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Eel Serum Synergistically Enhances Growth of Fish Cell Line GAKS

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Abstract

Generation of cell lines derived from fish is getting to increase. Generally, mammalian cell line culture needs appropriate media with approximately 5 to 10 % fetal calf serum. However, the optimal culture conditions for fish cell lines have not been established so far. We investigated the effect of exposure to inactivated fish serum on cell culture of goldfish scale cell line GAKS. The 5 % sera from gibel, carp, tilapia, and eel were toxic to GAKS cells. In addition, these fish serum were toxic to not only the GAKS, but also the mammalian cell lines HEK293 and SH-SY5Y. GAKS cells did not proliferate under the culture condition with 5% fish serum. Although it is known that fish serum contains thiaminase, the effect of supplemental addition of vitamin B1 was negligible for the proliferation of GAKS cells. Lower concentrations of fish sera, especially less than 5 % of eel serum stimulated the cell survival of GAKS compared with 5 % of each fish serum. The culture medium containing around 1 % eel serum coupled with 1% FCS was comparable to 5 % FCS conditions for proliferation of GAKS cells. Furthermore, the passage culture was successful under these culture conditions.

Culture techniques of animal-derived cell lines are very important in life science research. For mammalian cell lines, the liquid medium containing appropriate concentrations (5–10 %) of fetal calf serum (FCS) or newborn calf serum (NCS) is commonly used. Since, the FCS and NCS can supply trace amounts of essential components, including hormones, vitamins, and growth factors, and stimulate the cell proliferations[1)]. Recently, generated cell lines isolated from fish are getting to increase[2,3)], which culture medium commonly contains FCS in spite of non-mammalian cells. In addition, safety check before commercial distribution is necessary to avoid the risk of abnormal form prion infection on the bovine spongiform encephalopathy problem. However, the optimal culture conditions for fish cell lines have not been established so far. Insulin-like growth factor-I, insulin, growth hormone, and thyroxine were detected in the plasma of fish including gilthead seabream (*Sparus aurata*)[4)], coho salmon (*Oncorhynchus kisutch*)[5)], atlantic salmon (*Salmo salar*)[6)], and channel catfish (*Ictalurus punctatus*)[7)]. A probable fibroblast growth factor was obtained from the swim bladder of red seabream (*Pagrus major*)[8)]. Furthermore, primary cultures of cells from fish gills and kidneys were developed using serum from the North African catfish (*Clarias gariepinus*)[9)]. Therefore, fish serum maybe expected potential to have some growth-stimulating effects on fish cells.

This study was performed to investigate the effect of exposure to fish serum on cell culture of goldfish scale cell line GAKS. Although we demonstrated that fish serum was inferior to FCS in potential to proliferate GAKS cells, low concentration of eel serum synergistically enhanced the cell growth when given concomitantly with lower concentration of FCS.

Materials and Methods

Fish

Gibel (*Carassius auratus langsdorfii*), 20–25 cm in length

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weighing 65–70 g, and carp (*Cyprinus carpio*), 30–35 cm in length weighing 70–85 g were caught in Nagata river, Kagoshima. Tilapia (*Oreochromis niloticus*), 20–35 cm in length weighing 60–70 g were caught in Minato river, Ibusuki. Cultured eel (*Anguilla japonica*), 50–55 cm in length weighing 200–250 g were purchased from Sueyoshi Co. in Kagoshima. They were starved for several days before experiments.

Preparation of fish serum

After ice-cold anesthesia, each species fish blood was collected from a vein of 1–3 fishes using a 23 G syringe needle for a single experiment. The collected blood was pooled and was left to stand at 4 ˚C overnight. After that the fish blood was centrifuged at 800 x g, 4 ˚C for 15 min to remove clots, and the supernatant was filtered with a 0.2 μm-membrane filter after inactivation at 60 ˚C for 30 min. The filtrated fish serum was stored at –25 ˚C until medium preparation.

Cell viability studies

Colorimetric assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to assess the viability of the cells *in vitro* as described in our previous report[10]. Exponentially growing cells were trypsinized and harvested, and equal numbers of cells in 200 μl of several concentrations of fish serum-, FCS- or both serum-containing DMEM were inoculated into each well of a 96-well microplate and then they were incubated for 1 or 3 days. Thereafter, 50 μl of 1 mg/ml MTT solution was added to each well, and the plates were incubated for 3 h at 37 ˚C in a CO_2 incubator. After aspirating the culture medium, the resulting formazan was dissolved with dimethylsulfoxide. Plates were placed on a shaker for 5 min and read absorbance immediately at 570 nm with a microplate reader, SunRise, TECAN, Switzerland, and cell viability was determined.

