Hypoxia-Inducible Factor Inhibitors Derived from Marine Products Suppress a Murine Model of Neovascular Retinopathy

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Abstract: Neovascular retinal degenerative diseases are the leading causes of blindness in developed countries. Anti-vascular endothelial growth factor (VEGF) therapy is commonly used to treat these diseases currently. However, recent reports indicate that long term suppression of VEGF in the eye is associated with chorioretinal atrophy. Therefore, a physiological amount of VEGF is required for retinal homeostasis. Hypoxia-inducible factor (HIF) is a transcriptional factor upstream of VEGF. We previously reported that HIF regulated pathological angiogenesis in the retina of murine models of oxygen-induced retinopathy and laser-induced choroidal neovascularization. Most of the known HIF inhibitors are anti-cancer agents which may have systemic adverse effects in for clinical use; thus, there is a need for safer and less invasive HIF inhibitors. In this study, we screened marine products, especially fish ingredients, and found that six species of fish had HIF inhibitory effects. Among them, administration of *Decapterus tabl* ingredients significantly suppressed retinal neovascular tufts by inhibiting HIF expression in a murine oxygen-induced retinopathy model. These results indicate that particular fish ingredients can act as anti-angiogenic agents in retinal neovascularization diseases.

Keywords: HIF; marine products; retinal neovascularization; oxygen-induced retinopathy

1. Introduction

Pathological retinal angiogenesis is a major pathology of various eye diseases such as diabetic retinopathy (DR), which is one of the most common complications of diabetes, and retinopathy of prematurity (ROP), which is a complication in low birth-weight infants [1–3]. These diseases are leading causes of blindness worldwide [4,5]. Neovascular retinopathy has two pathological phases; the first phase is vessel loss leading to tissue ischemia and hypoxia, followed by upregulation of angiogenic factors including vascular endothelial growth factor (VEGF), which stimulates pathological neovascularization in the second phase [2,6–8]. Abnormal neovascularization can result in vision loss caused by edema, hemorrhage, retinal fibrosis, scarring, and retinal detachment. Anti-VEGF therapy has been established and is now commonly employed to treat this pathological angiogenesis [9]. However, local or systemic adverse events such as chorioretinal atrophy and renal

injury have recently been reported as resulting from potent long-term pharmacological VEGF antagonism [10–12]. This is supported by the biological evidence that VEGF is required to maintain physiological vascular homeostasis [13]. Therefore, there is a need to establish a novel therapy for suppressing pathological amount of VEGF without affecting the physiological amount.

We have focused on hypoxia-inducible factors (HIFs), which are transcriptional factors that regulate various genes to adapt to cellular hypoxia [14]. Under normoxic conditions, the subunit of HIFs (HIF-as) is immediately hydroxylated by prolyl hydroxylase (PHD) and ubiquitinated by von Hippel–Lindau protein (VHL) to be degraded in a proteasome-dependent manner [15]. Under hypoxic conditions, the activity of PHD decreases, resulting in HIF-as stabilization, then HIF-as translocate to the nucleus to bind to the hypoxia response element (HRE) in target genes such as VEGF, B-cell lymphoma 2 (BCL2) interacting protein 3 (BNIP3), and phosphoinositide-dependent kinase 1 (PDK1) [16]. We previously reported that pharmacological inhibition of HIFs suppressed retinal neovascularization in murine models of oxygen-induced retinopathy (OIR), known as a retinal neovascular degeneration model [17], and laser-induced choroidal neovascularization (CNV), known as an exudative age-related macular degeneration model [18]. On the other hand, most of the existing HIF inhibitors are anticancer agents [19] which may have systemic side effects in clinical use. Thus, we also need to develop safer and less invasive HIF inhibitors.

Recently, we examined 238 natural products to discover novel HIF inhibitors, and reported that halofuginone extracted from hydrangea has a retinal neuroprotective effect in a murine ischemia–reperfusion model [20]. In the study, fish ingredients such as fish protein from *Spratelloides gracilis* and bio-active shark cartilage powder were also found to suppress HIF activity. There have been some reports about the usefulness of fish ingredients to prevent various diseases. Omega-3 (w-3) polyunsaturated fatty acids (PUFA) from fish oil known as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are reported to suppress cardiovascular events [21], and these fatty acids also decreased the risk of sight loss in diabetic retinopathy in clinical research [22]. On the other hand, there has been no report about the effect of water-soluble components of fish on ophthalmic diseases.

In this study, we explored water-soluble ingredients from 68 marine species showing HIF inhibitory effects. We also evaluated the therapeutic effects of HIF inhibitors derived from fish on pathological angiogenesis in a murine retinal neovascular degeneration model.

2. Materials and Methods

2.1. Marine Product Preparation

The material extraction was performed by referring to the protocol previously described [23]. The materials used in this study are shown in Table A1 and A2. Almost all marine product samples were obtained in Shizuoka prefecture, Japan, except *S. gracilis*, which was obtained in Kagoshima prefecture, Japan. All samples were stocked in a freezer (−40 °C) until extraction. Samples were excised from the dorsal part, fillet, the headless body, and other parts described in Tables A1 and A2. Each muscle was minced using a knife, and two grams of mince were homogenized with 20 mL cold ultrapure water (generated from Direct-Q3UV, Merck KGaA, Darmstadt, Germany) using a blender (NS-50 and NS-10, Microtec co. ltd, Chiba, Japan) for 2 min. The homogenate was incubated for 30 min in boiling water. After cooling on ice, the homogenate was centrifuged at 1650× *g* for 20 min at 4 °C. The precipitate was homogenized with 10 mL ultrapure water using a glass rod and centrifuged as described above. These supernatants were filtered using a paper filter (Advantec No. 5A, Toyo Roshi, ltd, Tokyo, Japan) under reduced-pressure conditions, and then a small volume of the oil layer was removed from the filtrate with 10 ml *n*-Hexane. The filtrate was frozen and then dried in a vacuum.

2.2. Luciferase Assay for Fish Screening

The luciferase assay was performed as previously described [20]. Human retinal pigment epithelium cell line ARPE19 and murine cone photoreceptor cell line 661W were transfected with a HIF-luciferase reporter gene construct (Cignal Lenti HIF Reporter, Qiagen, Venlo, The Netherlands). The HIF-luciferase construct encodes firefly luciferase gene under the control of a hypoxia response

element which binds HIFs. These cells were also co-transfected with a cytomegalovirus (CMV)-renilla luciferase construct as an internal control. These cells were seeded at 1.0×10^4 cells/well/70 mL (ARPE19) or 0.8×10^4 cells/well/70 mL (661W) in an HTS Transwell®-96 Receiver Plate, White, tissue-culture (TC)-Treated, Sterile (Corning, NY, USA). At 24 h after seeding, $CoCl_2$ (200 mM, cobalt (II) chloride hexahydrate, Wako, Japan) or dimethyloxalylglycine (DMOG) (1 mM, N-(2-Methoxy-2-oxoacetyl) glycine methyl ester, Merck, Darmstadt, Germany) was administered to the cells in order to induce normoxic HIF activation. To evaluate the suppressive effect of fish ingredients against HIF activation, fish ingredients from 69 species were administered at the same time when $CoCl_2$ or DMOG was added. After incubation for 24 h at 37 °C in a 5% CO_2 incubator, the luminescence was measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA). Additionally, 1mM of topotecan (Cayman Chemical, Ann Arbor, MI, USA) or doxorubicin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was used as a positive control as known HIF inhibitors.

2.3. Real-Time PCR

Total RNA was isolated from the ARPE19 cell line using TRI reagent® (MRC Global, Cincinnati, OH, USA) and an Econospin column for RNA (GeneDesign, Osaka, Japan). The columns were washed with Buffer RPE and RWT (Qiagen, Hilden, Netherlands). RT-PCR was performed using ReverTra Ace® qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan). Real-time PCR was performed using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO, Osaka, Japan) with the StepOnePlus Real-Time PCR system (Applied Biosystems, Waltham, MS, USA). The primer sequences were as follows: HIF-1a forward TTCACCTGAGCCTAATAGTCC, HIF-1a reverse CAAGTCTAAATCTGTGTCCTG, HIF-2a forward CGGAGGTGTTCTATGAGCTGG, HIF-2a reverse AGCTTGTGTGTTCGCAGGAA, VEGF forward TCTACCTCCACCATGCCAAGT, VEGF reverse GATGATTCTGCCCTCCTCCTT, APO2 forward TCATTAGCCACTGAGTGTTGTTT, APO2 reverse CTCGAATACGATGACTCGGTG, EPO forward CCCTGCCAGACTTCTACGG, EPO reverse GGAGGCCGAGAATATCACGAC, BNIP3 forward GGACAGAGTAGTTCCAGAGGCAGTTC, BNIP3 reverse GGTGTGCATTTCCACATCAAACAT, PDK1 forward ACAAGGAGAGCTTCGGGGTGGATC, PDK1 reverse CCACGTCGCAGTTTGGATTTATGC, GLUT1 forward CGGGCCAAGAGTGTGCTAAA, GLUT1 reverse TGACGATACCGGAGCCAATG, GAPDH forward TCCCTGAGCTGAACGGGAAG, GAPDH reverse GGAGGAGTGGGTGTCGCTGT.

