of cytokines by RGCs. Culture supernatants and cell lysates from cultures exposed to normoxia or hypoxia were assayed for the expression of 29 cytokines, using a rat cytokine profiling array. Vascular endothelial growth factor (VEGF) levels were increased in culture supernatants and lysates of cells subjected to hypoxia (Figure 5a). Western blot analysis confirmed the hypoxia-induced increase in VEGF expression; however, the increase was not statistically significant (Figure 5b).

We further examined the effect of Sema3A knockdown on VEGF and the expression of VEGF receptors in RGCs under hypoxia. As shown in Figure 5c, the hypoxia-induced increase in VEGF expression was blocked by Sema3A siRNA

knockdown. Furthermore, the hypoxia-induced increase expression of Neuropilin 1 (the receptor for Sema3A and VEGF) was blocked by Sema3A siRNA knockdown; however, no change was observed with Plexin A (the receptor for Sema3A) (Figure 5d). This indicates that alterations in Sema3A expression are involved in regulating axon-specific protein expression under hypoxia and one of the mechanisms is the possibility of involvement in VEGF expression.

Discussion

In this study, we investigated whether Sema3A may function as a neuroprotective factor in RGCs. We examined the expression of class 3 Semaphorins in RGCs cultured in a chamber with 1% oxygen to model hypoxic conditions. Sema3A expression in RGCs cultured under hypoxia was increased at both the mRNA and protein levels, whereas the expressions of the other class 3 Semaphorins (Sema3B, Sema3C, Sema3F) did not change. Exposure of RGCs to hypoxia resulted in down-regulation of the RGC marker TUJ1 and neurite outgrowth. The hypoxia-induced down-regulation of TUJ1 expression and neurite outgrowth was blocked by siRNA-mediated knockdown of Sema3A. Additionally, hypoxia increased VEGF expression and release from RGCs. Thus, our data indicate that Sema3A may play an important neuroprotective function through interaction with VEGF.

The current first line treatments for glaucoma are aimed at reducing IOP. First choice anti-glaucoma drugs include prostaglandins and β-blockers (Weinreb et al., 2014; Cholkar et al., 2015; Prum et al., 2016). If medical treatment does not sufficiently reduce IOP, surgical intervention is an option (Weinreb et al., 2014; Cholkar et al., 2015; Prum et al., 2016). However, these drugs and surgical interventions do not block the death of RGCs, which is a key contributing factor to glaucoma pathology. Neuroprotective therapies that increase the survival of RGCs are lacking. A

neuroprotective effect of a particular factor for some in vitro forms of stress-induced cell death have been reported (Marcic et al., 2003; Foxton et al., 2013; Flachsbarth et al., 2018). Therefore, we decided to investigate whether members of the neural guidance factor family of Semaphorins exhibited a neuroprotective effect.

Semaphorin family members are well-characterized regulators of nerve guidance. Each Semaphorin exhibits diverse functions, and many have not been completely characterized. We recently revealed that Sema3F is involved in neuroprotection in experiments using a rat nerve crush model (Ko et al., 2016). Other studies showed that Sema3A is involved in RGC apoptosis (Shirvan et al., 1999; Shirvan et al., 2002). In the present study, we found that while the expressions of Sema3F, Sema3C, and Sema3B were not affected by hypoxic stress, Sema3A expression was up-regulated in hypoxia-stressed RGCs. Moreover, siRNA-mediated knockdown of Sema3A blocked the down-regulation of TUJ1 and neurite outgrowth in hypoxia-stressed RGCs. We further found that the VEGF cytokine is up-regulated in hypoxia-stressed RGCs. The receptor for VEGF is neuropilin 1, which is the same receptor used by Sema3A (Gagnon et al., 2000). We initially predicted that reducing Sema3A expression by siRNA knockdown would increase VEGF expression because of competition for the neuropilin 1 receptor. However, the results were opposite to our expectation; suppressing Sema3A expression decreased VEGF expression. The mechanism underlying this phenomenon has not been explored and whether Sema3A and VEGF directly interact is unknown. Thus, future studies are required to elucidate Sema3A signal transduction mechanisms that affect the expression of VEGF and the neuropilin 1 receptor.