Dialysis and ultrafiltration of fish serum

To evaluate the effect of macromolecules in gibel serum and eel serum on the cell survival rate of GAKS, these sera were dialyzed with ice-cold PBS overnight. The dialyzed fish serum consisting of macromolecules, which molecular weight was larger than approximately 12,000, were filtrated with 0.2 μm-membrane filter and used immediately.

To evaluate the effect of small molecules in the gibel serum and eel serum on the cell survival rate of GAKS, these sera were centrifuged in a Spin-X UF6® (Corning) at 10,000 x g for 30 min at 4 ˚C. The pass-through fraction consists of small molecules, which molecular weight was less than approximately 5,000, were filtrated with 0.2 μm-membrane filter and used immediately.

Morphological analysis after passage

Exponentially growing GAKS cells were harvested into centrifugation tube after trypsinization, and centrifuged at 800 x g for 3 min at room temperature. Cell pellets were re-suspended in 5 ml of DMEM containing the several concentrations of inactivated eel serum, and then inoculated to the fresh flask and cultured in a CO_2 incubator. After the cells were confluently grown, the cells were re-seeded as described in above. Cells were repeated the passage once more. Cells (1 x 10^6 cells/25 cm^2 flask) were seeded and cultured for 24 h, the morphology was analyzed using phase-contrast microscopy.

Statistical Analysis

Differences between groups were analyzed using Wilcoxon-Mann-Whitney test. $P < 0.05$ was considered significant.

Results

Effect of gibel serum treatment on growth of cell lines

We investigated whether supplemental treatment with gibel serum for 24 h accelerates proliferation of GAKS cells (Fig.1). Contrary to expectation, 5 % gibel serum induced considerable cytotoxicity compared with the under serum-free condition. In contrast, GAKS cells proliferated well under the

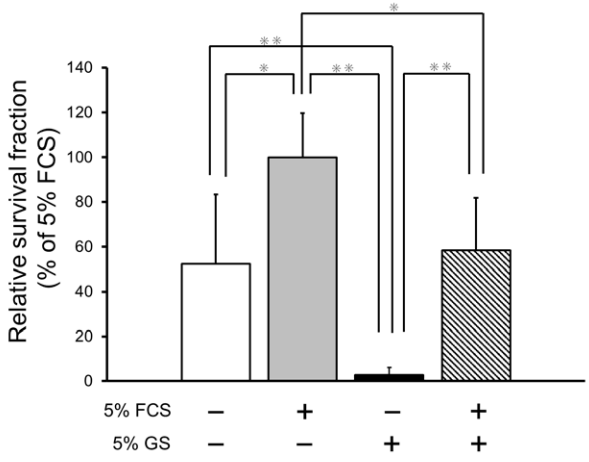

Fig.1. Effect of supplemental addition of gibel serum on cell growth of GAKS.
GAKS cells were cultured with DMEM containing 5 % FCS, 5 % gibel serum (GS), or both sera. Cell survival fraction was determined by MTT assay. The data represent the mean values ± SD of five independent experiments each performed in triplicate. *p<0.05 and **p<0.01 were considered significant.

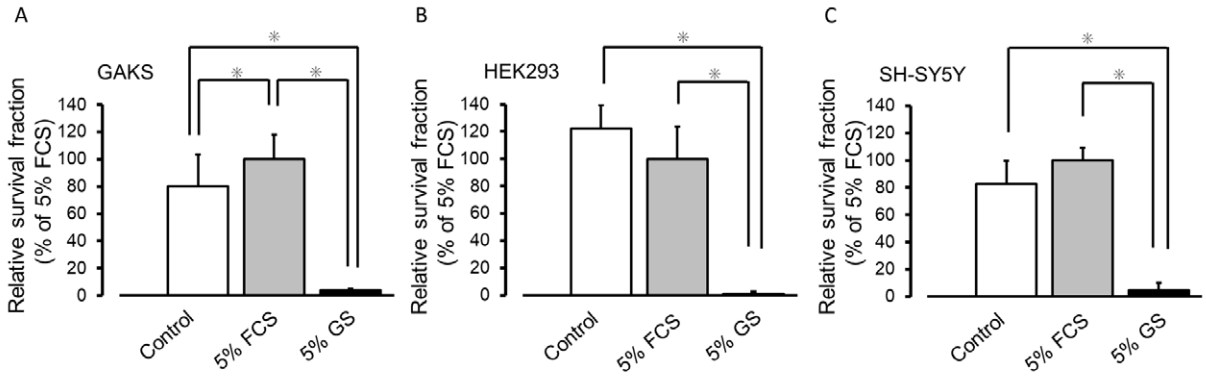

Fig. 2. Effect of supplemental addition of gibel serum on growth of mammalian cell lines.
HEK293 (B) and SH-SY5Y (C) as well as GAKS (A) cells were cultured with DMEM containing 5 % FCS or 5 % gibel serum (GS). Control indicates the survival fraction under serum-free conditions. Cell survival fraction was determined by MTT assay. The data represent the mean values ± SD of five independent experiments each performed in triplicate. *p<0.05 was considered significant.

conditions of 5 % FCS.