2.4. Western Blotting

661W or ARPE19 cells were collected in radioimmunoprecipitation (RIPA) Buffer (Thermo Fisher Scientific, Waltham MA, USA) containing protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Each sample was fractionated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane, then blocked with 5% nonfat dry milk for 1 h at room temperature. The membranes were incubated with primary antibodies: rabbit monoclonal antibodies against HIF-1a (1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal antibodies against HIF-2a (1:1000, NOVUS Biologicals, Centennial, CO, USA) over two nights or mouse monoclonal antibodies against b-actin (1:10,0000, Sigma-Aldrich, St Louis, MO, USA) overnight at 4°C. After washing with tris buffered saline and Tween 20 (TBS-T), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody goat anti-rabbit IgG (1:5000, GE Healthcare, Princeton, NJ, USA) for HIF-1a and HIF-2a or with sheep anti-mouse IgG (1:10,000, GE Healthcare, USA) for b-actin for 1 h at room temperature. The signals were detected using EzWestLumi plus (Atto, Tokyo, Japan). Protein bands were visualized via chemiluminescence (ImageQuant LAS 4000 mini, GE Healthcare, Chicago, IL, USA).

2.5. Animals

All procedures related to animal experiments were performed in accordance with the National Institutes of Health (NIH) guidelines for work with laboratory animals, the Association for Research

in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and Animal Research: Reporting In Vivo Experiments (ARRVIVE) guidelines, and were approved by the Institutional Animal Care and Use Committee of Keio University. C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan).

2.6. Oxygen-Induced Retinopathy Model and Administration of Fish Gradients

The OIR model was produced as previously described [17,24]. Postnatal day 8 (P8) mice were exposed to 85% O_2 for 72 h in an oxygen supply chamber with their nursing mothers. After oxygen exposure, mice were placed back in room air until P17. Pups received oral administration of *S. gracilis* (1.2 g/kg/day), *D. tabl* (3 g/kg/day), or ultrapure water as vehicle once a day from P12 to P16. At P17, the mice were sacrificed, and the eyes were enucleated. The eyes were fixed for 15 min in 4% PFA (paraformaldehyde) solution. Retinal wholemounts were post-fixed in 4% PFA for 1 h. After washing, the tissues were stained with isolectin GS-IB4 from *Griffonia simplicifolia* conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA) at 4 °C for 3 days. After encapsulation, retinal vessels were observed with a fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan). We measured the number of pixels in neovascular tufts and vaso-obliteration using the lasso tool and the magic wand tool of Photoshop (Adobe, San Jose, CA, USA), respectively [25].

2.7. Statistical Analysis

We used a two-tailed Student's *t*-test for comparison of two groups and ANOVA-Turkey for the comparison of three or more groups, respectively. We considered $p < 0.05$ as being statistically significant. All results in this paper are expressed as the mean + standard deviation.

3. Results

3.1. In Vitro Screening for Hypoxia-Inducible Factor (HIF) Inhibitors from Marine Products

We prepared 68 types of marine products for screening. All the marine products were water soluble and were dissolved in ultrapure water for use in the experiment. As the first screening, the murine retinal cone cell line (661W) was used to evaluate suppression of HIF activity via HIF luciferase dual assay as previously reported [20]. Cobalt chloride ($CoCl_2$) was used to inhibit prolyl hydroxylase (PHD), resulting in an induction of HIF activity, and the suppressive effects of these marine products were then evaluated (Tables A1, A2). In the first screening, 27 species showed HIF suppressive effects when compared to vehicle administration under $CoCl_2$ exposure (Table A1). These marine products were further examined at the second screening (Tables 1,A3). Since it was possible that sequestration of cobaltous ions by chelation with fish ingredients was the cause of HIF inhibition [26], we used dimethyloxalylglycine (DMOG) as another PHD inhibitor in the second screening. Through the second screening, four species of fishes, *Selar crumenophthalmus*, *Spratelloides gracilis*, *Seriola dumerili*, and *Decapterus macarellus* showed significant HIF inhibitory effects when compared with vehicle administration under DMOG stimulation (Figure 1A). The human retinal pigment epithelium cell line (ARPE19) was also used to evaluate the effects of these fish and genealogically related species of fish, *Decapterus muroadsi* and *Decapterus tabl* (Figure 1B). As a result, the screened four and related two species of fish ingredients significantly inhibited HIF activity induced by DMOG. We also evaluated the HIF inhibitory effects of *S. gracillis* at various concentration using the murine embryo fibroblast cell line (NIH-3T3) (Figure A1). *S. gracilis* inhibited HIF activity induced by 1% oxygen in a dose-dependent manner. Further, *S. gracilis* showed a significant HIF inhibitory effect only at a concentration of 1 mg/mL.

Table 1. The list of fishes showing hypoxia-inducible factor (HIF) inhibitory effects in the second screening with statistical analysis and the rate of change of HIF activity compared with dimethyloxalylglycine (DMOG)-administrated controls (n = 3). (†Positive control chemicals) *** p < 0.001, † p < 0.0001, ‡ p < 0.00001 compared with DMOG.

Species of fish	Japanese name	Sampling parts	Rate of change (%)	p value
Topotecan†	-	-	−54.4	0.0000000‡
Doxorubicin†	-	-	−54.4	0.0000000‡
Selar crumenophthalmus	Meaji	skinless fillet	−47.2	0.0000001‡
Seriola dumerili	Kanpachi	white muscle, dorsal	−29.0	0.00003†
Spratelloides gracilis	Kibinago	headless	−27.9	0.0003***
Decapterus macarellus	Kusayamoro	skinless fillet	−24.4	0.0001***
Panulirus japonicus	Ise-ebi	muscle, abdomen	−17.2	0.069
Sulculus diversicolo supertexta	Tokobushi	Muscle, foot	−12.2	0.283
Trachurus japonicus	Maaji	skinless fillet	−8.8	0.127
Dried bonito	Ara-bushi	-	−3.8	0.999
Scomberoides lysan	Ikekatsuo	skinless fillet	−2.9	0.999
Rhabdosargus sarba	Hedai	white muscle, dorsal	−1.4	0.999
DMOG	-	-	0	-

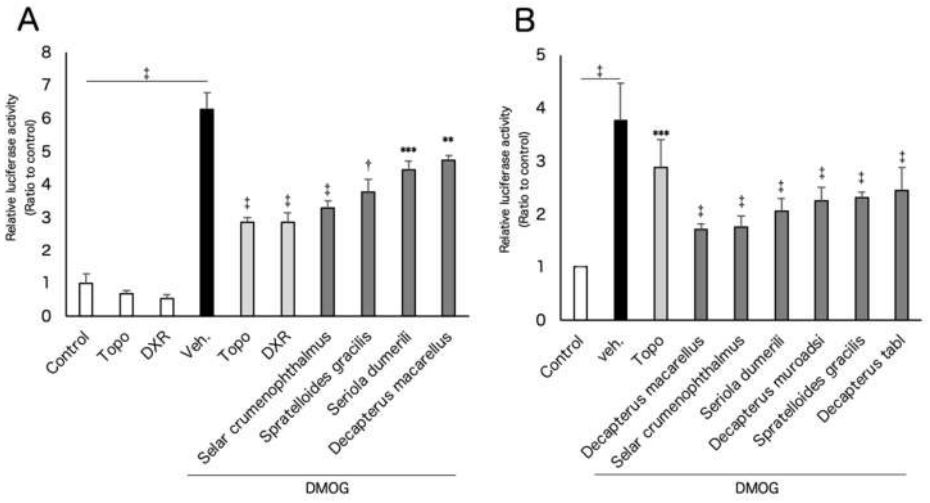


Figure 1. Fish ingredients show inhibitory effects on hypoxia-inducible factor (HIF) activation in vitro. HIF-reporter luciferase assay was performed using the murine retinal cone cell line (661W) (**A**) and the human retinal pigment epithelium cell line (ARPE19) (**B**) cell lines (n = 3). Topotecan, doxorubicin, and fish ingredients were administrated in dimethyloxalylglycine (DMOG)-induced culture conditions. Note that six species of fish ingredients significantly inhibited HIF activity induced by DMOG. ** p < 0.01, *** p < 0.001, † p < 0.0001, ‡ p < 0.00001 compared with DMOG-Veh. Error bars indicate mean plus SD. Veh., Vehicle; Topo, topotecan; DXR, doxorubicin.

3.2. Screened Fish Ingredients Inhibit HIF and HIF Target Genes In Vitro

In order to determine how the fish ingredients affect HIF and HIF target genes, ARPE19 cells incubated in 1% oxygen conditions and four species of fish ingredients were added simultaneously. In the ARPE19 cells, the gene expression level of *hif-1a*, decreased by hypoxia (possibly due to a negative feedback [27]), and *hif-2a* was suppressed by fish ingredients (Figure 2A). Expression of HIF target genes such as *vegf*, *epo*, and *pdk1* was upregulated under 1% O_2 conditions and was significantly suppressed by fish ingredient administration (Figure 2B). Western blotting showed that the protein levels of HIF-1α and HIF-2α in ARPE19 cells, increased by $CoCl_2$ (Figure 3A–C), were suppressed by fish ingredient administration. The protein level of HIF-1α in ARPE19 cells, increased by 1% O_2 (Figure 3D,E), or in 661W cells, increased by $CoCl_2$ (Figure 3F,G) or 1% oxygen (Figure 3H,I), was also suppressed by fish ingredient administration. These results indicated that the screened fish

ingredients had inhibitory effects on the stabilized HIF expression in pseudo and real hypoxic conditions.

