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科学研究費助成事業　　研究成果報告書

令和　７ 年　 ６ 月 １９ 日現在

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研究課題名（和文）魚類の幹細胞培養系とin vitro ゲノム編集による生殖幹細胞の未分化維持機構

研究課題名（英文）Invitro genome editing in aquaculture

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：本研究は、魚類のin vitro配偶子産生のためマサバの生殖幹細胞（GSC）の培養・分化誘導、ゲノム編集、クローン選抜及びGSCの代理親魚への移植法を確立した。またin vitro GSC培養において、生殖細胞への分化誘導を行うことで複数の生殖細胞分化因子を同定した。GSC培養において、生殖細胞への分化誘導を行うことでfshを含む複数の生殖細胞分化因子を同定した。しかしGSCから機能的配偶子への誘導は未だ達成されておらず、さらなる研究が必要である。また今回同定された因子の一つであるFSHは魚類卵成熟における必須因子の一つでもあり、GSCにおける機能維持、分化に対する詳細な機能解析が必要である。

研究成果の学術的意義や社会的意義
We have establish the culture, in vitro genome editing techniques for chub mackerel GSC and produced genome edited porgeny. These, expectedly will improve and fast track the breeding of new fish strains, ease the species conservation and contribute towards future food and biodiversity security.

研究成果の概要（英文）：In vitro breeding, i.e. production of fish egg and sperm in vitro has ann enormous potential for aquaculture in future. Therefore, in this research, I have established the protocol for culture and differentiation, genome editing, clone selection and transplantation of chub mackerel germ line stem cell (GSC). I have created host for transplantation, performed transplantation and produce next generation from genome edited GSC and established that cultured GSC can produce progeny with the aid of appropriate surrogate. I have also differentiated the GSC into germ cell invitro and identified several differentiation factors, including FSH. Though Germ cell differentiation from GSC was achieved but complete differentiation of GSC into functional gamete needs future research. Additionally importance of FSH in fish GSC maintenance or differentiation requires indepth analysis.

研究分野：Reproduction

キーワード：stem cell　fish　culture　pluripotent　chub mackerel　differentiaiton　in vitro　FSH

１．研究開始当初の背景

With the rapid expansion of aquaculture, there are high expectations for the breeding of farmed fish. With the development of new breeding technologies such as genome editing and stem cell manipulation, if the proliferation and differentiation of germ stem cells, which are the origin of eggs and sperm, can be properly controlled in vitro (in vitro), breeding technology can be dramatically accelerated.

２．研究の目的

in this research, I aimed to elucidate the control mechanism of germline stem cell (GSC) proliferation and differentiation and establish an in-vitro genome editing methodology for future realization of in vitro fish breeding.

To achieve this, the objective of this research were:

1. Analysis of GSC maintenance, proliferation and differentiation mechanism.
2. In vitro genome editing of fish GSC and production of mutant individual
3. Identification of factors critical for GSC physiology.

３．研究の方法

Adult fish gonadal cell suspensions were sorted using antibody coated magnetic bead and used for culture using our patent pending protocol. Passages were done in every 3 days with daily media exchange. Periodic observation via fluorescent microscopy, immunohistochemistry and transcriptional analysis was conducted to assess the stemness and differentiation status. Low cell RNA sequencing analysis of fresh, cultured and differentiated GSC were performed using Illumina protocol. Electroporation or chemical transfection was used for Invitro genome editing of cultured GSC followed by serial-dilution culture for selection of pure clone. At each stage DNA was isolated and confirmed by HMA analysis and sequencing. Germ cell free surrogates were produced by DND gene editing through CRISPR/Cas9 and used for transplantation of cultured and in vitro genome edited GSC. Transplantation was carried out in immunosuppressant-treated germ cell-free adults and periodically observed for gonadal development and maturation. Mature fish were bred to raised progeny. Randomly collected progenies / fin clips were used for DNA based genotyping.

４．研究成果

We have isolated germline stem cell (GSC) from female chub mackerel and successfully cultured them under feeder free and serum free condition for 210 days (70 passage) (Figure 1). We performed comparative NGS analysis of freshly isolated GSC and cultured GSC, and found that the transcription of pluripotent (e.g. oct4, sox2, etc.) and other DNA methylation related gene markers increases while the differentiation markers (*Nanog, rar, rxr,* etc.) were significantly downregulated after culture (Figure 2). The candidate genes were confirmed using realtime PCR analysis of several marker gene. We also analyzed the cell morphology using antibody staining and found that GSC