We analyzed the specificity of the potent cytotoxicity of gibel serum to GAKS cells. We compared the cytotoxicity of gibel serum to GAKS cells with HEK293 and SH-SY5Y cells. Cytotoxicity was detected in HEK293 cells and SH-SY5Y cells as well as GAKS cells, suggesting gibel serum possesses cytotoxicity to wide spectrum, at least not only fish cell line but also mammalian cell lines (Fig. 2).

Next, we verified the effect of thiamine (vitamin B1) treatment on attenuation of gibel serum cytotoxicity, since gibel serum contains potent thiaminase activity[11]. Although cell viability seemed to be recovered slightly but negligible after addition of vitamin B1 at double-concentration in DMEM (Fig.3), suggesting the major causative agent of the toxicity in gibel serum was not thiaminase.

Comparison of cytotoxicity among the fish serum from several species and its concentration dependency

We verified whether the toxicity of gibel serum is specific to this species or not. Then, we compared the cytotoxicity of gibel serum with fish serum from other species such as carp, tilapia, and eel. In addition, we analyzed the concentration dependency on the cytotoxicity of the fish serum. Interestingly, the cytotoxicity of gibel serum was increased in a concentration dependent manner up to 2.5 %. The higher concentrations, more than 2.5 % up to at least 10 %, of gibel serum induced equivalent cytotoxicity to GAKS cells (Fig. 4-A). Survival fraction under the 0.1 % gibel serum condition was higher than that of serum-free condition, but its survival fraction was lower than that of 5 % FCS condition (Fig. 4-A). In the case of carp serum, the tendency was similar, but was higher toxicity than that of gibel serum (Fig. 4-B). Tilapia serum was also potently toxic, and the concentration dependency of toxicity was broad (Fig. 4-C). Eel serum was most effective for proliferation of GAKS cells. The concentration of 1.0 % eel serum was most effective to proliferation of GAKS cells. Over 5 % up to 10 % eel serum was toxic (Fig. 4-D).

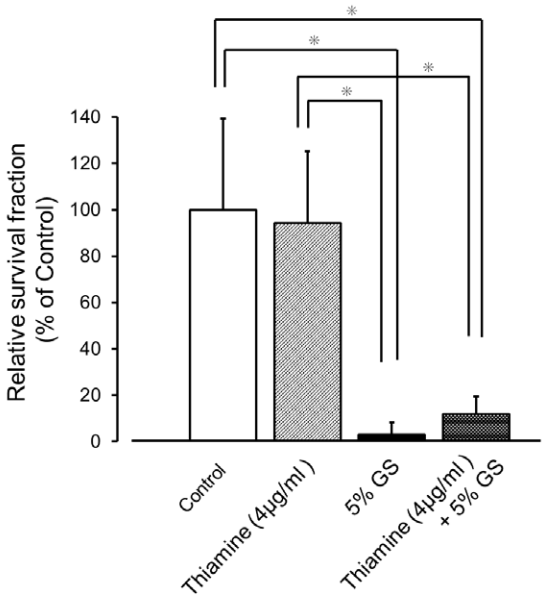

Fig. 3. Effect of supplemental addition of thiamine on cell growth of GAKS.
GAKS cells were cultured with DMEM with or without 5 % gibel serum (GS) and thiamine (4 μg/ml). Cell survival fraction was determined by MTT assay. The data represent the mean values ± SD of five independent experiments each performed in triplicate. *p<0.05 was considered significant.