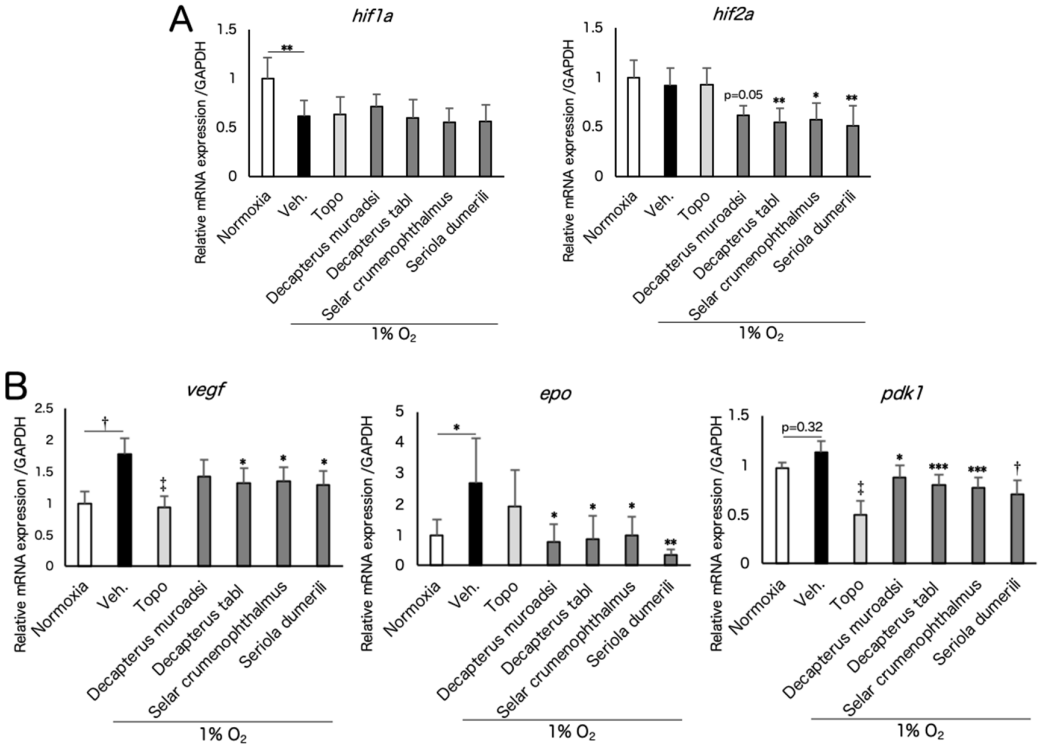
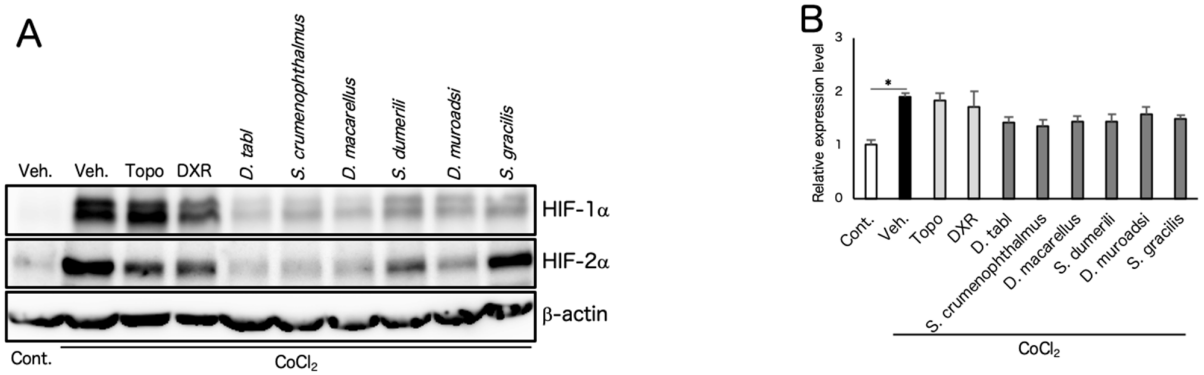


Figure 2. Fish ingredients suppress HIFs and their target genes associated with angiogenesis in vitro. Real-time PCR was performed for *hif-1a* and *hif-2a* (**A**) and their target genes, including *vegf*, *epo*, and *pdk1*, under 1% O_2 conditions in ARPE19 cells (**B**). Note that gene expression of *hif-2a* was suppressed by fish ingredients. *Vegf*, *epo*, and *pdk1* were upregulated under 1% O_2 conditions and significantly suppressed by fish ingredients administration. Fish ingredients were added at 1 mg/ml and the hypoxic conditions were maintained for 12 h. n = 6/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † $p < 0.0001$, ‡ $p < 0.00001$ compared with 1% O_2/vehicle. Error bars indicate mean plus SD. Veh., vehicle; Topo, topotecan; epo, erythropoietin.



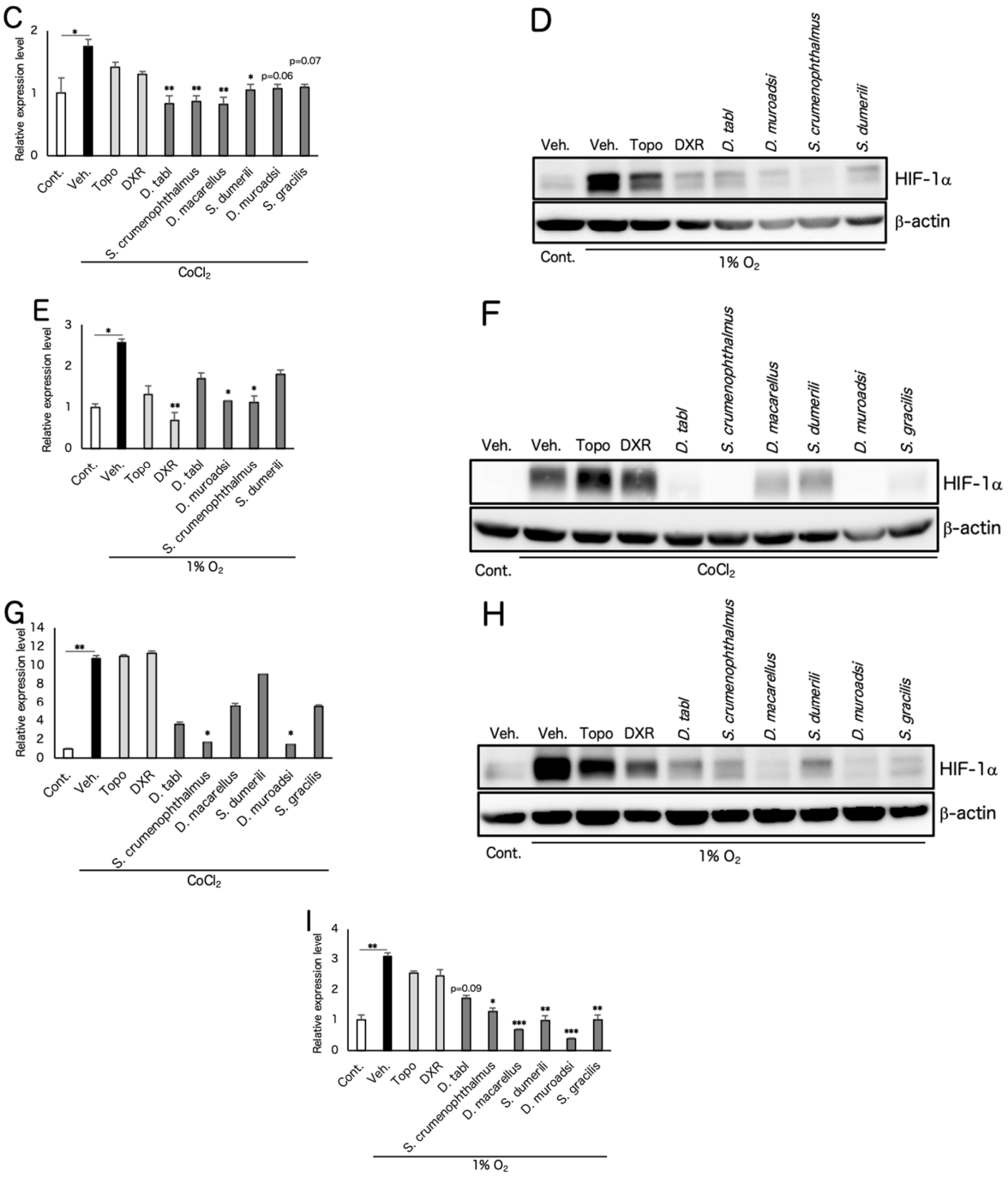
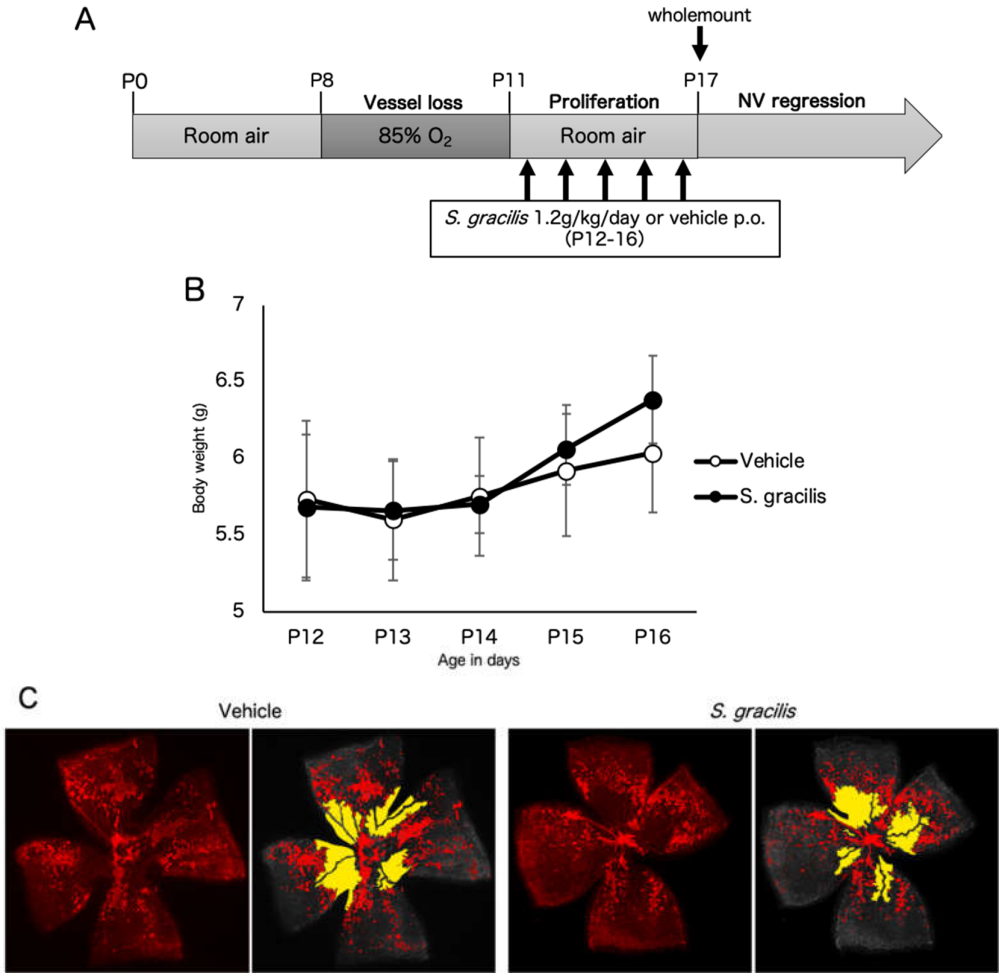