Conclusions

Our results show that secreted Sema3A plays an important role in the survival and function of RGCs. Further studies are required to elucidate how Sema3A and related factor(s) play important roles in mediating neuroprotection of RGCs.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Figure Legends

Figure 1. Purification of retinal ganglion cells (RGC) and culture under hypoxia. (**A**) Fluorescence images of RGCs purified using a two-step immunopanning method. Immunofluorescence staining was performed using an antibody against TUJ1 (green) and GFAP (red). (**B**) Fluorescence images of RGCs cultured in 21% O_2 (control; left) or 1% O_2 (hypoxia; right). Immunofluorescence staining was performed using an antibody against TUJ1 (green) and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (**C**) Western blot analysis of TUJ1 and α-tubulin (loading control) in lysates of RGCs cultured as in Figure 1B; TUJ1 abundance was quantified by western blot densitometry and normalized to the corresponding amount of α-tubulin. *$p<0.05$

versus the corresponding value of RGCs cultured in hypoxic conditions (Student's t-test).

Figure 2. Effect of hypoxia stress on the expression of Semaphorins in RGCs. (A) Representative RT-PCR analysis of Sema3A, Sema3B, Sema3C, Sema3F, and GAPDH (internal standard) mRNAs in RGCs cultured for 5–6 days in 21% O_2 (cont) or 1% O_2 conditions. (B) Quantification of Semaphorin mRNA from densitometric scanning of ethidium bromide-stained gels was performed after normalization to GAPDH mRNA. (C) Western blot analysis of Sema3A, Sema3F, and α-tubulin (loading control) in RGCs after culture for 5–6 days in 21% O_2 or 1% O_2 conditions. (D) Sema3A and Sema3F levels were quantified by densitometric scanning of immunoblots and normalized to α-tubulin levels. *$p<0.05$ versus the corresponding value of RGCs cultured in hypoxic conditions (Student's t-test).

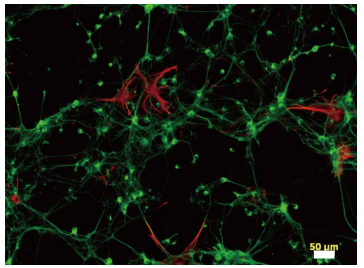
Figure 3. Effect of siRNA Sema3A knockdown on hypoxia-induced changes in protein levels. Hypoxia-cultured RGCs were treated with siRNA targeting Sema3A, or negative control siRNA for 48 h. Cell lysates were immunoblotted to assess the expression of Sema3A, TUJ1 and α-tubulin.

Figure 4. Effect of hypoxia stress on the expression of the dendrite marker, MAP2, in RGCs.

(A) Fluorescence images of RGCs cultured in 21% O_2 (control; upper), and 1% O_2 (hypoxia; bottom). Immunofluorescence staining was performed using antibodies against TUJ1 (green) and MAP2 (red). (B) Immunoblot analysis of TUJ1, MAP2 and α-tubulin (loading control) in lysates of RGCs cultured as in (A). (C) TUJ1 and MAP2 levels were quantified by densitometric scanning of western blots and normalized to α-tubulin levels. *$p<0.05$ versus the corresponding value of RGCs cultured in hypoxic conditions (Student's t-test).

Figure 5. Effects of hypoxia stress and Sema3A expression on the expression and release of VEGF from RGCs. (A) RGCs were incubated under control (21% O_2) or hypoxia (1% O_2) conditions for 48 h. Culture supernatants and cell lysates were then assayed for cytokines using a profiling array. (B) Western blot analysis of VEGF and α-tubulin (loading control) in lysates of RGCs cultured as in (A) (left). Quantification of VEGF levels by densitometric scanning of the western blot and normalized to α-tubulin levels (right). (C) Hypoxia-cultured RGCs were transfected with siRNA targeting Sema3A or negative control siRNA for 48 h. Cell lysates were assessed for the expression of Sema3A, TUJ1, VEGF and α-tubulin by immunoblotting. (D) Hypoxia-cultured RGCs were transfected with siRNA targeting Sema3A or negative control siRNA for 48 h. Cell lysates were assessed for the expression of Neuropilin 1, Plexin A and α-tubulin by immunoblotting.