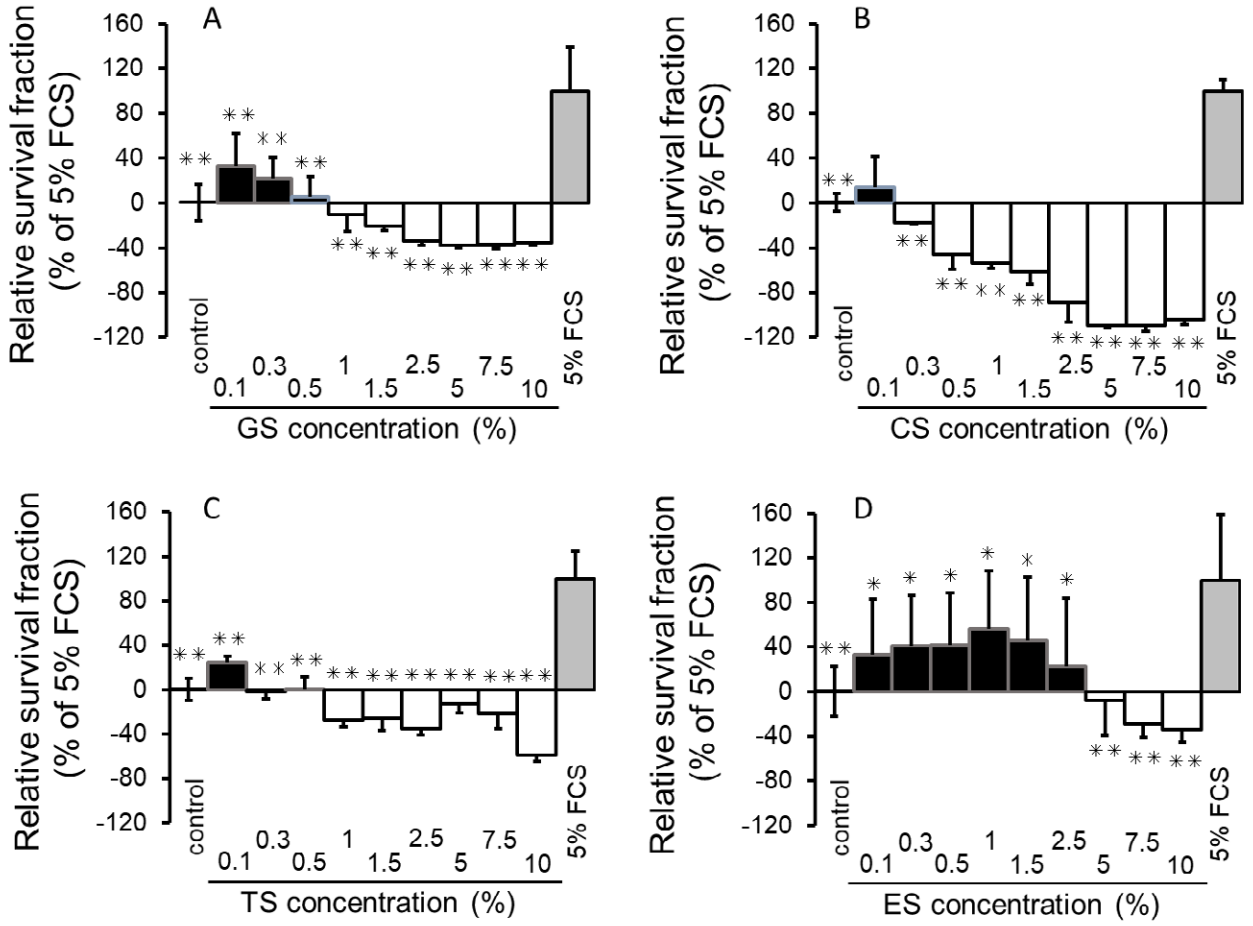

Fig. 4. Effect of several concentrations of fish serum on cell survival.
GAKS cells were cultured with DMEM medium containing several concentrations of serum from gibel (GS) (A), carp (CS) (B), tilapia (TS) (C), and eel (ES) (D) between 0 to 10%. Cell survival fraction was determined by MTT assay. The data represent the mean values ± SD of five independent experiments each performed in triplicate. *p<0.05 and **p<0.01 were considered significant.