Figure 3. Fish ingredients suppress HIF-1a protein expression in vitro. Western blotting for HIF-1α and HIF-2α was performed under $CoCl_2$ condition in ARPE19 cells (**A**), and for HIF-1α under 1% O_2 condition in ARPE19 cells (**D**), under CoCl2 (**F**) or 1% O2 conditions (**H**) in 661W cells. Quantification of the blots showed that the administration of fish ingredients suppressed increased HIF-1α protein expression under $CoCl_2$ (**B**) or 1% O_2 conditions (**E**) in ARPE19 cells and under $CoCl_2$ (**G**) or 1% O_2 conditions (I) in 661W cells ($n = 3$). Quantification of the blots also showed that the administration of fish ingredients suppressed the increased HIF-2α protein expression under $CoCl_2$ in ARPE19 cells (**C**) ($n = 3$). $CoCl_2$ was administered at a concentration of 200 mM, fish ingredients were added at 1 mg/ml simultaneously, and cells were incubated for 24 h. The hypoxic conditions were maintained for 48 h. Note that the fish ingredients inhibited HIF-1α and HIF-2α expression induced by $CoCl_2$ or hypoxia. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with 1% O_2/vehicle or $CoCl_2$/vehicle. Error bars indicate mean plus SD. Veh., vehicle; Topo, topotecan; DXR, Doxorubicin.

3.3. Fish Ingredients Suppressed Neovascularization in a Murine Oxygen-Induced Retinopathy (OIR) Model

To assess the effect of the fish ingredients on retinal neovascularization, we orally administrated them to OIR mice and analyzed neovascular tufts and vaso-obliteration via retinal wholemount staining. Firstly, *S. gracilis* was assessed as a candidate screened in the previous study [20]. Vehicle ($n = 6$) or *S. gracilis* ingredient (1.2 g/kg/day, $n = 5$) was orally administered according to the schedule shown in Figure 4A. There was no significant difference in body weight between the two groups throughout the administration period (Figure 4B). Administration of *S. gracilis* showed little change in neovascular tufts compared to the control ($p = 0.2$) (Figure 4C,4D), probably due to technical limitations to increasing the dose of the active ingredient in the crude sample. Thus, in the following experiment, we analyzed *D. tabl*, which was screened in the current study and showed a more potent inhibitory effect on HIF1-α. Vehicle ($n = 4$) or *D. tabl* ingredient (3 g/kg/day, $n = 4$) was orally administered according to the schedule shown in Figure 5A. There was no significant difference in body weight between the two groups throughout the administration period (Figure 5B). Administration of *D.tabl* significantly ($p < 0.05$) suppressed neovascular tufts compared to the control, while no significant difference was observed in vaso-obliteration (Figure 5C,5D).



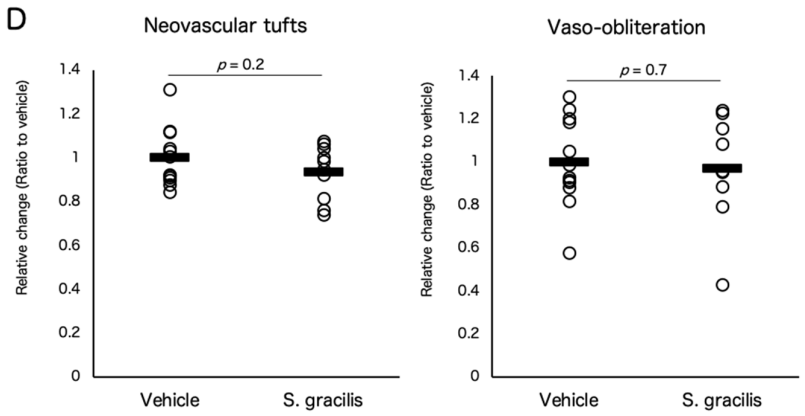
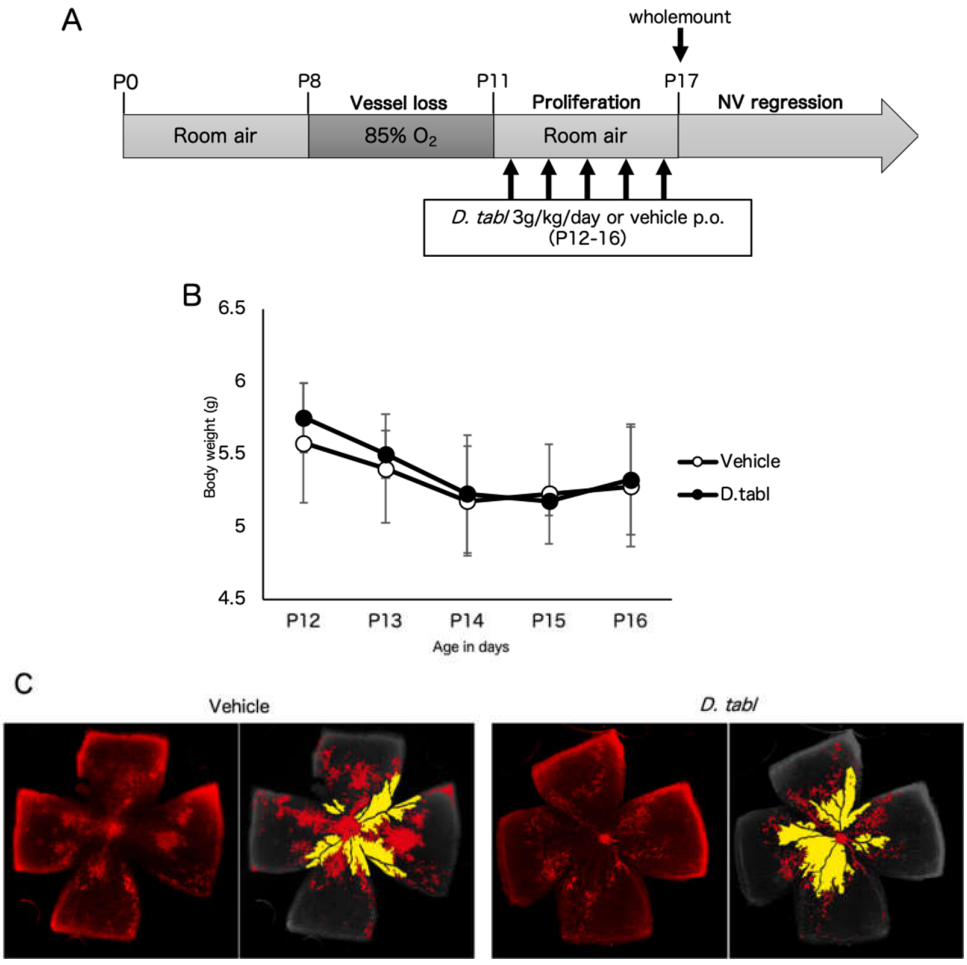


Figure 4. Oral administration of *Spratelloides gracilis* ingredients show a tendency to suppress retinal neovascularization in a murine oxygen-induced retinopathy (OIR) model. (**A**) A schematic illustration of the oxygen-induced retinopathy (OIR) procedure. Vehicle (n = 6) or *S. gracilis* (n = 5) was orally administered from P12 to P16. (**B**) Mean body weight change of mice. The average body weight of the *S. gracilis* group did not differ from that of the vehicle group through the administration period. (**C**) Representative images of retinal vasculature staining with isolectin B4. Areas of neovascular tufts (red) or vaso-obliteration (yellow) are highlighted. (**D**) Quantification of neovascular tufts and vaso-obliteration (right) in OIR as a percentage of the total retinal area. Note that *S. gracilis* tended to suppress neovascular tufts while no significant difference was observed in vaso-obliteration. n.s., not significant. Error bars indicate mean plus SD.