maintain stemness throughout, except for cell stiffness which might be associated with excessive actin filamentation of cell membrane. We have tested bio-laminin as a substitute of vitronectin for 2D culture and found no significant differences in stem cell proliferation and maintenance. However, we found that, addition of Autophagy (rock) inhibitor significantly reduces the spontaneous differentiation. Simultaneously, when GSC were cultured on collagen-1 matrix or alginic acid bead differentiation was induced at 30^{0}C, evidenced by nanog expression and 40± 11.34% reduction in Alkaline phosphatase positive cells. Additionally, I observed that, a short period of temperature shock followed by treatment with retinoic acid and BMP2, 4 and 15 for a period of 1 week accelerates the GSC differentiation, reduces Oct4 expression and increases vasa (germ cell marker) expression in the cultured condition. However, the rate of differentiation was 20± 19.76%. Differentiation was significantly induced to 54± 6.11% by addition of follicle stimulating hormone (FSH). Therefore, in the next experiment, I tested SDS-clarified Japanese anchovy gonad as 3D collagen matrix to induce differentiation followed by treatment with retinoic acid, Bone morphogenic proteins and FSH and successfully induced Germ line stem cell (GSC) differentiation into germ cells expressing vasa, dazl (germ cell marker and even Sycp3 (meiotic marker) within 21 days, however no further development was observed (Figure 3). Simultaneously, low cell transcriptomic analysis of freshly isolated, cultured GSC and differentiated GSC were conducted (Figure 2) and data suggests that culture of GSC induces the expression of demethylases, alkaline phosphatase and cell differentiation related gene transcription while differentiated GSCs abundantly expresses above mentioned marker along with high expression in TGFb family transcription factors. Several TGFb transcription factors are critical for gonial differentiation in animals and thus it will be interesting to study which specific genes are critical for chub mackerel germ cell differentiation through functional analysis.

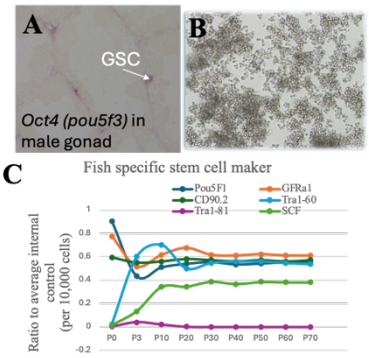

Figure 1: Culture and evaluation of chub mackerel GSC. (A) in situ hybridization showcasing GSC localization in the gonad. (B) GSC after 60 days of culture. (C) transcription of various stem cell specific genes in different passages. Note: P= passage.

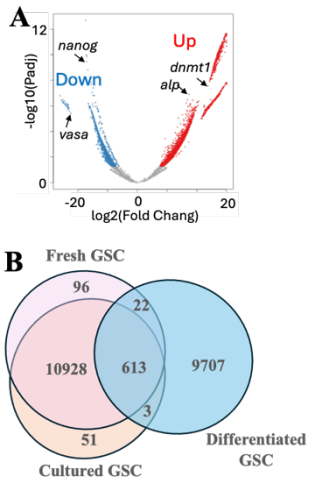

Figure 2: Comparative transcriptomic analysis of different GSC population. (A) fresh and cultured GSC. (B) volcano plot showing the commonness and unique transcriptional profiles of undifferentiated and differentiated GSC.

To develop an in vitro genome editing protocol and establish a methodology for Further we designed three different Crispr probe and tested transfection and electroporation of cultured GSC using cas9-GFP and performed HMA analysis of each colony. We found that 66-70% of the cells are mutated after 1 week of culture. Different dilution protocol were tested to obtain homogeneous population of mutant cells but failed to obtain a pure

FSH-KO GSC line. Moreover, most of the FSH-KO cell showed certain growth abnormality, especially failed to differentiate when treated with retinoic acid and BMP cocktail, though Nanog, rar, and rxr were significantly elevated, compared to their non-treated control. However, when synthetic FSH were added along with, differentiation into gonia (confirmed by expression of *vasa* and *dazl*) was observed. This, along with the differentiation induction results, suggests that FSH is a potential differentiation regulator in chub mackerel.

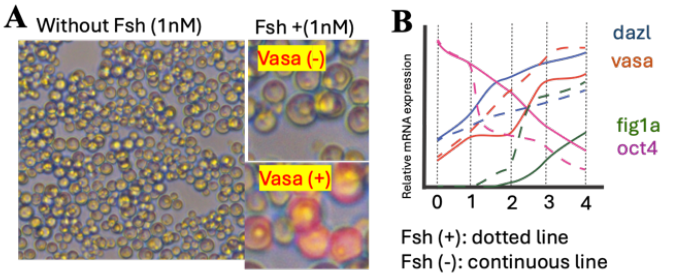

Figure 3: GSC differentiation in vitro. (A) photomicrographs and (B) qPCR analysis of germ cell related genes depicting the effects of Fsh on GSC differentiation.