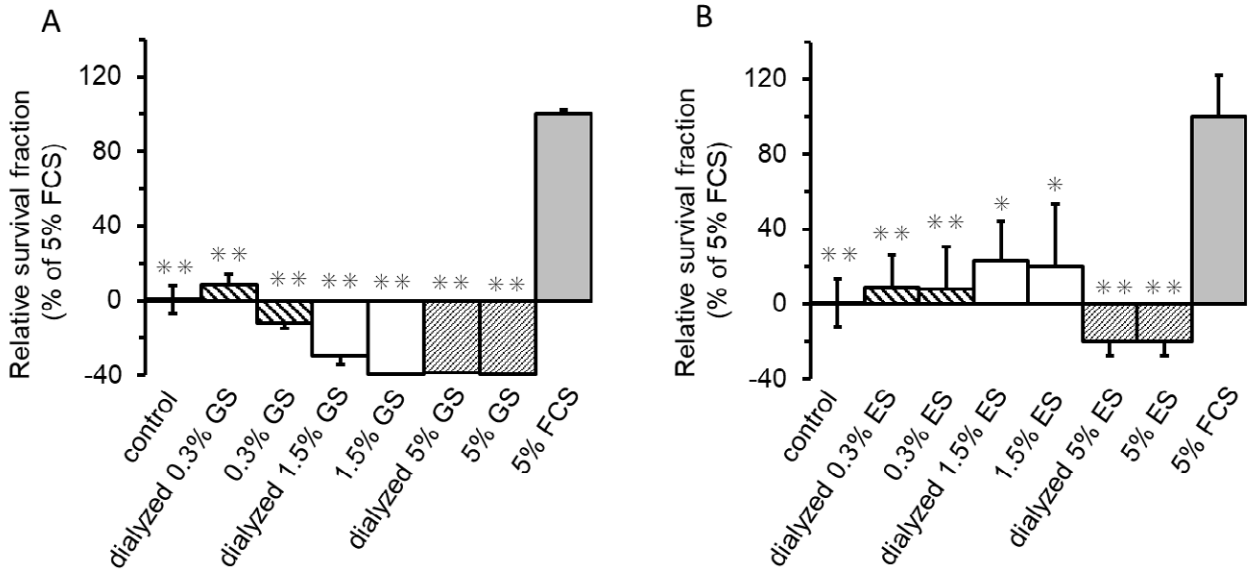

Fig. 5. Effect of supplemental addition of macromolecular fraction from gibel serum (GS) and eel serum (ES) on cell growth of GAKS.
Several concentrations of gibel and eel serum were added to GAKS cells after dialysis. Cell survival fraction was determined by MTT assay. The data represent the mean values ± SD of five independent experiments each performed in triplicate. *p<0.05 and **p<0.01 were considered significant.

Effect of dialysis and ultrafiltration of fish serum on the proliferation of GAKS cells

To verify whether the major causative agent of toxicity in gibel serum and eel serum was macromolecules such as proteins, lipids, and so on, or small molecules such as peptides, amino acids, ions, and chemical compounds, and so on, these fish sera were dialyzed or applied to ultrafiltration before viability assay. The effects of dialysis both of gibel and eel both sera were biologically negligible on the acceleration of GAKS proliferation (Fig. 5). As shown in Fig. 6-A, after ultrafiltration, pass-through small molecular fraction of gibel serum increased the cell survival rate of GAKS compared with Fig. 4-A. In contrast, the pass-through fraction of eel serum decreased cell survival rate compared with whole intact one in Fig. 4-D (Fig. 6-B).

Passage culture of GAKS cells using eel serum containing DMEM

We attempted to perform passage culture of GAKS cells using DMEM containing 1 % eel serum. However, the cells did not proliferate any more (data not shown). Next, we tried to perform passage culture of GAKS cells using DMEM supplementary contains combination of lower concentration of FCS and 1 % eel serum. Combination of 1 % FCS and 1 % eel serum was equivalently enhanced the proliferation of GAKS cells to 5 % FCS-containing DMEM (Fig. 7-A). Furthermore, it was confirmed that passage culture was successfully performed using DMEM containing the 1 % FCS coupled with 1 % eel serum using by cell morphology observation (Fig. 7-B).

Discussion

Cell culture technology on life science is commonly useful tool. Generally, FCS has been supplementary added to culture medium for not only mammalian cells but also other cell lines including fish. We investigated whether fish serum can replace to FCS for cell culture of fish and mammalian cell line. While optimal temperature for teleost cell culture is lower than that of mammalian cell lines in general, gold fish scale fibroblast-derived cell line GAKS is established for 37 ˚C culture as well as mammalian cell lines[12]. At first, we attempted to improve the culture condition for GAKS cells using gibel serum instead of FCS. Interestingly, gibel serum was toxic to GAKS cells. The gibel serum was also toxic to mammalian cells, HEK293 and SH-SY5Y. While teleost possess complement components as well as mammalian, alternative pathway is functionally more critical than that of mammalian[13,14]. Yano et al.[13] reported that unlike other teleost, eel possess more potent complement activity via another system in addition to