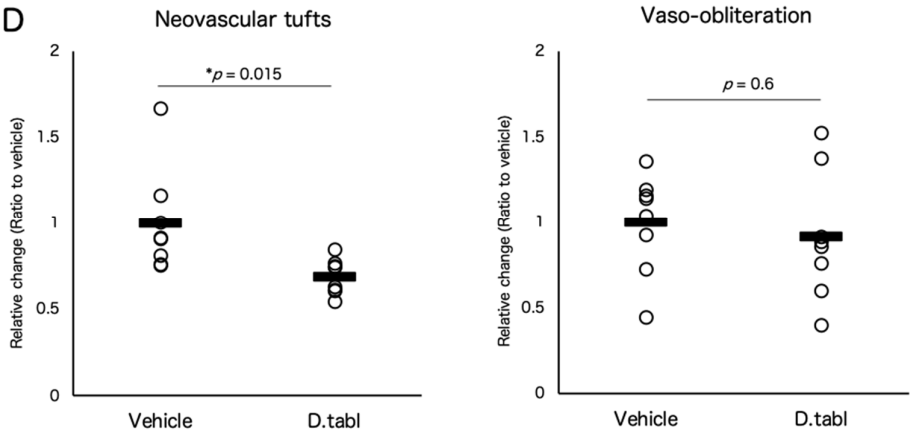


Figure 5. Oral administration of *Decapterus tabl* ingredient suppress retinal neovascularization in a murine oxygen-induced retinopathy (OIR) model. (**A**) Schematic illustration of the OIR procedure. Vehicle or *D. tabl* was orally administered from P12 to P16. (**B**) Mean body weight change of mice. The average body weight of the *D. tabl* group did not differ from that of the vehicle group throughout the administration period. (**C**) Representative images of retinal vasculature staining with isolectin B4. Areas of neovascular tufts (red) or vaso-obliteration (yellow) are highlighted. (**D**) Quantification of neovascular tufts (left) and vaso-obliteration (right) in OIR as a percent of the total retinal areas. Note that *D. tabl* significantly ($p < 0.05$) suppressed neovascular tufts, while no significant difference was observed in vaso-obliteration. $n = 4$/group. * $p < 0.05$; n.s., not significant. Error bars indicate mean plus SD.

4. Discussion

In this study, among marine products from 68 species, we found fish ingredients from four species which had HIF inhibitory effects by luciferase assay (Tables 1,A1–A3, Figure 1A). Additionally, two species of fish genealogically related to the four species also had HIF inhibitory effects (Figure 1B). These fish ingredients suppressed gene expression of *hif-2a*, followed by suppression of their downstream angiogenic factors and others in vitro (Figure 2A,2B), and the fish ingredients also inhibited HIF-1α and HIF-2α protein expression induced by $CoCl_2$ or hypoxia (Figure 3). The activity of HIF-as can be inhibited at the levels of transcription, translation, translocation to the nucleus, and DNA binding [16]. In this study, we found that the fish ingredients suppressed mRNA expression of *hif-2a*. In contrast, the gene expression of *hif-1a* had already been decreased by hypoxia possibly due to a negative feedback [27], and the suppression of *hif-1a* mRNA expression by the fish ingredients could not been seen. At this point, we could confirm that the fish ingredients inhibited HIF-1α and HIF-2α protein expression; however, further mechanism should be investigated in the future studies.

The in vivo experiment revealed that administration of *D. tabl* in an OIR model had a significant suppressive effect on pathological retinal neovascularization (Figure 5). On the other hand, *S. gracilis* showed little change in neovascular tufts and none in vaso-obliteration (Figure 4). In this study, oral administration of ingredients from *S. gracilis* and *D. tabl* was performed at the highest concentration and volume as much as possible according to the procedure previously described [28]. These fish ingredients were crude, and the dosage of *S. gracilis* may not have been sufficient for this model. VEGF is the primary factor driving the formation of neovascular tufts in the OIR model. Although these fish products showed *vegf* suppressive effects in vitro concomitantly with HIF inhibition as well as topotecan which showed a significant suppression of upregulated *vegf* in OIR retinas [17], the changes of VEGF expression level in vivo need to be investigated in the future studies.

The six species of fish are classified into two families: *Spratelloides gracilis* belongs to the Herring family, and *Selar crumenophthalmus*, *Seriola dumerili*, *Decapterus macarellus*, *Decapterus muroadsi*, and *Decapterus tabl* belong to the Carandiae family. Since the fish have similar properties in the same

families, it is possible that any characteristic compounds contained in these fishes inhibit HIF activity. There are some reports regarding the disease-preventive effects of w-3 PUFAs derived from fish oil by inhibiting HIF-1a and its downstream pathway. For instance, in a murine model of lung carcinoma, DHA suppressed expression of the HIF-1a/VEGF axis and decreased tumor size with cisplatin treatment [29]. Another report suggested that DHA and EPA attenuated HIF-dependent inflammation and reduced neuronal damage in stroke [30]. In this study, the fish ingredients were incubated in boiled water, then oil in the fish was removed by hexane extraction. We examined HIF activity of each ingredient from some of fishes with or without degreasing with *n*-Hexane using the HIF-reporter luciferase assay, and confirmed that the ingredients containing oil component showed no change in HIF inhibitory effect compared with the oil-free ingredients (Figure A2). Therefore, it is inferred that oil components excluded by this extraction methods have no HIF inhibitory effect, whereas oil-free and water-soluble components contain the biologically active substances. Additionally, the active ingredients contained in these fishes are considered to be small molecules such as dipeptides, amino acids, nucleic acids, and minerals. Further purification of these fish ingredients will be needed. It is also suggested that these water-soluble components do not affect the postnatal growth in OIR mice, as indicated that no change in body weight was observed with administration of either fish ingredients (Figures 4B,5B). Further studies are needed in order to assess the other physiological responds to these ingredients.

Although anti-VEGF drugs are the main pharmacological approach for macular edema and neovascularization in DR and retinal vein occlusion, and for exudative age-related macular degeneration, long-term VEGF antagonism may induce photoreceptor and Retinal pigment epithelium (RPE) cell atrophy [10,11]. Furthermore, VEGF gene deletion in RPE was shown to induce photoreceptor and choroidal degeneration [18,31]. On the other hand, HIF gene deletion in the retina in adult mice showed no phenotypic change [18], while HIF-as gene deletion in RPE suppressed laser-induced CNV in mice [18]. These data suggest that anti-VEGF drugs may suppress the physiological amount of VEGF required to maintain normal vasculatures and metabolism of cells in the retina and choroid, and that inhibition of HIFs prevents only pathological angiogenesis. Moreover, frequent intravitreal injection of anti-VEGF agents is invasive and of high cost for patients. Therefore, fish ingredients and their active components are readily accepted because of their safety and accessibility for oral intake, and they can be used as a preventive medicine or supplement for proliferative retinopathy.

5. Conclusions

We found six types of fish ingredients as novel HIF inhibitors. *D. tabl* had a suppressive effect against pathological retinal neovascularization in a murine OIR model. In conclusion, our results indicate that administration of these fish ingredients may be a possible approach to cure retinal angiogenic diseases by inhibiting HIFs in the retina.

6. Patents

The current data includes patents applied for Keio University for a therapeutic or prophylactic agent for ischemic disease, glaucoma, optic nerve disease, retinal degenerative disease, angiogenic retinal disease, cancer, neurodegenerative or autoimmune disease, and a hypoxia inducing factor inhibitor (application no. PCT/JP2017/040884) and by Keio University and Shizuoka Prefectural Research Institute of Fishery for control of hypoxic response by components from marine products (application no. PCT/JP2019/68141, PCT/JP2019/145435).

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix

Table A1. The list of fishes showing HIF inhibitory effects in the first screening and the rate of change of HIF activity compared with $CoCl_2$-administrated controls. (†Positive control chemicals).

Species of fish	Japanese name	Sampling parts	Rate of change (%)
Scomber australasicus	Gomasaba	white muscle, dorsal	−75.0
Fermented dried bonito	Honkare-bushi	-	−70.9
Dried bonito	Ara-bushi	-	−70.9
Katsuwonus pelamis	Katsuo	white muscle, dorsal	−68.0
Scomber japonicus	Masaba	white muscle, dorsal	−68.0
Thunnus obesus	Mebachi	white muscle, dorsal	−65.0
Etrumeus teres	Urumeiwashi	skinless fillet	−56.5
Cypselurus agoo	Tobiuo	white muscle, dorsal	−55.8
Dried mackerel	Saba-bushi	-	−50.5
Spratelloides gracilis	Kibinago	headless	−49.5
Thunnus obesus	Mebachi	head muscle	−49.5
Trachurus japonicus	Maaji	skinless fillet	−47.6
Topotecan†	-	-	−40.0
Thunnus obesus	Mebachi	Pectoral fin muscle	−39.8
Scomberomorus niphonius	Sawara	white muscle, dorsal	−38.8
Doxorubicin†	-	-	−37.5
Selar crumenophthalmus	Meaji	skinless fillet	−35.5
Sardinops melanostictus	Maiwasi	whole	−33.3
Decapterus macarellus	Kusayamoro	skinless fillet	−31.0
Seriola dumerili	Kanpachi	white muscle, dorsal	−26.5
Coryphaena hippurus	Siira	white muscle, dorsal	−25.0
Pagrus major	Madai	white muscle, dorsal	−24.3
Dried sardine	Iwashi-bushi	-	−24.3
Rhabdosargus sarba	Hedai	white muscle, dorsal	−23.7
Panulirus japonicus	Ise-ebi	muscle, abdomen	−14.5
Seriola quinqueradiata	Buri	white muscle, dorsal	−11.5
Haliotis diversicolor aquatilis	Tokobushi	muscle, foot	−10.0
Scomberoides lysan	Ikekatsuo	skinless fillet	−4.9
Marsupenaeus japonicus	Kuruma-ebi	muscle, abdomen	−0.0
$CoCl_2$	-	-	0

Table A2. The list of fishes showing no HIF inhibitory effect in the first screening and the rate of change of HIF activity compared with $CoCl_2$-administrated controls.

