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研究課題名（和文）魚類の給餌を最適化する新たなアプローチ：オートファジーによる生体防御機構の解明

研究課題名（英文）A New Approach to Optimize Feeding of Fish: Elucidation of Organism Defense Mechanism by Autophagy

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交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：本研究は、魚病に関連したプロオートファジーヘキソキナーゼ(HK)の機能解析を目的に行った。まず4つのHK遺伝子(I, II, IIIおよびIV) を同定後、肝臓組織の培養によりそれら発現を確認した。またプロモーター活性、魚病細菌暴露およびクロマチン免疫沈降を実施した。魚のHKは、哺乳類のHKと高い配列類似性を持ち、肝臓やステロイド産生組織で強く発現し、飢餓や魚病細菌の感染に発現応答することを明らかにした。またHKII活性には性差があり、いくつかの抗 およびプロ オートファジー遺伝子を介したエストロゲン依存性を示した。さらに、HKIIが性依存的に雌のオートファジーに重要であると推測された。

研究成果の学術的意義や社会的意義
本研究で明らかにしたヘキソキナーゼとその関連因子は、魚類の健康状態を調べるための優れた指標となることから、環境に配慮した養殖魚の疾病予防対策および養殖生産性の向上に貢献するとともに、消費者に対するより安心で安全な水産物の提供に繋がる。また、魚類において見出したオートファジー機構の性差は、人を含めた脊椎動物における疾患や病態の性による差異にかかわる分子・細胞機構の理解に寄与すると考えられる。

研究成果の概要（英文）：Autophagy, cells own repair mechanism, promotes health and longevity in various animals. In this study, we aimed to understand the role of autophagy, especially the pro-autophagic hexokinase (HK) genes, in fish disease management. We identified four HKs (HKI, HKII, HKIII & HKIV), characterized their effects in in vitro and ex vivo liver culture, analyzed the promoter activity in vitro, conducted Edwardsiella tarda challenge experiment, and performed the Chromatin immunoprecipitation analysis. We found that, fish HKs have high sequence similarity with mammalian ones, prevalent in liver and other steroidogenic tissues and was affected by starvation and early stages of E. tarda infection. Interestingly, only HKII showed a sex-biased and estrogen-dependent activity via several anti (mTOR) and pro(AMPK and ULK)-autophagic genes. Further analysis confirmed that HKII-mediated mitophagy are also sex biased, confirming the fact that HKII has significance in gender dependent female autophagy.

研究分野：Fish physiology

キーワード：Autophagy　Disease　Liver　fish　hexokinase

１．研究開始当初の背景

Feeding status plays a major role in the transmission of the disease and feeding restrictions are highly effective against fish diseases such as red sea bream Iridoviridae and Edwardsiellosis. On the other hand, undernourishment can be a factor for poor health and other illnesses. Therefore, optimal feeding has become an extremely important proposition in controlling the growth and disease of farmed fish, and there is a need to support an appropriate feeding method based on scientific evidence. So far, many studies have been conducted on the relationship between feeding, metabolism, and biological defense, mainly in mammals. Furthermore, dietary restrictions have been shown to have health benefits not only in mammals but also in fish. Moreover, previously I found that short-term starvation is effective in improving immunity and disease resistance using small model fish and farmed fish, and thus hypothesizing the existence of a universal health protection mechanism through dietary restriction.

Interestingly, in recent years, autophagy, a process by which the cells self-degrade under nutrient deprivation or stress, is gaining tremendous importance in health and longevity. In case of nutritional deficiency, the cells are deprived of nutrients by themselves and are thus actively involved in the maintenance of cell function. Autophagy has also been shown to function in infection control, disease healing and promoting longevity. Although such information is considered to be extremely useful in optimizing the feeding amount of farmed fish, but unfortunately not much studies have been conducted on fish and fish health. So, in this research, we have focused to understanding the importance of autophagy and disease prevention mechanisms in fish.