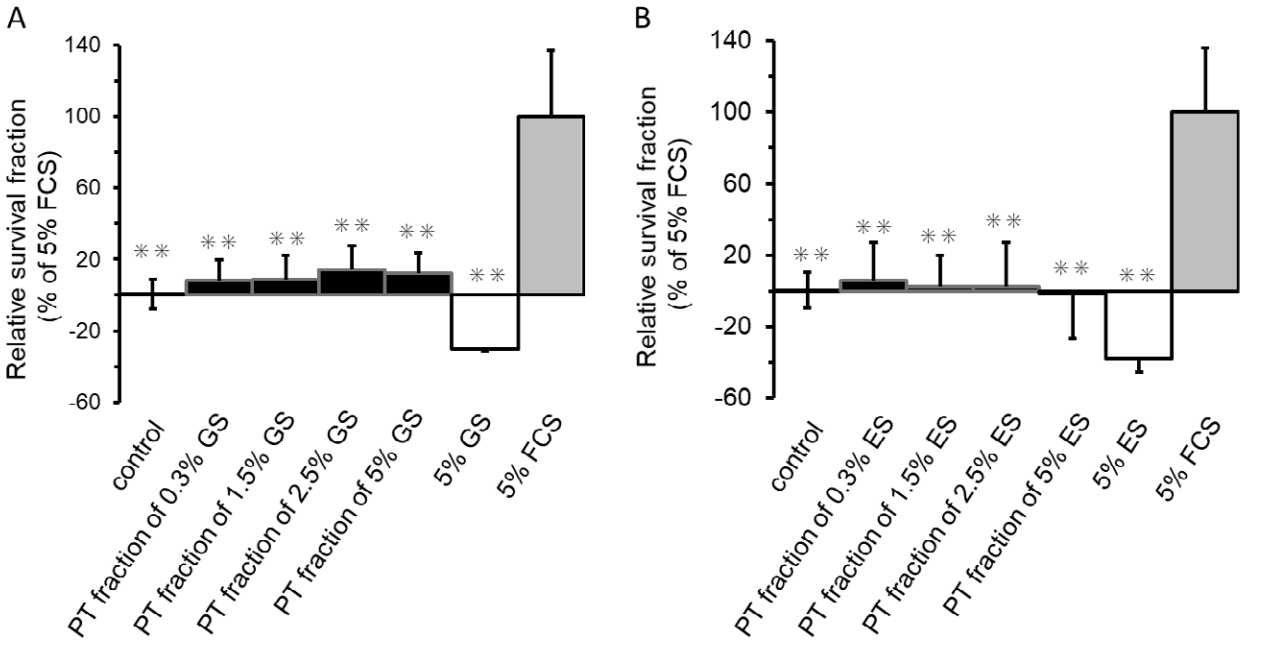

Fig. 6. Effect of supplemental addition of small molecules from gibel serum (GS) and eel serum (ES) on cell growth of GAKS.
Several concentrations of gibel serum (A) and eel serum (B) were applied to ultrafiltration, and consequent pass through (PT) fraction was added to GAKS cells. Cell survival fraction was determined by MTT assay. The data represent the mean values ± SD of five independent experiments each performed in triplicate. **$p<0.01$ was considered significant.

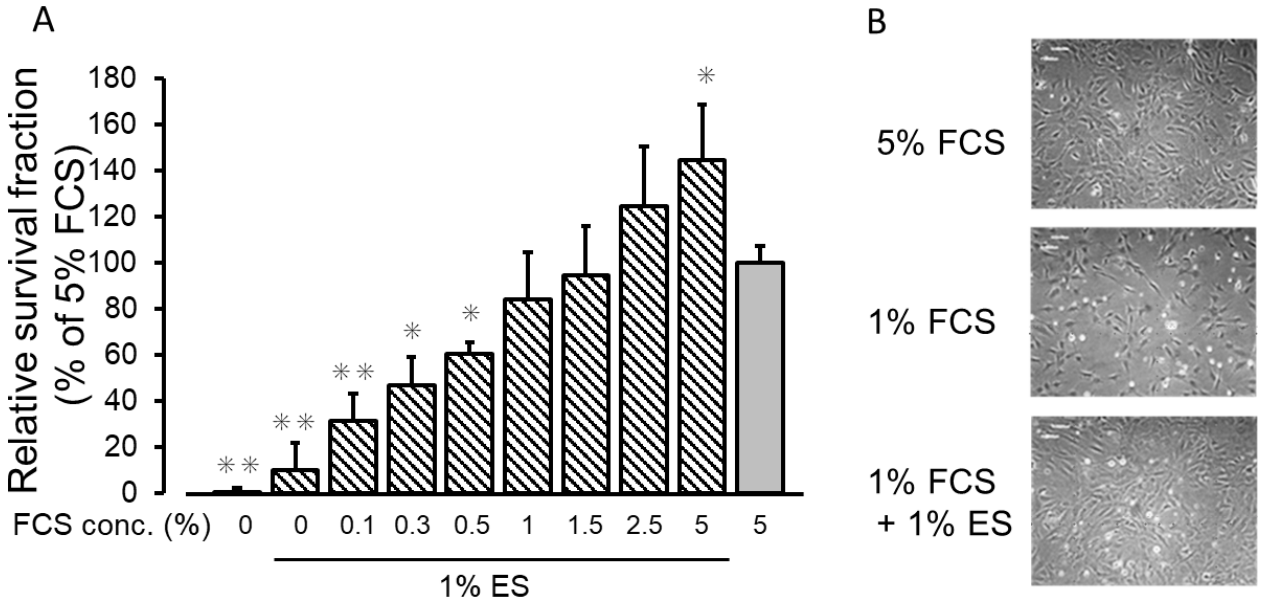

Fig. 7. Effect of supplemental addition of FCS coupled with eel serum (ES) on cell growth of GAKS.
GAKS cells were cultured with DMEM medium containing several concentrations of FCS coupled with 1 % ES. Cell survival fraction was determined by MTT assay (A). The data represent the mean values ± SD of five independent experiments each performed in triplicate. *p<0.05 and **p<0.01 were considered significant. Cell morphologies of GAKS under the culture conditions such as 5 % FCS, 1 % FCS, and 1 % FCS coupled with 1 % ES were observed with microscope (B).

alternative pathway. In this study, we used fish serum after inactivation to avoid the effect of complement.