Species of fish	Japanese name	Sampling parts	Rate of change (%)
$CoCl_2$	-	-	0
Oncorhynchus mykiss	Nijimasu	white muscle, dorsal	1.9
Lateolabrax latus	Hirasuzuki	white muscle, skinless fillet	4.4
Crassostrea gigas	Magaki	edible part	8.0
Haliotis gigantea	Megai-awabi	muscle, foot	9.5
Gerres equulus	Kurosagi	white muscle, skinless fillet	12.5
Caranx sexfasciatus	Gingameaji	skinless fillet	16.9

Plecoglossus altivelis altivelis	Ayu	skinless fillet	17.4
Dasyatis akajei	Akaei	white muscle, dorsal	24.5
Caranx ignobilis	Rounin-aji	skinless fillet	29.9
Charonia lampas sauliae	Boushu-bora	muscle, foot	30.5
Oplegnathus punctatus	Ishigakidai	white muscle, dorsal	36.5
Plectorhinchus cinctus	Koshodai	white muscle, skinless fillet	39.5
Guinusia dentipes	Shojin-kani	muscle, cheliped and ambulatory leg	39.5
Girella punctata	Mejina	white muscle, dorsal	44.0
Stephanolepis cirrhifer	Kawahagi	white muscle, skinless fillet	47.0
Lateolabrax japonicus	Suzuki	white muscle, skinless fillet	53.0
Scombrops boops	Mutu	headless	75.0
Paralichthys olivaceus	Hirame	white muscle, skinless fillet	77.0
Turbo sazae	Sazae	muscle, foot	80.0
Meretrix lusoria	Hamaguri	edible part	83.0
Sphyraena japonica	Yamatokamasu	white muscle, dorsal	87.5
Oncorhynchus masou ishikawae	Amago	skinless fillet	88.5
Ruditapes philippinarum	Asari	edible part	96.5
Anguilla japonica	Nihon-unagi	skinless fillet	98.1
Sphyraena pinguis	Akakamasu	white muscle, dorsal	104.2
Siganus fuscescens	Aigo	white muscle, dorsal	104.2
Heteropriacanthus cruentatus	Gomahirekintoki	white muscle, skinless fillet	105.5
Trichiurus japonicus	Tachiuo	white muscle, skinless fillet	108.5
Prionurus scalprum	Nizadai	white muscle, dorsal	117.5
Diaphus suborbitalis	Senhadaka	headless	127.7
Stichopus japonica	Manamako	muscle	129.5
Beryx splendens	Kinmedai	white muscle, dorsal	133.0
Chelidonichthys spinosus	Houbou	white muscle, skinless fillet	134.0
Siganus fuscescens	Aigo	ovary	142.5
Parapristipoma trilineatum	Isaki	white muscle, skinless fillet	147.5
Goniistius zonatus	Takanohadai	white muscle, dorsal	147.5
Calotomus japonicus	Budai	white muscle, dorsal	149.0
Engraulis japonica	Katakuchi-iwashi	whole (larva)	180.0
Konosirus punctatus	Konoshiro	white muscle, skinless fillet	182.0
Lucensosergia lucens	Sakura-ebi	whole	215.8
Sphyraena pinguis	Akakamasu	testis	230.8

Table A3. The list of fishes showing no HIF inhibitory effect in the second screening with statistical analysis and the rate of change of HIF activity compared with DMOG-administrated controls ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † $p < 0.0001$, ‡ $p < 0.00001$ compared with DMOG.

Species of fish	Japanese name	Sampling parts	Rate of change (%)	*p* value
DMOG	-	-	0	-
Fermented dried bonito	Honkare-bushi	-	1.0	0.999
Seriola quinqueradiata	Buri	white muscle, dorsal	3.8	0.871
Coryphaena hippurus	Siira	white muscle, dorsal	6.3	0.826
Thunnus obesus	Mebachi	white muscle, dorsal	7.7	0.919
Cypselurus agoo	Tobiuo	white muscle, dorsal	8.2	0.882
Dried sardine	Iwashi-bushi	-	10.9	0.039
Scomber australasicus	Gomasaba	white muscle, dorsal	11.1	0.529
Sardinops melanostictus	Maiwasi	whole	11.5	0.464
Katsuwonus pelamis	Katsuo	white muscle, dorsal	13.9	0.206
Pagrus major	Madai	white muscle, dorsal	15.9	0.011*

Scomber japonicus	Masaba	white muscle, dorsal	21.6	0.004**
Dried mackel	Saba-bushi	-	26.9	0.0002***
Scomberomorus niphonius	Sawara	white muscle, dorsal	28.4	0.0001***
Etrumeus teres	Urumeiwashi	skinless fillet	69.0	0.00004†
Thunnus obesus	Mebachi	head muscle	70.4	0.00002†
Thunnus obesus	Mebachi	pectoral fin muscle	79.5	0.000005‡

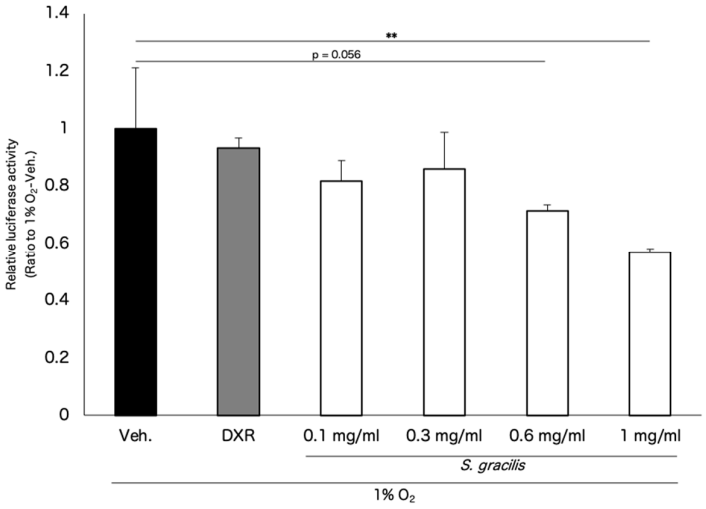

Figure A1. *S. gracilis* ingredients show inhibitory effects on HIF activation in a dose-dependent manner *in vitro*. HIF-reporter luciferase assay was performed using the murine embryo fibroblast cell line (NIH-3T3). *S. gracilis* inhibited HIF activity induced by 1% oxygen in a dose-dependent manner. Note that *S. gracilis* showed significant HIF inhibitory effect only at a concentration of 1 mg/ml. ** $p <$ 0.01 compared with 1% O_2-Veh. Error bars indicate mean plus SD. Veh., Vehicle; DXR, doxorubicin.

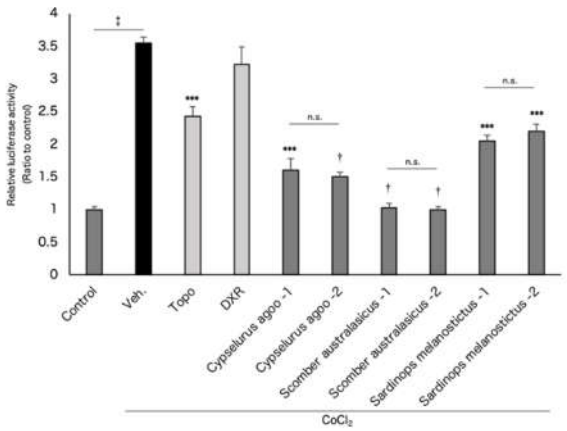

Figure A2. Oil components of Fish ingredients show no effect on HIF activity in vitro. HIF-reporter luciferase assay was performed using 661W ($n = 3$). *C. agoo*, *S. australasicus*, and *S. melanostictus* ingredients were extracted with (*fish*-2) or without (*fish*-1) *n*-Hexane. Note that oil-free ingredients showed no change in HIF inhibitory effect compared with the ingredients containing oil component. *** $p < 0.001$, † $p < 0.0001$, ‡ $p < 0.00001$ compared with $CoCl_2$-Veh. Error bars indicate mean plus SD. Veh., Vehicle; Topo, topotecan; DXR, doxorubicin. *Fish*-1, fish ingredient containing oil component; *Fish*-2, oil-free fish ingredient.

