細胞表面抗原を用いた魚類生殖細胞操作法の開発

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博士学位論文

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平成 29 年度
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目次

諸言

　生殖細胞は初期の分化段階において、始原生殖細胞から発生し、生殖腺の性に同調する形で、メスでは卵原細胞、オスではA型精原細胞へと分化することが知られている（Schulz et al., 2010; Lubzens, et al., 2010）。これら生殖細胞の性分化は、周囲の体細胞による影響を受けることで引き起こされる。すなわち、始原生殖細胞は周囲の体細胞の微細環境により卵、精子のどちらにも分化する能力を持つといえる。さらに、卵原細胞やA型精原細胞も性分化前の生殖腺に移植すると、始原生殖細胞と同様に周囲の体細胞の影響をうけ、体細胞の性に従い、卵と精子の両者に分化する能力を持ち合わせている（Okutsu et al., 2006; Yoshizaki et al., 2010, 2012）。本研究室ではこれらの性質を利用してニジマス（*Oncorhynchus mykiss*）の始原生殖細胞、卵原細胞、精原細胞等の未分化生殖細胞群をヤマメ（*Oncorhynchus masou masou*）の孵化稚魚腹腔内に移植し、宿主ヤマメを成熟させることで、ヤマメからニジマス由来の機能的な配偶子を作出することに成功している。この生殖細胞の異種間移植技術は代理親魚技術と呼ばれ、様々な魚種への応用が期待されている（Takeuchi et al., 2004; Okutsu et al., 2007; Yoshizaki et al., 2010）。

　その１つにタイヘイヨウクロマグロ（*Thunnus orientalis*; 以下クロマグロ）の配偶子生産への応用が挙げられる。クロマグロは高級魚として市場で取引されているが、近年過剰漁獲の影響により漁獲量が減少している。これらの対策として、クロマグロ種苗の放流による資源量の回復が考えられる。しかしクロマグロは、その完全養殖に成功しているものの、親魚の体重が 100kg 程度、年齢が５歳以上になって初めて成熟するため、飼育管理に莫大なスペース、コスト、労力を必要とする（Masuma et al., 2011）。一方、同じサバ科魚類であるがマサバ（*Scomber japonicus*）やゴマサバ（*Scomber australasicus*）は体サイズがはるかに小型であり、飼育環境下では、体重約 200g 以上、満１年程度で採卵可能である（石橋, 2007）。そこでクロマグロの未分化

生殖細胞をマサバやゴマサバ等の小型のサバ科魚類に移植し、クロマグロ生殖細胞を保育した小型サバを代理親として成熟させることで、サバからクロマグロ由来の機能的な配偶子を作出可能となると期待されている。これにより、飼育管理にかかるスペース、コスト、労力を大幅に削減することが可能となる。また、小型サバ類は調温、調光が可能な陸上水槽で成熟までの飼育が可能である（石橋, 2006）。そのため、クロマグロ生殖細胞を移植した宿主を人為的に催熟することで、周年を通してクロマグロ種苗の供給も可能になると期待される。

　代理親魚を実際に作出するには、まずドナーとなる魚の生殖腺を摘出し、酵素を用いて組織片を分散し、生殖腺の細胞懸濁液を調整する。次にその細胞懸濁液をマイクロインジェクターにより、孵化仔魚腹腔内に顕微注入するという操作が必要となる。これまでの研究により、この顕微注入時に使用する細胞懸濁液の中に含まれる一部の始原生殖細胞、A型精原細胞、卵原細胞のみが宿主生殖腺に生着する能力を有することが明らかとなっている（Takeuchi et al., 2004; Yano et al., 2008; Yoshizaki et al., 2010）。つまり代理親魚技術においては、ドナー生殖腺に含まれる一部の未分化生殖細胞をレシピエントとなる魚の腹腔内に移植することが操作の本質である。クロマグロ代理親魚技術を樹立する際は、これら一連の操作をクロマグロおよび宿主となるサバ科魚類を用いて構築しなければならないが、現段階では効率的な移植技術を樹立するうえでいくつかの課題が残されている。