２．研究の目的

Aquaculture industry has tremendous potential for food security and nutritional supply for ever growing human population, and it is expected that aquaculture production will be strengthened and further expanded in near future. However, at fish farming production sites, high production costs and mortality due to fish diseases are significantly reducing the fisherman's profitability. In fish farming, majority of the production cost is forage (about 60% for red sea bream and 70-80% for yellowtail; Ministry of Agriculture, Forestry and Fisheries "Fishery Management Survey Report"). Therefore, there is a strong demand to reduce wasteful feeding and curtail the cost of feeding. On the other hand, with regard to fish diseases, the annual nationwide loss is about 10 billion yen (2013 Fisheries White Paper), which is a huge disadvantage. Damage caused due to fish diseases not only results in loss of products, but also requires a great deal of cost for medicines such as vaccination. In a study conducted earlier, I found that short-term feeding is effective in improving immunity and disease resistance using small model fish and farmed fish and suggested that autophagy may play a central role in this cascade. In addition, the autophagy cascade is known to be universal among fish species and vertebrates. Therefore, by elucidating this mechanism, it is possible to understand the related mechanism of feeding metabolism-biological defense for the first time in fish, and to obtain a physiological index for deciphering the appropriate feeding state.
So, the purpose of this study is to construe the autophagic actions in fish, at molecular and cellular level, and understand the self defense mechanisms in fish.

３．研究の方法

A. Identification of hexokinases and characterization: Genes were scouted from available red seabream database and confirmed by gene specific PCR using liver CDNA. Various tissues were collected for RNA analysis, and Realtime PCR was used to measure the transcription. Simultaneously, a hepatocyte culture system for red seabream was established, serum and glucose starvation were conducted, and transcriptional alterations were measured using Realtime PCR.
B. Elucidation of the hexokinase dependent autophagy induction: Hexokinase ORFs were cloned into expression vector and overexpressed in hepatocyte cultures using electroporation protocol. The autophagic gene expression were analyzed and localization of various autophagic proteins were assessed using live cell immunohistochemistry and fluorescent

microscopy. Immunoprecipitation (IP) was performed to identify directly linked proteins.
C. Elucidation of upstream regulatory factors of hexokinases (HK): 5kb promoters were isolated from red seabream and medaka genomes, cloned and used for dual luciferase assay using HEK293 cells. Either Estrogen, androgen or estrogen receptor (ER) were added/overexpressed simultaneously to determine promoter activity. Estrogen receptor knockout medaka were used to identify the ER-HK associations and Realtime, Chromatin Immunoprecipitation (ChIP) and immunohistochemistry data were obtained.
D. Analyzing the fasting dependent disease improvement mechanisms
Adult Medaka and red seabream were either starved, exposed to fish pathogenic bacteria (*Edwardsiella tarda*) and virus (Iridovirus), or concomitantly starved and exposed, and liver and serum samples were collected. Analysis was performed using Realtime PCR, immunohistochemistry and biochemical analysis of both female and male fish. Mitochondria were isolated from cultured hepatocytes, subjected to various stressors, and analyzed using specific staining and microscopy.

４．研究成果
A. Identification of hexokinases and characterization:
I have isolated four homologues of Hexokinase (HK), i.e., HKI, HKII, HKIII and HKIV and one alternatively spliced truncated isoform of HKI (hereafter named as HKIb) from red seabream liver cDNA.

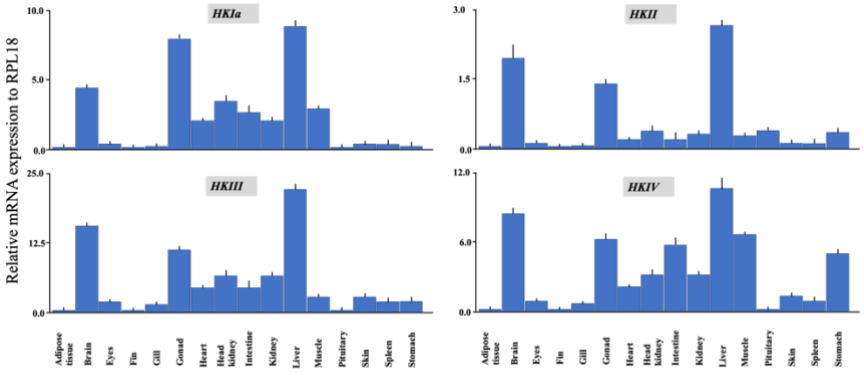

Figure 1: *Tissue distribution of various HK. Note: Data were plotted as mean; Error bar= Standard error; Number of fish=10*

Interestingly, all the full-length HK showed high similarity (>70%) with their mammalian counterpart and depicted similar domain characteristics. Tissue distribution analysis showed that HK were most abundant in liver, followed by gonad or brain (Figure 1). Additional analysis showed that, HK1a and HK1b were both abundant in liver but the truncated form was relatively more abundant in the male. Further, 12 hours of serum and glucose starvation of primary hepatocyte culture depicted significant increase in HK transcription (Figure 2).