So, to avoid any knockout associated germ cell physiology alteration, subsequently, cultured female GSC were transfected with SJ-AVTRV2- platinum-TALEN mRNA using Lipofectamine Stem cell Transfection Reagent, performed 3 rounds of serial dilution and homogeneous knockout cell population of AVTR-KO were obtained (Figure 4). Sanger sequencing analysis to confirm the homogeneity of the final cell population and 18 different homogeneous lines were obtained. A 11bp deletion mutant line was processed further for transplantation into DND-KO mackerel. Notably, host DND-KO mackerel were largely devoid of germ cell, evidenced by histology, immunohistochemical analysis of vasa and canulations were suggestive of gonadal sterility. Progeny were obtained 3 months after transplantation and progeny analysis using AVTR-KO specific PCR confirmed that at least 30% of them were homozygous for 11 bp AVTR deletion (Figure 5). Further, donor derived AVTR$^{\Delta 11}$ mutant progenies were grown, periodically analyzed and compared with previously generated AVTR$^{\Delta 5}$ mutants. No difference in growth, feeding behavior, gut physiology, and gonadal development was observed. The reproductive capabilities of donor derived AVTR$^{\Delta 11}$ mutant will be tested in future.

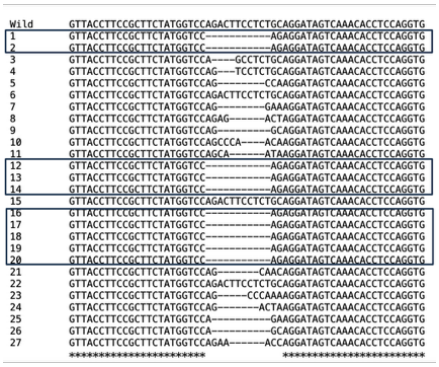

Figure 4: In vitro genome editing of cultured GSC. Note: Boxed clones are 11 base mutations.

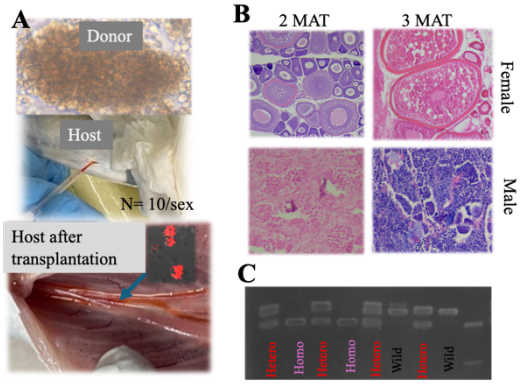

Figure 5: Production of AVTRv1a2$^{\Delta 11}$ mutant using invitro genome editing. (A) transplantation into adult gonad. (B) assessment of gonadal progression at 2 and 3 months of transplantation, MAT. (C) DNA based mutation analysis of progeny.

In summary, the research has progress more smoothly than initially planned. I have established the protocol for culture and differentiation, genome editing, clone selection

and transplantation of GSC. I have created host for transplantation, performed transplantation and produce next generation from genome edited GSC and established that cultured GSC can produce progeny with the aid of appropriate surrogate. I have also differentiated the GSC into germ cell invitro and identified several differentiation factors, including FSH. In future, I will also continue researching on increasing the differentiation of GSC, perform NGS analysis of various stages of differentiated GSC as well as different types of germ cells, identify the detailed differentiation mechanism, elucidated the FSH influences on early germ cell development and continue to decipher the fish germ cell biology.