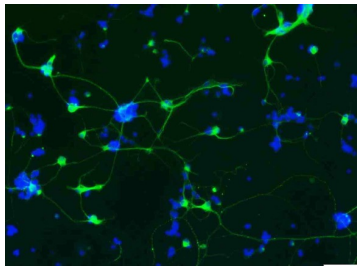
A



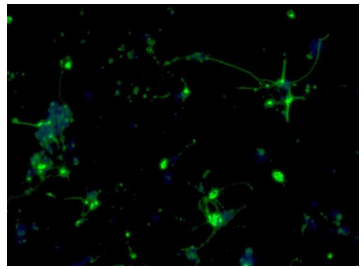
RGC culture

B

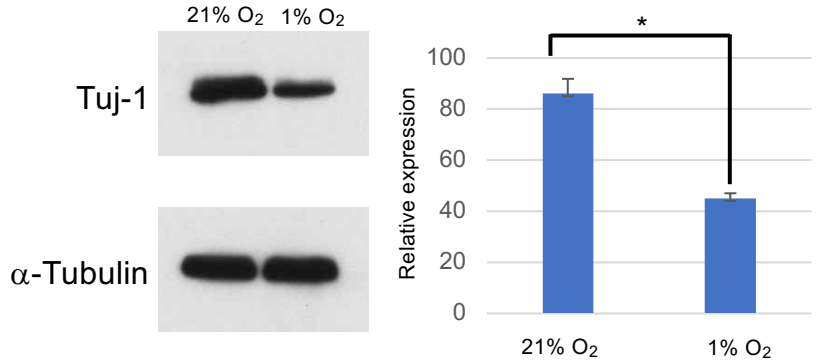
21% O_2

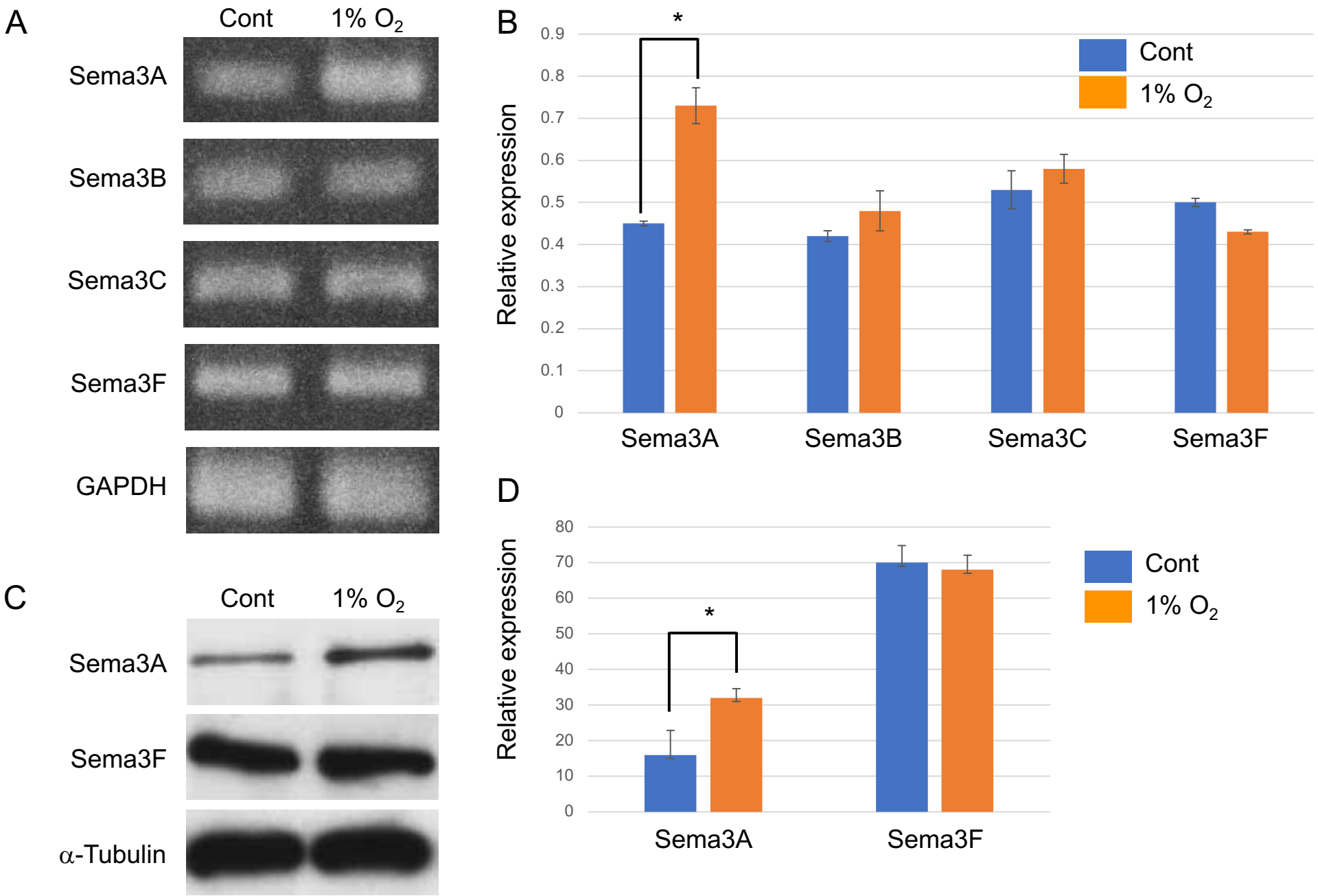


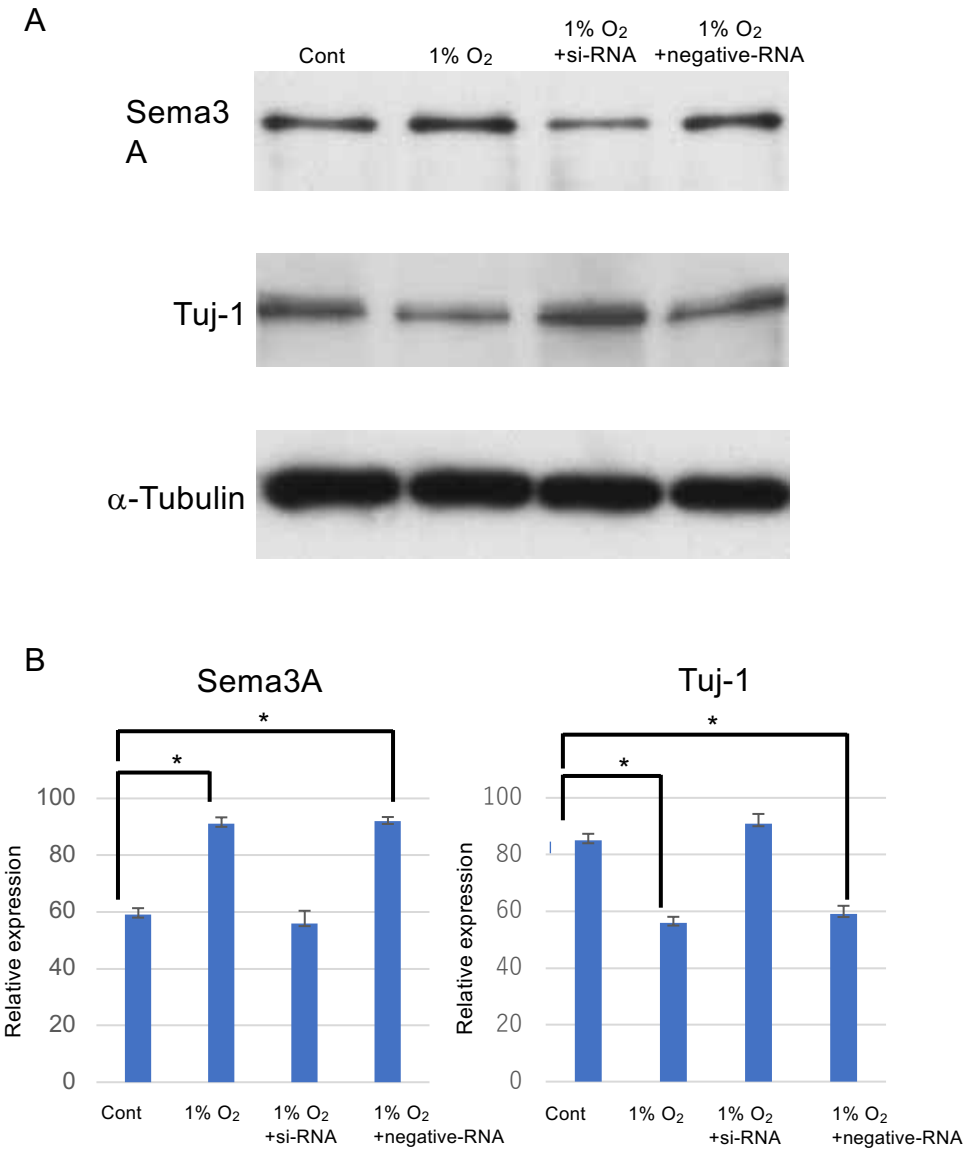
1% O_2