B. Elucidation of the hexokinase dependent autophagy induction:
HKIa and HKII were cloned into mammalian expression vector and transfected to primary hepatocyte culture. We found that, HKIa overexpression aggravated the starvation effects (Figure 2). Further analysis showed that subsequent pro-autophagic genes (AMPK, ULK1 and LC3) were significantly upregulated in all groups, while the anti-autophagic genes (mTOR) were drastically reduced. The data were further confirmed using immunohistochemistry.

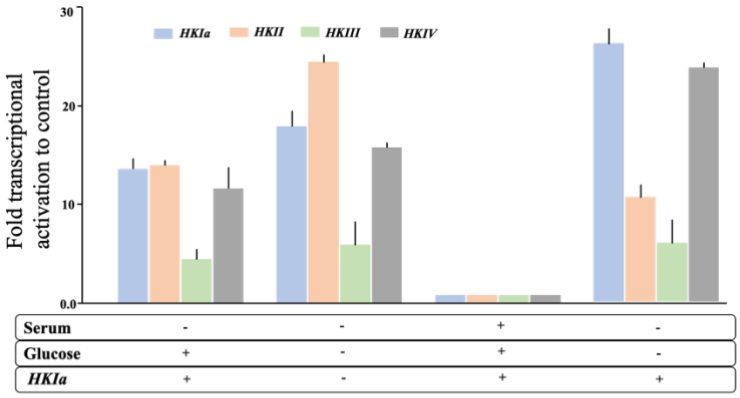

Figure 2: *Invitro effects of serum and glucose stravation, HKIa overexpression on HK proliferation in primary cultured hepatocyte. Note: Serum (10%) and glucose (0.2mg/ml) supplimented groups were used as control; Data were plotted as mean; Error bar= Standard error; N=12*

IP analysis showed that both mTOR and HKI are the direct targets of HKII, while HKIV is the direct target for only HKI. Our study suggests that HKII regulates the expression of HKI, so in our subsequent analysis, we focused on HKII only.

C. Elucidation of upstream regulatory factors of hexokinases:

HKII was isolated from both medaka and red seabream and cloned into a 5KB promoter sequence. It was found that 17b-estradiol significantly increased the HKII promoter activity *in vitro*. The activities were further induced by co-transfection with ERs (Figure 3). Later, using two Estrogen receptor Knockout (ER-KO) medaka line and ChIP analysis, it was confirmed that HKII is a direct target of ERs (Mohapatra et al., 2020).

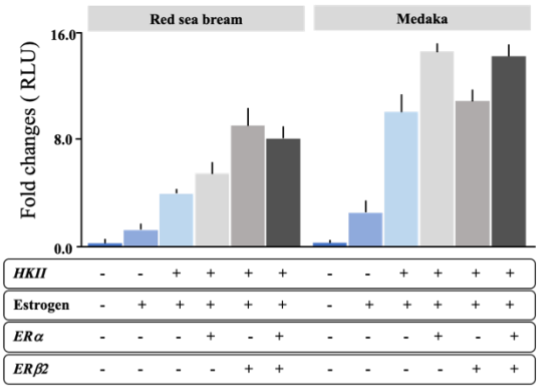

Figure 3: *Red seabream and medaka HKII promoter analysis by dual luciferase assay using HEK293 cells. Data were plotted as mean RLU; Error bar= Standard error; experimental replicates(N)= 6*

D. Analyzing the fasting dependent disease improvement mechanisms

Several experiments using medaka or red seabream, i.e., starvation, 17b-estradiol manipulation, high temperature, bacterial and viral treatment were performed to decipher the HKI mediated autophagy mechanisms *in vivo*. Importantly we found that, starvation induced HKII-AMPK-ULK pathway while steroid manipulation or temperature stress bypassed the AMPK action and shifted them to HKII-ULK pathway. We also determined that HKII pathway was accelerated during early stages of *E. tarda* infection in both species, but not from iridoviral infection. Further in-depth analysis confirmed that, starvation mediated HKII modulations were highly corelated with circulating steroid concentration (Figure 4). To validate the estrogen-hexokinase association hypothesis, we conducted a comparative starvation analysis using ER-KO fish and found a strong female biasness in HKII mediated autophagy in medaka liver (Mohapatra et al., 2020) .