５．主な発表論文等

〔雑誌論文〕　計7件（うち査読付論文　6件／うち国際共著　6件／うちオープンアクセス　4件）

１．著者名 Yahiro Issei、Barnuevo Kyle Dominic Eguid、Sato Oga、Mohapatra Sipra、Toyoda Atsushi、Itoh Takehiko、Ohno Kaoru、Matsuyama Michiya、Chakraborty Tapas、Ohta Kohei	４．巻 15
２．論文標題 Modeling the SDF-1/CXCR4 protein using advanced artificial intelligence and antagonist screening for Japanese anchovy	５．発行年 2024年
３．雑誌名 Frontiers in Physiology	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fphys.2024.1349119	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 Yahiro Issei、Sato Oga、Mohapatra Sipra、Mukai Koki、Toyoda Atsushi、Itoh Takehiko、Matsuyama Michiya、Chakraborty Tapas、Ohta Kohei	４．巻 351
２．論文標題 SDF-1/CXCR4 signal is involved in the induction of Primordial Germ Cell migration in a model marine fish, Japanese anchovy (Engraulis japonicus)	５．発行年 2024年
３．雑誌名 General and Comparative Endocrinology	６．最初と最後の頁 114476～114476
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.ygcen.2024.114476	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 Chakraborty Tapas、Yamamoto Yume、Hanai Shoma、Hirano Mayumi、Mohapatra Sipra、Yamaguchi Akihiko、Takeda Tatsusuke、Matsuyama Michiya、Ohta Kohei	４．巻 9
２．論文標題 Divulging the social sex change mechanism in a unique model system for studying the sexual plasticity of protogynous hermaphrodite fish, three bamboo leaf wrasse (Pseudolabrus sieboldi)	５．発行年 2022年
３．雑誌名 Frontiers in Marine Science	６．最初と最後の頁 NA
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fmars.2022.1048506	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 Chakraborty Tapas、Mohapatra Sipra、Matsuyama Michiya、Nagahama Yoshitaka、Ohta Kohei	４．巻 23
２．論文標題 Sex Lethal Gene Manipulates Gonadal Development of Medaka, Oryzias latipes, through Estrogenic Interventions	５．発行年 2022年
３．雑誌名 International Journal of Molecular Sciences	６．最初と最後の頁 15496～15496
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/ijms232415496	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 Roy Suvra、Kumar Vikash、Behera Bijay Kumar、Parhi Janmejay、Mohapatra Sipra、Chakraborty Tapas、Das Basanta Kumar	４．巻 9
２．論文標題 CRISPR/Cas Genome Editing?Can It Become a Game Changer in Future Fisheries Sector?	５．発行年 2022年
３．雑誌名 Frontiers in Marine Science	６．最初と最後の頁 NA
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fmars.2022.924475	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 Mukai Koki、Mohapatra Sipra、Matsuyama Michiya、Ohta Kohei、Chakraborty Tapas	４．巻 361
２．論文標題 Exposure effects of benzalkonium chloride (BAC) on gonadal physiology and fertility suppression in medaka (Oryzias latipes)	５．発行年 2024年
３．雑誌名 Environmental Pollution	６．最初と最後の頁 124778～124778
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.envpol.2024.124778	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 Galotta Mariel、Dam Anh Tuan、Eto Yuhei、Toyoda Atsushi、Itoh Takehiko、Mohapatra Sipra、Ogino Yukiko、Saitou Marie、Matsuyama Michiya、Chakraborty Tapas、Ohta Kohei	４．巻 11
２．論文標題 Insights into epigenetic regulation of cyp19a via comparative analysis using the scombrid chub mackerel as model	５．発行年 2024年
３．雑誌名 Frontiers in Marine Science	６．最初と最後の頁 1408561
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fmars.2024.1408561	査読の有無 無
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15件（うち招待講演　2件／うち国際学会　13件）

１．発表者名 江口瑛・八尋逸清・佐藤桜雅・Mohapatra Sipra ・ 向井幸樹・伊藤武彦・長野直樹・松山倫也・Chakraborty Tapas ・太田耕平
２．発表標題 マサバ黄体形成ホルモン（Lh）のノックアウトによる不妊化－妊性回復系統の作出の試み
３．学会等名 The Japanese society of fisheries sciences, spring meeting, 2024（国際学会）
４．発表年 2024年

１．発表者名
Chakraborty Tapas, Kawao Sota, Mohapatra Sipra, Matsuyama Michiya, Ohta Kohei, Nagahama Yoshitaka

２．発表標題
Role of Oct4 in gonadal sex maintenance of fish.

３．学会等名
The Japanese society of fisheries sciences, spring meeting, 2024（国際学会）

４．発表年
2024年

１．発表者名
佐藤桜雅・Chakraborty Tapas・加藤洋教・八尋逸清・ 入路光雄・伊藤武彦・長野直樹・ 松山倫也・太田耕平

２．発表標題
ゲノム編集と組換え濾胞刺激ホルモンによる マサバの生殖統御の試み

３．学会等名
水産増殖学会, 2023

４．発表年
2023年

１．発表者名
Chakraborty Tapas, Mohapatra Sipra, Ohta Kohei, Matsuyama, Michiya, Nagahama Yoshitaka

２．発表標題
Oct4 and sexuality in fish gonad; an understanding using Medaka, Oryzias latipes

３．学会等名
12th International Symposium on Reproductive Physiology of Fish, Greece（国際学会）

４．発表年
2023年

１．発表者名
Ohta Kohei, Chakraborty Tapas, Tateyama Kazuki, Hanai Shoma, Mizumura Kodai, Yamamoto Yume, Mohapatra Sipra, Yamasaki Yumiko, Toyoda Atsushi, Itoh Takehiko, Matsuyama Michiya, Nagahama Yoshitaka

２．発表標題
Molecular mechanism of sexual plasticity in a natural sex changing fish.