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和文要約・解説

1. 背景

網膜病的血管新生は糖尿病網膜症や未熟児網膜症等の眼疾患における主要な病態であり、失明に至る主な要因である。網膜病的血管新生は、正常血管の脱落による網膜虚血と、そこから血管内皮増殖因子(VEGF)を含む血管新生因子が産出されることで起こる病的血管新生の二病相からなり、異常血管からの浮腫、出血、線維化、瘢痕化による視力低下を引き起こす。現在、抗 VEGF 薬硝子体内注射が血管新生性網膜疾患への治療として確立されているが、長期投与による網脈絡膜萎縮や腎機能障害の報告がある。VEGF は生理的な血管恒常性維持に必要であることから、病的に増加した血管新生因子のみを抑制する新規治療法が求められている。

低酸素誘導因子(HIF)は生体の低酸素応答を担う転写因子である。HIF は α と β のサブユニットからなるヘテロダイマーで転写因子として機能しており、そのうち α サブユニットは、哺乳類では、HIF-1α、HIF-2α、HIF-3α の 3 種類が存在している。通常酸素下では、これらのサブユニット(HIF-α)は、プロリン水酸化酵素(PHD)による水酸化を経てフォン・ヒッペル・リンドウタンパク(VHL)によるユビキチン化を受け、プロテアソームにて直ちに分解される。一方低酸素条件下では、PHD 活性が低下することで HIF-α が安定化し、核内移行して低酸素応答を担う標的遺伝子の低酸素応答エレメントに結合する。我々はこれまでに、網膜病的血管新生モデルとして知られる酸素誘導性網膜症(OIR)や滲出性加齢黄斑変性モデルとして知られるレーザー誘導性脈絡膜血管新生(CNV)モデルにおいて、HIF の薬理学的阻害が網膜または脈絡膜病的血管新生を抑制することを報告した。一方、既存の HIF 阻害剤の多くは抗がん剤であり、臨床では全身的な副作用を伴う可能性がある。従って、より安全で侵襲性の低い HIF 阻害剤の開発が必要である。

加齢黄斑変性においては、ルテインやゼアキサンチンといった天然色素であるカロテノイドの予防効果が報告され、(Age-Related Eye Disease Study Research Group. *Arch Ophthalmol*. 2001, The Age-Related Eye Disease Study 2(AREDS2) Research Group. *JAMA*. 2013)、サプリメントとして通常診療に用いられている。よって長期の予防的効果を試みる上で、天然物由来の HIF 阻害作用を持つ物質を探索することは一つのふさわしいアプローチであると考えた。

近年我々は、238 種類の天然物スクリーニングにより新規の HIF 阻害作用を持つ物質を発見した。このうち、キビナゴ由来成分やサメ軟骨抽出物に HIF 活性を抑制する効果が認められた。オーストラリアで行われた加齢黄斑変性に関するコホート研究では、49 歳以上の成人において魚の摂取回数が月 1 回未満の集団に対し、週 1 回以上摂取している集団で AMD 発症リスクのオッズ比が 0.5 であったという報告がある（Smith W. et al. *Arch Ophthalmol*. 2000)。また、血漿オメガ 3 多価不飽和脂肪酸(PUFA)の濃度が高いほど、晩期加齢黄斑変性進行のリスクが低いという報告がある（Merle BM. *J Nutr*. 2013)。糖尿病網膜症においても、魚の摂取回数が多いほど、後期糖尿病網膜症のリスクが低下すること（Chua J. et al. *Sci Rep*. 2018)、特に魚油由来の PUFA は視力低下リスクを減少させることが報告されている。一方でこれらの研究は油性成分の効果について論じており、魚の水溶性成分が眼疾患に及ぼす影響については報告がない。

よって本研究では、HIF 阻害作用を示すキビナゴを含む魚類 68 種の水溶性成分を抽出し探索した。また、マウス網膜病的血管新生モデルを用いて、魚類由来 HIF 阻害剤の病的血管新生に対する治療効果を評価した。

2. 方法

魚類抽出物：キビナゴは鹿児島県産、その他の魚類は静岡県産である。それぞれ熱水抽出し、減圧下で濾過後、n-ヘキサンにより濾液から油層を除去した。濾液を凍結乾燥した。

魚類抽出物のスクリーニング：ルシフェラーゼアッセイにより魚類のスクリーニングを行った。ヒト網膜色素上皮細胞株(ARPE19)とマウス網膜錐体細胞株(661W)、マウス胚線維芽細胞株(NIH-3T3)に HIF 活性依存的-Luciferase (HIF が結合する低酸素応答エレメント(HRE)の下流に TATA Box を含む人工合成 miniP 領域、ホタル由来 luciferase 遺伝子、ヒト PGK プロモーター、Streptomyces alboniger 由来の Puromycin 耐性遺伝子を有する(図 1A)) と内在性コントロール CMV-Renilla-Luciferase (サイトメガロウィルスの CMV プロモーター下流にウミシイタケ由来 *Renilla* luciferase 遺伝子、ヒト PGK プロモーターの下流に大腸菌由来の Hygromycin 耐性遺伝子を有する(図 1B)) を共に遺伝子導入し安定発現株を作製した。これらの細胞に PHD 阻害剤である塩化コバルト($CoCl_2$)またはジメチルオキサリルグリシン(DMOG)を添加することで通常酸素下で HIF を安定化すると同時に、68 種の魚類成分を添加した。24 時間後にルシフェリンによる発光を測定した。ポジティブコントロールとして既知の HIF 阻害剤であるトポテカンまたはドキソルビシンを使用した。

Real time PCR：ARPE19 から Total RNA を単離した。逆転写酵素を用いて cDNA 合成を行い、real time PCR を行った。

ウェスタンブロッティング：661W または ARPE19 よりタンパク抽出を行った。電気泳動による分画、転写後、HIF1α、HIF2α、βactin の各一次抗体、二次抗体との反応後、化学発光法にて可視化を行った。

動物実験：実験動物の使用は動物実験に関連する種々のガイドラインを遵守し行った。

OIR モデルと魚類抽出物の投与：C57BL/6J マウスを用いて OIR モデルを作成した。生後 8 日目のマウスを 85%高酸素下に 72 時間暴露し、生後 11 日目から室内通常酸素下で飼育した。生後 12 日目から 16 日目までキビナゴ(1.2g/kg/日)またはオアカムロ(3g/kg/日)、対照群として超純水を経口投与した。生後 17 日目に眼球を摘出し、網膜ホールマウントを作成した。アイソレクチン B4 で組織染色を行い、蛍光顕微鏡で網膜病的新生血管および無血管領域の割合を測定した。

統計解析：2 群間比較には両側ステューデント t 検定を、3 群間以上の比較には ANOVA-Turkey 検定をそれぞれ用いた。$p < 0.05$ を統計学的に有意とした。

3. 結果

3.1 魚類抽出物のスクリーニング：

全ての魚類抽出物は水溶性であり、超純水に溶解し使用した。まず一次スクリーニングでは、661W を用いて $CoCl_2$ 曝露下でおこなった。その結果、27 種の魚類で HIF 阻害効果を示した。魚類成分と $CoCl_2$ イオンのキレート作用による見かけの HIF 活性低下を除外するため、二次スクリーニングは DMOG 暴露下で行った。その結果、メアジ、キビナゴ、カンパチ、クサヤモロの 4 種が有意な HIF 阻害効果を示した。4 種のうちキビナゴを除く 3 種はアジ科の魚類であったことから、ARPE19 を用いて、その類縁魚 2 種（ムロアジ、オアカムロ）にも同様の効果があるか追加検証を行った。その結果、6 種の魚類成分は、DMOG によって誘導される HIF 活性を有意に阻害した。また、NIH-3T3 を用いて、キビナゴの濃度依存的 HIF 阻害効果を評価した結果、キビナゴは 1%酸素によって誘導される HIF 活性を濃度依存的に阻害し、1 mg/mL の濃度でのみ有意な

HIF 阻害効果を示した。

3.2 魚類抽出物による HIF と HIF 標的遺伝子の抑制：

魚類抽出物の HIF および HIF 標的遺伝子への影響を調べた。ARPE19 に、4 種の魚類抽出物を添加し 1%酸素条件下でインキュベートした。ARPE19 では、低酸素によって *HIF1A* の遺伝子発現レベルが低下し、魚類抽出物により *EPAS1* の発現が抑制された。ここで、網膜に発現し血管新生に関与していることが知られ（Watanabe D et al. *N Engl J Med.* 2005)、OIR モデルマウス網膜にて発現増加することが確認されている（文献[17]）*epo* を含め、1%酸素条件下で増加した HIF 標的遺伝子(*VEGFA*、*EPO*、*PDK1)*の発現は、魚類抽出物により有意に抑制、または抑制傾向を認めた。ウェスタンブロッティングでは、ARPE19 おいて、$CoCl_2$ により増加した HIF-1α および HIF-2α のタンパク質量が、魚類抽出物により有意に減少、または統計学的有意差はないものの減少傾向を示した。また、661W において、$CoCl_2$ により増加した HIF-1α のタンパク質量は、魚類抽出物により有意に減少、または統計学的有意差はないものの減少傾向を示した。ARPE19 および 661W ともに、1%酸素条件下で増加した HIF-1α のタンパク質量が、魚類抽出物により有意に減少、または統計学的有意差はないものの減少傾向を示した。これらの結果から、擬似低酸素状態および実際の低酸素状態において安定化した HIF 発現に対し、6 種の魚類が抑制傾向を有することが示された。

3.3 マウス OIR モデルにおける魚類抽出物による網膜病的血管新生抑制効果

網膜病的血管新生に対する魚類成分の効果を評価するために、OIR マウスに対し 2 種の魚類抽出物の経口投与を行った。ニシン科魚類であるキビナゴ投与群では、対照群と比較して血管新生に変化が見られなかった。一方、アジ科の魚類のうちオアカムロ投与群では、対照群と比較して有意に病的血管新生を抑制した。一方、無血管領域にはどちらも有意差は認められなかった。