　まず1つ目は、精原細胞をはじめとする未分化生殖細胞群の迅速な可視化ができないことである。ドナー生殖腺から調整した細胞懸濁液には、未分化生殖細胞の他に生殖細胞を保育する生殖腺体細胞、さらに分化した生殖細胞や血球等様々な細胞種が数多く含まれている。さらに、生殖腺中における未分化生殖細胞の存在比は雌雄差、成熟段階、季節、性別により大きく異なり、状況次第では未分化生殖細胞の存在比が多い生殖腺を選んで使用しなければならない。例えば、ニジマスにおいては、未成熟オスにおける全精巣細胞中のA型精原細胞の存在比は30〜50%程度であるのに対し、

未成熟メスにおける全卵巣細胞中の卵原細胞の存在比は 3〜5％とオスの 10 分の 1 以下である（Kise et al., 2012; 市川, 2008）。またドナーが成熟している場合オスの精巣は大量の精子で満たされており、A 型精原細胞の存在比が 1％未満になるケースも少なくない。このように未分化生殖細胞の存在比が低いドナーを移植に用いると、必然的に宿主腹腔内に移植可能な未分化生殖細胞数も減少することにより、その移植効率が不安定になってしまうケースが存在する。そのため、ドナー細胞中に含まれる未分化生殖細胞をリアルタイムで可視化しながら、適切なドナーを選抜することが極めて重要となる。

　2 つ目はクロマグロ未分化生殖細胞の特異的な単離ができないことである。クロマグロのように飼育、種苗生産の困難な種においては特定の成熟段階のドナー生殖腺を安定的に調達することが難しく、必ずしも未分化生殖細胞の存在比が高い生殖腺をドナーとして使用できるとは限らない。さらに、宿主魚にも制約が存在する。本法では移植した細胞が免疫拒絶により宿主腹腔内で消失することを防ぐために、移植には免疫機能が未発達な孵化稚魚、仔魚を用いなくてはならない。魚類の孵化稚魚、仔魚は一般的に小型であるため、腹腔内の容積も非常に小さい。そのため移植可能な細胞数には物理的な限界が存在する。例えばサケ科魚類の場合は一匹当たり 10 万程度までの細胞を移植可能だが、孵化仔魚の体長が小さい海産魚においては、1 匹あたり 1〜2 万細胞程度しか移植ができない（Yazawa et al., 2010; Morita et al., 2012）。こうした背景より、未分化細胞の存在比が低い生殖腺をドナーとして用いる際に、移植する細胞数を増やすことで移植効率をあげるという戦略には限界がある。そこで、全生殖腺細胞中から未分化生殖細胞のみを特異的に単離することで、移植に用いる全細胞中に占める未分化生殖細胞の存在比を上げることが可能となれば、上記のような状況であっても、高い移植効率を維持できると期待される。

　3 つ目は移植したクロマグロ未分化生殖細胞を宿主腹腔内で追跡することができないことである。クロマグロ生殖細胞の移植後に移植された細胞と宿主自身がもともと

保持している生殖細胞を宿主の腹腔内で識別するのは非常に困難である。さらに移植細胞を PKH26 などの蛍光色素で標識したとしても、それら蛍光色素は処理に供した全細胞を標識してしまうため、宿主生殖腺へと生着した細胞が確かに未分化生殖細胞であるかを判断することが難しい。そのため、現段階ではドナー細胞を宿主に移植した場合、その移植効率は宿主が成熟するまで飼育し、交配実験を行うまでは分からない。すなわち移植成功の可否が明らかになるのは移植を行ってからおよそ 1 年後になる。そのためドナー細胞特異的な追跡、検出法を樹立しこれらの細胞の、宿主生殖腺へ移動、取り込み、生着、増殖を経時的に観察しながら各種条件の至適化を行うことが極めて重要となる。