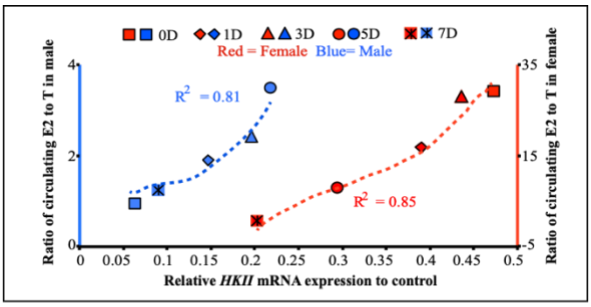

Figure 4: *Invivo analysis of Starvation, circulating steroids and HKII correlation in Red seabream*

Further, we isolated the mitochondria from female and male red sea bream liver hepatocytes, subjected it to various stressors (starvation, high temperature and steroid manipulation) and infections, and found a clear sex specific differences in mitochondria HKII expression in all the samples except for the iridovirus infected hepatocytes. Notably, our *in vivo* data suggests that all these events reduce the glucose content in mitochondria. Cumulatively, our finding denotes that HKII, ER regulated glycolytic molecule, can sense mitochondrial glucose derangements, and modulate its intracellular localization and thus can regulate mitophagy and cellular autophagy in fish, and therefore can be used as a universal health status marker for fish.

５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　2件／うちオープンアクセス　1件）

１．著者名 Sipra Mohapatra, Tapas Chakraborty, Sonoko Shimizu, Kayoko Ohta, Yoshitaka Nagahama, Kohei Ohta	４．巻 27
２．論文標題 Estrogen and estrogen receptors chauffeur the sex-biased autophagic action in liver	５．発行年 2020年
３．雑誌名 Cell Death & Differentiation	６．最初と最後の頁 3117-3130
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41418-020-0567-3	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 2.Tapas Chakraborty, Sipra Mohapatra, Lin yan Zhou, Takahiro Matsubara, Taisen Iguchi, Yoshitaka Nagahama	４．巻 13
２．論文標題 Estrogen receptor β2 oversees germ cell maintenance and gonadal sex differentiation in medaka, Oryzias latipes	５．発行年 2019年
３．雑誌名 Stem Cell Reports	６．最初と最後の頁 419-433
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.stemcr.2019.07.013	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Noman Reza Mohammad Ali、Mohapatra Sipra、Shimizu Sonoko、Kitamura Shin-Ichi、Harakawa Shogo、Kawakami Hidemasa、Nakayama Kei、Sawayama Eitaro、Matsubara Takahiro、Ohta Kohei、Chakraborty Tapas	４．巻 82
２．論文標題 Molecular cloning, characterization and expression analysis of complement components in red sea bream (Pagrus major) after Edwardsiella tarda and red sea bream Iridovirus (RSIV) challenge	５．発行年 2018年
３．雑誌名 Fish & Shellfish Immunology	６．最初と最後の頁 286～295
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.fsi.2018.08.027	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計3件（うち招待講演　2件／うち国際学会　3件）

1．発表者名 Sipra Mohapatra, Tapas Chakraborty, Takahiro Matsubara, Kohei Ohta
2．発表標題 Sex and sex steroids are eminent regulators of fish autophagy
3．学会等名 10th International Conference on Fisheries and Aquaculture, Toronto, Canada（招待講演）（国際学会）
4．発表年 2019年

1．発表者名 Tapas Chakraborty, Sipra Mohapatra, Sonoko Shimizu, Takahiro Matsubara, Kiyoshi Naruse, Kohei Ohta
2．発表標題 Comparative transcriptomics and Edwardsiellosis detection in fish: a study using medaka and Red Sea bream
3．学会等名 10th International Conference on Fisheries and Aquaculture, Toronto, Canada（招待講演）（国際学会）
4．発表年 2019年

1．発表者名 Tapas Chakraborty, Sipra Mohapatra, Dipak Pandey, Takahiro Matsubara, Kohei Ohta
2．発表標題 Exploring the potentiality of autophagy in fish fertility.
3．学会等名 11th International Symposium on Reproductive Physiology of Fish (ISRPF), Manaus, Brazil, 2018.（国際学会）
4．発表年 2018年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
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６．研究組織（つづき）

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究協力者	太田　耕平 (Ohta Kohei)		
研究協力者	清水　園子 (Shimizu Sonoko)		

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関