Fish serum including gibel serum contains high amount of thiaminase[11]. We speculated that gibel serum onset toxicity to the cell lines caused by depletion of vitamin B1. We supplementary added vitamin B1 to the cells at the same amount of that in the culture medium. However, the effect of vitamin B1 was negligible for cell growth of GAKS cells.

We investigated the effect of serum from several species of fish including carp, tilapia, and eel. Carp and tilapia sera were toxic which could be contained cell cycle arrest and cellular senescence to GAKS cells as same as gibel serum. Hashimoto et al.[15] reported that carp serum stimulated growth of primary cultured cells from goldfish fin, however, in our study, the carp serum was toxic to cell line from goldfish scale. In this study, small molecular fraction, which molecular weight was less than approximately 5,000, of gibel serum after ultrafiltration increased cell survival rate of GAKS. In contrast, the effect of dialysis which means macromolecules (larger than approximately 12,000) was negligible. These results suggested that the macromolecule fraction of gibel serum contained toxic components. In contrast, eel serum was more effective to cell growth stimulation. Dialysis was not effective to excrete the major causative component, in addition, small molecules fraction after the ultrafiltration decreased cell survival rate of GAKS. These results suggested that middle range molecule fraction of eel serum between MW 5,000 to 12,000, pertinent to e.g. peptides maybe possess growth factor activity. Eel serum contains proteinaceous toxin, which can be inactivated by heat treatment at 60 ˚C for 30 min[16,17]. Only the inactivated eel serum was most appropriate supplement for GAKS cell culture among the fish sera used in the present study. Around 2 % of inactivated-eel serum from some individual was completely comparable to 5 % inactivated-FCS for cell growth of GAKS cells (data not shown). Japanese eel possesses very low-density lipoprotein (VLDL), but not high-density-lipoprotein (HDL), as the main component in the blood plasma, unlike most teleosts where HDL is the main component[18]. In primary cultures of goldfish caudal fin in a medium containing a carp serum, cell proliferation activity was detected in HDL fraction[19]. In addition, the eel blood plasma is held in low concentration of bilirubin and in high concentration of biliverdin, unlike mammalian and other teleost fish plasma[20, 21]. Furthermore, eel possess a novel green fluorescent protein (eelGFP) in the muscle cells[22,23]. The eelGFP needs bilirubin as a ligand to express fluorescence[24,25]. Further study to clarify the component in the unique teleost eel and the functional

mechanism to stimulate cell growth is needed.

In conclusion, eel serum was potently effective to proliferation of GAKS cells. Furthermore, combination of 1 % eel serum coupled with 1 % FCS was good enough for the culture of GAKS cells. Further studies to identify the growth factor in the eel serum and mechanisms of its proliferative stimulation activity on the balance against the toxicity are expected.

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References

1) Hayashi, S. and M. Komatsu (1998). Primary culture of eel hepatocytes - Synthesis and secretion of lipoprotein. In: Cell Tissue Culture:Laboratory Procedures. (A. Doyle, J.B. Griffiths and D.G. Newell eds.) ECACC, Centre for Applied Microbiology and Research, John Wiley and Sons LTD, Baffins Lane, Chichester, England, 23A:2.1-2.10.

2) Fujiwara, M., R. Tsukada, I. Shioya, and M. Takagi (2009). Effects of heat treatment and concentration of fish serum on cell growth in adhesion culture of Chinese hamster ovary cells. *Cytotechnology*, 59, 135–141.

3) Radošević, K., M. Cvjetko, N. Kopjar, R. Novak, J. Dumić, and V.G. Srček (2013). In vitro cytotoxicity assessment of imidazolium ionic liquids: Biological effects in fish Channel Catfish Ovary (CCO) cell line. *Ecotoxicology and Environmental Safety*, 92, 112–118.

4) Funkenstein, B., A. Silbergeld, B. Cavari, and Z. Laron (1989). Growth-hormone increases plasma-levels of insulin-like growth-factor (IGF-1) in a teleost, the gilthead seabream (*Sparus-aurata*). *J. Endocrinology*, 120, R19-R21.

5) Larsen, D.A., B.R.Beckman, and W.W. Dickhoff (2001). The effect of low temperature and fasting during the winter on metabolic stores and endocrine physiology (insulin, insulin-like growth factor-I, and thyroxine) of Coho Salmon, *Oncorhynchus kisutch*. *General and Comp. Endocrinol*., 123, 308–323.

6) Nordgarden, U., T. Hansen, G-I. Hemre, A. Sundby, and B.T. Björnsson (2005). Endocrine growth regulation of adult Atlantic salmon in seawater: The effects of light regime on plasma growth hormone, insulin-like growth factor-I, and insulin levels. *Aquaculture*, 250, 862–871.