4. 考按

本研究では、全 70 種の水産物の中からルシフェラーゼアッセイによる HIF 阻害作用を有する 6 種の魚類を発見した。また、これらの魚類抽出物が *EPAS1* の mRNA 発現を抑制することを見出した。一方、*HIF1A* の遺伝子発現は低酸素により既に低下しており(負のフィードバックが作用したと考えられる)、魚類抽出物による *HIF1A* の転写レベルでの抑制は見られなかった。魚類抽出物は HIF-1α および HIF-2α タンパク質の発現を抑制していることが確認できた。HIF 活性の抑制ポイントとしては、転写、翻訳、タンパク分解、核内移行、HRE への結合などがある（文献[16])。本研究で Positive Control として使用した、Topotecan、Doxorubicin はそれぞれ HIF mRNA レベル、HRE への結合を阻害するとされている。しかし、本研究では、Topotecan にて mRNA、タンパク発現量の低下が見られない細胞種・培養条件が見られ、逆に Doxorubicin にてタンパク発現量の低下を示した細胞種・培養条件が見られた。よって、Topotecan が核内移行や HRE への結合阻害作用をも有しており、一方で、Doxorubicin が mRNA やタンパク発現レベルでの阻害作用をも有していることが示唆される。また、NIH-3T3 に対する Doxorubicin の HIF 抑制効果は他の 2 種の細胞に比して少ないことが本研究結果より確認されており、細胞種によって HIF に対する作用が異なることが示唆される。同様に、魚類抽出物も HIF 活性の様々な抑制ポイントに働き、またそれは細胞種によって異なる可能性がある。魚類抽出物が HIF-α の機能をどのポイントで阻害しているか、メカニズムの検討が今後の課題である。

また、魚類抽出物が細胞の生理的な機能に与える影響を確認するため、$CoCl_2$ や DMOG、低酸

素状態などの負荷を与えない条件下で、魚類抽出物による HIF 活性、VEGF の発現変化を確認する必要がある。

本研究では OIR モデルへのオアカムロの投与により、網膜病的血管新生を有意に抑制したが、キビナゴでは有意な抑制は見られなかった。魚類抽出物は可能な限り高濃度・高用量で行ったが、魚類抽出物は未精製であり、このモデルではキビナゴに含まれる有効成分量が十分ではない可能性がある。また、オアカムロによる血管新生抑制の程度を、既存の抗 VEGF 薬と比較検討する必要があると考える。

OIR モデルマウスへの魚類抽出物投与時には、*D. tabl*、*S. gracillis* の投与群どちらも高酸素解除後初期に仔マウスの体重が減少する傾向にあった。OIR モデルにおける高濃度酸素とその解除という実験条件が影響した可能性や、母親の高酸素解除後の授乳状況が悪化した可能性が考えられる。

6 種の魚類はニシン科とアジ科に属する。科により類似の性質を有することから、今回の抽出成分に含まれる特徴的な化合物が HIF 阻害活性を持つと考えられる。本研究で使用した魚類抽出物のヘキサン抽出前後の HIF 活性を調べたところ、油分を含む成分は、油分を含まない成分に比して HIF 阻害効果に変化がなかったことから、水溶性成分に生理活性物質が含まれていると推察できる。また、有効成分はジペプチド、アミノ酸、核酸、ミネラルなどの低分子であると考えられ、有効成分の精製・同定が必要となる。また、これらの水溶性成分はマウスの成長に影響を与えないことを確認したが、他の生理学的応答の評価が必要である。

低酸素状態におかれた細胞には、HIF による様々な防御機構が存在する。例えば、HIF 下流因子である glucose transporter 1(GLUT1)や PDK1 の増加による ATP 産生の維持(Majmundar AJ. et al. *Mol Cell*. 2010, Kim JW. et al. *Cell Metab*. 2006)や、NADH dehydrogenase 1α subcomplex, 4-like 2(NDUFA4L2)や cytochrome C oxidase(COX)4-2 の誘導による ROS 産生抑制(Tello D. et al. *Cell Metab*. 2011, Fukuda R. et al. *Cell*. 2007)がある。これらの細胞防御機構とは別に、EPO による赤血球増加、VEGF や platelet-derived growth factor β polypeptide(PDGFB)による血管増殖により組織における酸素環境を維持している(Rey S. et al. *Cardiovasc*. 2010)。一方で、糖尿病網膜症や加齢黄斑変性等の慢性眼疾患においてはこれらの増殖因子の増加が病期の進行・予後に大きく関与する。血管内皮細胞の形態変化や異常血管新生、細胞外マトリックスの増生は、周辺組織障害や線維性瘢痕を引き起こし視機能に影響を及ぼす。よってこの２つの慢性眼疾患において、これらの増殖因子を制御する HIF を治療標的とすることは妥当である。

一方、低酸素のみならず炎症性サイトカインによっても HIF 転写は亢進する。炎症急性期反応を担う nuclear factor-kappa B(NF-kB)や IL-6 等のサイトカインによって誘導される signal transducer and activator of transcription(STAT3)は HIF1a を誘導することが知られている(Rius J. et al. *Nature*. 2008, JE Jung. et al. *FASEB J*. 2005)。特に加齢黄斑変性では、加齢、光障害、喫煙等による酸化ストレスによって引き起こされる網膜色素上皮細胞(RPE)の炎症性変化が一因と報告されている(Anderson DH. et al. *Am J Ophthalmol*. 2002, Donoso LA. et al. *Surv Ophthalmol*. 2005)。ここでは炎症性転写因子を介した HIF の活性化が存在すると考えられ、加齢黄斑変性においても HIF が治療標的となりうる。

現在、糖尿病網膜症や加齢黄斑変性の標準治療は VEGF 抑制であるが、長期的な抗 VEGF 薬投与による視細胞や RPE 細胞の萎縮が報告されている。また、成体マウスの RPE における VEGF 遺伝子欠損は、視細胞や脈絡膜の変性を誘発することが知られている。一方で網膜における HIF

遺伝子欠損は生理的な異常を示さずに、RPE における HIF 遺伝子欠損がマウスレーザー誘導 CNV を抑制したという報告がある。VEGF 抑制は、網膜や脈絡膜の生理的な血管恒常性を維持するために必要な VEGF をも減少させる可能性があり、一方で HIF 抑制は病的な VEGF のみを減少させることを示唆している。

また、頻回の抗 VEGF 薬硝子体注射は侵襲的であり、患者にとっては高額な費用がかかる。したがって、魚類抽出物およびその有効成分は安全性が高く経口摂取が可能であり、血管新生性網膜疾患の予防または治療として使用できる可能性がある。一方で、本研究でマウスに投与を行ったオアカムロ抽出物溶解液の量を 60kg のヒトに換算した場合、概算で 1 日あたり 12 個体に相当する。今後、抽出方法の検討、有効成分の同定、精製が必要である。最小有効投与量に関しては検討を行っておらず、今後追実験が必要と考える。

最後に、本研究の Limitation として、In vivo での HIF 動態の確認がなされていないことがあげられ、今後検討を行うべきであると考える。In vivo では、OIR マウスモデルを作成し魚類抽出物の投与を行い、網膜からの mRNA 抽出やタンパク抽出により HIF-αs と HIF 下流因子の発現変化を確認することができる。この際、1 匹の仔マウス網膜から採取できる mRNA、タンパクは微量であることから、十分量の匹数を用意する必要がある。In vitro での血管新生実験として、血管内皮細胞を用いた Tube formation assay、Migration assay、Proliferation assay 等が挙げられ、これらの実験系で魚類抽出物を添加することにより血管新生性変化の評価を行うことが可能である。しかし、網膜での病態を考慮すると、網膜神経組織の低酸素感知がパラクライン作用を持って血管内皮細胞へ与える影響が重要であり、血管内皮細胞を用いた培養実験系では病態生理を模倣できない可能性がある。このことから、In vivo 実験系でのタンパク・遺伝子発現変化を確認する必要があると考える。

5. 結論

本研究では新規 HIF 阻害剤として 6 種類の魚類を見出し、オアカムロはマウス OIR モデルにおいて網膜病的血管新生を抑制した。これらの魚類成分の投与は、網膜の HIF 阻害により網膜病的血管新生疾患を治療できる可能性があることが示唆された。

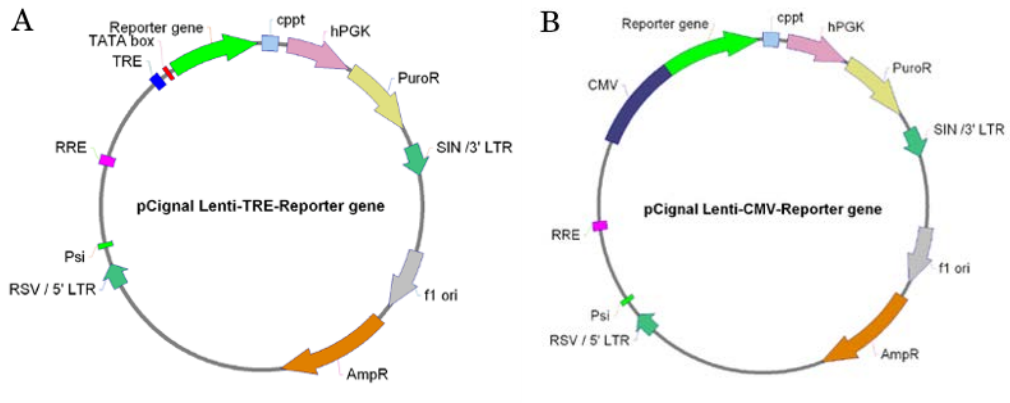

図 1　Transfer vector 概略図（Cignal Lenti Reporter Handbook, Qiagen, 2012 より抜粋）A) Cignal Lenti-TRE-Reporter gene. 特定の転写応答エレメント(TRE)に HIF が結合する低酸素応答エレメント(HRE)配列が相当する。B) Cignal Lenti-CMV-Reporter gene. CMV-Reporter gene として Renilla ルシフェラーゼ発現配列を含む。