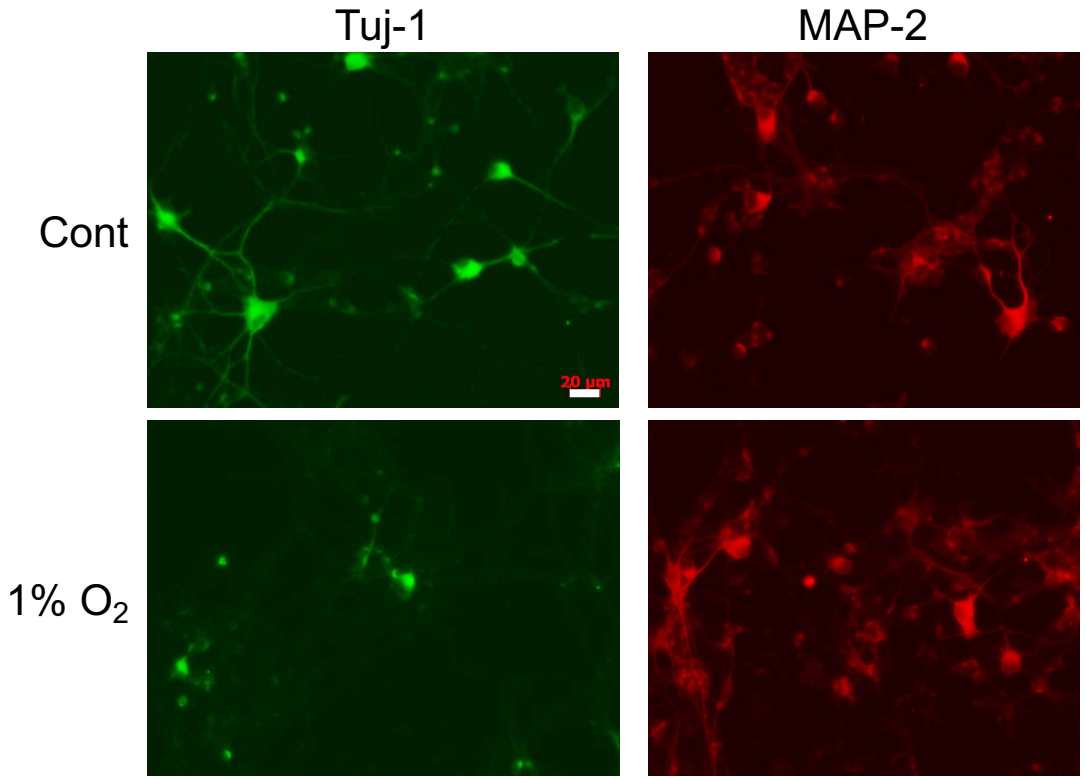
C



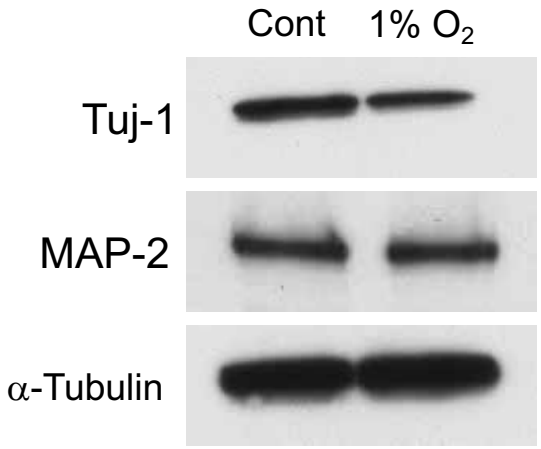

A
Cont
1% O2
Sema3A
Sema3B
Sema3C
Sema3F
GAPDH
B
*
Relative expression
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cont
1% O2
Sema3A
Sema3B
Sema3C
Sema3F
C
Cont
1% O2
Sema3A
Sema3F
α-Tubulin
D
*
Relative expression
80
70
60
50
40
30
20
10
0
Cont
1% O2
Sema3A
Sema3F


A
Cont
1% O2
1% O2 +si-RNA
1% O2 +negative-RNA
Sema3A
Tuj-1
α-Tubulin
B
Sema3A
Tuj-1
Relative expression
100
80
60
40
20
0
*
*
Cont
1% O2
1% O2 +si-RNA
1% O2 +negative-RNA