7) Small, B.C., and B.C. Peterson (2015). Establishment of a time-resolved fluoroimmunoassay for measuring plasma insulin-like growth factor I (IGF-I) in fish: effect of fasting on plasma concentrations and tissue mRNA expression of IGF-I and growth hormone (GH) in channel catfish (*Ictalurus punctatus*). *Domestic Animal Endocrinology*, 28, 202–215.

8) Suzuki, T., T. Kurokawa, and M. Asashima (1994). Identification of a heparin-binding, mesoderm-inducing peptide in the swim-bladder of the red seabream, *pagrus-major* – A probable fish fibroblast growth-factor. *Fish Physiology and Biochemistry*, 13, 343-352.

9) Rathore, G., N. Sood, and R. Swaminathan (2001). Primary cell culture from fish gills and kidney using fish serum. *Indian Journal of Experimental Biology*, 39, 936–938.

10) Komatsu, M., T. Sumizawa, M. Mutoh, Z. Chen, K. Terada, T. Furukawa, Yang, X.L., H. Gao, N. Miura, T. Sugiyama, and S. Akiyama (2000). Copper-transporting P-type adenosine triphosphate (ATP7B) is associated to the base *Cancer Res*., 60, 1312–1316.

11) Suomalainen, P. and A.M. Pihlgren (1995). On the thiaminase activity of fish and some other animals and on the preservation of thiaminase in silage made from fish. *Acta Agralia Fennica*, 83, 221–229.

12) Chigwechokha, PK., M. Tabata, S. Shinyoshi, K. Oishi, K. Araki, M. Komatsu, T. Itakura, and K. Shiozaki (2015). Recombinant sialidase NanA (rNanA) cleaves α2-3 linked sialic acid of host cell surface N-linked glycoprotein to promote Edwardsiella tarda infection. *Fish Shellfish Immunology*, 47, 34–45.

13) Yano, T., Y. Hatayama, H. Matsuyama, and M. Nakao (1988). Titration of the alternative complement pathway activity of representative cultured fishes. *Nippon Suisan gakkaishi,* 54, 1049–1054.

14) Nonaka, M., and S.L. Smith (2000). Complement system

of bony and cartilaginous fish. *Fish and Shellfish Immunology*, 10, 215–228.

15）Hashimoto, H., H. Toyohara, Y. Yokoyama, M. Sakaguchi, K. Ozato, and Y. Wakamatsu (1997). Effects of carp serum on the growth of goldfish fin cells in early passage. *Journal of Fish Biology*, 50, 201–207.

16）Halstead, B.W. (1967). Poisonous and venomous marine animals of the world, vol 2. United States Government Printing Office, Washington DC, pp 951–981.

17）Yoshida, M., S. Sone, and K. Shiomi (2008). Purification and Characterization of a proteinaceous toxin from the serum of Japanese eel *Anguilla japonica*. *Protein J.* 27, 450–454.

18）Ando, S., and M. Matsuzaki (1996). A unique lipoprotein profile found in the plasma of cultured Japanese eel Anguilla japonica: very low-density lipoprotein, but not high-density lipoprotein, is the main component of plasma. *Fish Physiology and Biochemistry*, 15, 469–479.

19）Kondo, H., and S. Watabe (2006). Growth promoting effects of carp serum components on goldfish culture cells. *Fisheries Science*, 72, 884–888.

20）Ellis, M.J., and J.J. Poluhowich (1981). Biliverdin concentrations in the plasmas of fresh and brackish water eels, *Anguilla Rostrata*. *Comp. Biochem. Physiol*., 70A, 587–589.

21）Endo, M., T. Sakai, T. Yamaguchi, and H. Nakajima (1992). Pathology of jaundice in the cultured eel Anguilla japonica. *Aquaculture*, 103, 1–7.

22）Hayashi, S., Toda, Y., (2009). A novel fluorescent protein purified from eel muscle. *Fisheries Science*, 75, 1461–1469.

23）Funahashi, A., M. Komatsu, T. Furukawa, Y. Yoshizono, H. Yoshizono, Y. Orikawa, S. Takumi, K. Shiozaki, S. Hayashi, Y. Kaminishi, and T. Itakura (2016). Eel green fluorescent protein is associated with resistance to oxidative stress. *Comp. Biochem. Physiol*., 181-182C, 35–39.

24）Kumagai, A., R. Ando, H. Miyatake, P. Greimel, T. Kobayashi, Y. Hirabayashi, T. Shimogori, and A. Miyawaki (2013). A bilirubin-inducible fluorescent protein from eel muscle. *Cell*, 153, 1602–1611.

25）Funahashi, A., T. Itakura, A.A.I. Hassanin, M. Komatsu, S. Hayashi, and Y. Kaminishi (2017). Ubiquitous distribution of fluorescent protein in muscles of four species and two subspecies of eel (genus *Anguilla*). *J. Genetics*, 96, 127–133.