３．学会等名
12th International Symposium on Reproductive Physiology of Fish, Greece（国際学会）

４．発表年
2023年

１．発表者名
Tapas Chakraborty

２．発表標題
Genome Editing And Future In Fisheries

３．学会等名
CAS traininng in biotechnology, Central agricultural university, India（招待講演）

４．発表年
2023年

１．発表者名
Tapas Chakraborty, Sota Kawao, Kodai mizumura, Akira Eguchi, Sipra Mohapatra, Takehiko Itoh, Atsushi Toyoda, Naoki Nagano, Kohei Ohta, Michiya Matsuyama

２．発表標題
Gonadal stem cell: a possible solution of futuristic aquaculture.

３．学会等名
令和 5 年度日本水産学会春季大会（国際学会）

４．発表年
2023年

１．発表者名
Kodai Mizumura, Tapas Chakraborty, Kazuki Tateyama, Shoma Hanai, Yoshihara Yuya Yume Yamamoto, Sipra Mohapatra, Yumiko Yamasaki, Atsushi Toyota, Ito Takehiko, Michiya Matsuyama, Yoshitaka Nagahama, Kohei Ohta

２．発表標題
Gonadal stem cells in a sex changing fish, Pseudolabrus sieboldi.

３．学会等名
The International Symposium “Totipotency and Germ cell Development” （国際学会）

４．発表年
2022年

１．発表者名
水村航大・Chakraborty Tapas・八尋逸清・太田耕平・ 長濱嘉孝・ 松山倫也

２．発表標題
海産魚における生殖幹細胞の長期培養法

３．学会等名
JSFS autumn meeting（国際学会）

４．発表年
2022年

１．発表者名
Ohta K, Chakraborty T, Tateyama K, Hanai S, Mizumura K, Kazui Y, Yoshihara Y, Mohapatra S, Matsuyama M, Nagahama Y

２．発表標題
Molecular mechanism of sexual plasticity in fish: a viewpoint from a natural sex changing fish.

３．学会等名
Joint Conference of the 30th European Society for Comparative Endocrinology and the 9th International Society for Fish Endocrinology. Faro, Portugal.（国際学会）

４．発表年
2022年

１．発表者名
Chakraborty T, Mohapatra S, Ohta K, Matsuyama M, Nagahama Y

２．発表標題
Oct4 and sexual plasticity in fish gonad.

３．学会等名
Joint Conference of the 30th European Society for Comparative Endocrinology and the 9th International Society for Fish Endocrinology. Faro, Portugal.（国際学会）

４．発表年
2022年

１．発表者名
Tapas Chakraborty, Sipra Mohapatra, Kohei Ohta, Michiya Matsuyama, Yoshitaka Nagahama.

２．発表標題
Molecular mechanism of sexual plasticity in fish: a perspective from gonadal stem cell.

３．学会等名
31st Conference of European Comparative Endocrinologists (CECE), Ancona, Italy（国際学会）

４．発表年
2024年

１．発表者名
Sipra Mohapatra. Tapas Chakraborty, Yoshitaka Nagahama, Kohei Ohta, Michiya Matsuyama

２．発表標題
Autophagy and gonadal sexuality in fish.

３．学会等名
31st Conference of European Comparative Endocrinologists (CECE), Ancona, Italy（国際学会）

４．発表年
2024年

１．発表者名
Tapas Chakraborty, Sipra Mohapatra, Takehiko Itoh, Atsushi Toyoda, Kohei Ohta, Michiya Mat

２．発表標題
Gonadal stem cell: a possible solution of continuous seed production and futuristic aquaculture.

３．学会等名
National Seminar on "Advances in Environment Management for Sustainable Fisheries and Livestock Production (AEMS-2024), Kishanganj, India（招待講演）（国際学会）

４．発表年
2024年

１．発表者名
Tapas Chakraborty, Sipra Mohapatra, Hajime Nakagawa, Akira Eguchi, Issei Yahiro, Hirofumi Ohga, Naoki Nagano, Yoshitaka Nagahama, Michiya Matsuyama, Kohei Ohta

２．発表標題
Development of feeder-free, long-term culture of gonadal stem cell and evaluation of their progeny production capability in chub mackerel

３．学会等名
2024 Japanese Society of Fisheries Science (JSFS) spring Meeting, Tokyo, Japan（国際学会）

４．発表年
2025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

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