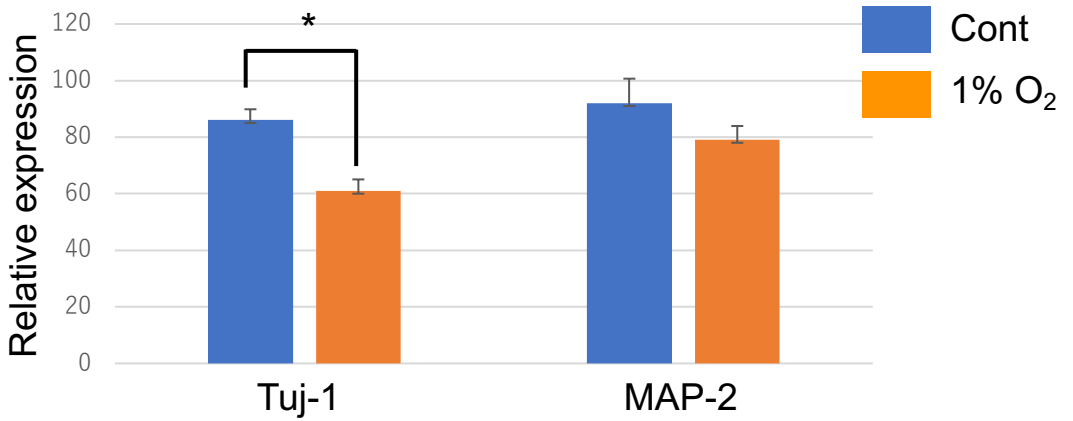
A


Tuj-1
MAP-2
Cont
1% O2
20 μm

B


Cont
1% O2
Tuj-1
MAP-2
α-Tubulin

C


Relative expression
120
100
80
60
40
20
0
*
Cont
1% O2
Tuj-1
MAP-2

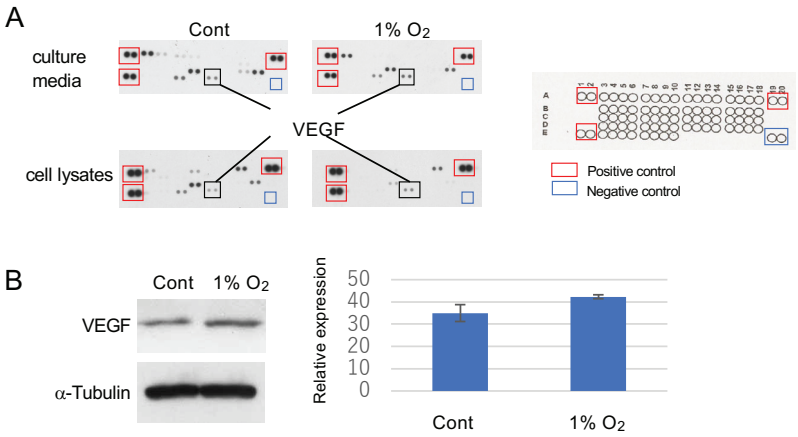

A
Cont
1% O2
culture media
VEGF
cell lysates
Positive control
Negative control
B
Cont
1% O2
VEGF
α-Tubulin
Relative expression
50
40
30
20
10
0
Cont
1% O2
C
Cont
1% O2
1% O2 +si-RNA
1% O2 +negative-RNA
Sema3A
Tuj-1
VEGF
α-Tubulin
D
Cont
1% O2
1% O2 +si-RNA
1% O2 +negative-RNA
Npn-1
Plexin A
α-Tubulin

Table 1

Table 1 Sequences of the PCR primers

Semaphorin 3A	Forward;	5'-3'	ATGTTCATCGGAACGGATGTTGGA
	Reverse;	5'-3'	GGCTCTCTGTGACTTCGGACTG
Semaphorin 3B	Forward;	5'-3'	CCTTGGGTCAGAATCCGAGTCT
	Reverse;	5'-3'	TGATGTGTAGGCCCCTCTTTGT
Semaphorin 3C	Forward;	5'-3'	TGTTGTCACTTTCATTCGGAACCA
	Reverse;	5'-3'	TCTTGTCTTCGGCTCCTTCGT
Semaphorin 3F	Forward;	5'-3'	AGGCTCTGTGTTCCGAGGATC
	Reverse;	5'-3'	GTTTTAACAGGTGCTGGCTCCTT
G3PDH	Forward;	5'-3'	GCTCTCTGCTCCTCCCTGTTC
	Reverse;	5'-3'	CGCCAGTAGACTCCACGCATA

(* G3PDH; glyceraldehyde-3-phosphate dehydrogenase)