　以上、クロマグロ代理親魚技術を効率的に樹立するために、ドナー細胞の可視化、単離、追跡といった各種細胞操作が極めて重要であることを概説した。従来、本研究室では、遺伝子導入魚を使用することでこの課題を解決してきた。本研究室が樹立した p*vasa-gfp* 遺伝子導入ニジマスは未分化生殖細胞で特異的に発現する *vasa* 遺伝子の発現制御領域に *gfp* 遺伝子を接続した（Yoshizaki et al., 2000; Takeuchi et al., 2002)コンストラクトが導入されており、未分化生殖細胞で特異的に GFP 蛍光を発する。そのため、蛍光観察のみで未分化生殖細胞を識別することが可能である。本系統を用いることで、ドナー細胞に含まれる未分化生殖細胞の割合の評価や移植後に宿主生殖腺内での未分化生殖細胞の追跡を容易に行うことができる（Takeuchi et al., 2004; Okutsu et al., 2006; Okutsu et al., 2007; Yoshizaki et al., 2010)。また、個々の細胞の大きさ、内部構造、蛍光強度などを指標に高速で細胞を分取できるフローサイトメーターと呼ばれる装置を用いることで、GFP 蛍光を指標に未分化生殖細胞を分取、濃縮することも可能である（Takeuchi et al., 2002; Kobayashi et al., 2004; Yano et al., 2008; Yoshizaki et al., 2010)。

　上記のように p*vasa-gfp* 遺伝子導入魚は代理親魚技術構築において大きく貢献したが、p*vasa-gfp* 遺伝子導入魚を新たな魚種で樹立するのは容易ではない。本系統を樹

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立するには、まず受精卵に外来遺伝子を注入する操作が必要になる。次に、これらの操作を施した親世代個体中から遺伝子が導入された個体を探し出し、野生型の個体やヘテロ個体との 1 対 1 交配を繰り返すことで、外来遺伝子が導入されているアリルをホモに持つ個体を作出しなければならない。これらの操作を行うためには、受精卵を自在に得ることができる技術、外来遺伝子が導入されている個体を成熟するまで維持管理できる安定した飼育技術、1 対 1 交配を自在に行うことができる技術が必要である。そのため、クロマグロのような親魚養成、産卵誘発、種苗生産の困難な魚種において遺伝子導入魚を樹立することは現実的ではない。

こうした背景から遺伝子導入技術を用いずにクロマグロ未分化生殖細胞の可視化、単離、追跡といった細胞操作を可能とする技術が必要とされている。そこで本研究ではこれらの細胞操作を行うために、細胞表面抗原に着目した。一般的に細胞は細胞膜表面に細胞種特異的なタンパク質や糖鎖が局在している。そこで、その細胞種特異的なタンパク質や糖鎖に対して抗体を作製し、その抗体に対して様々な物質を結合させることで、遺伝子導入技術を用いることなく抗体陽性細胞の可視化や単離、追跡といった一連の細胞操作が可能となると考えた。

哺乳類の研究において細胞表面抗原の利用の歴史は古く、造血幹細胞の研究においては CD34、CD33、c-kit、Thy1 (Berenson et al., 1988; Andrews et al., 1989; Spangrude et al., 1889; Orlic et al., 1993; Sidney et al., 2014)等を用いることで、その分画化や単離が行われてきた。また精原幹細胞の研究においても CD9、 ITGB1、ITGA6、GFRA1、EPCAM、NCAM1、THY1、CDH1、MCAM (Oatley et al., 2008; Kanatsu-Shinohara et al., 2012)などを用いることで大幅にその分画を絞り込むことに成功している。しかしながら魚類の精原細胞あるいは精原幹細胞における細胞表面抗原の報告は非常に少なく、上記のマーカーもその多くが魚類では使用できないことが明らかとなっている (Nagasawa et al., 2010)。これまで本研究室の長澤らにより Ly75/CD205 が同定されているが、生きた細胞を染色したという報告はない (Nagasawa et al., 2010; 2012)。また林

らがニジマスの精原細胞の細胞表面抗原を認識可能なモノクローナル抗体群の作製に成功しているが、その抗原が明らかになっていないことに加え、糖鎖を認識する抗体が多いことが示唆されており、抗原のオルソログに対する抗体作成を行うのが現段階では難しいと考えられる (林ら，未発表)。

　そこで本研究では、新たにクロマグロ精原細胞を認識可能なモノクローナル抗体を作製し、その抗体に蛍光色素を標識することでクロマグロ精原細胞の可視化、単離、追跡などの細胞操作法の樹立を目指した。まず第 1 章では抗体を作製するための免疫抗原に使用するために、フローサイトメーターの散乱光を指標にクロマグロ精原細胞の濃縮技術の樹立を行った。次に第 2 章では第 1 章で用いた濃縮技術を用いてクロマグロ精原細胞の細胞表面抗原を認識する抗体の作製およびそれを用いたクロマグロ精原細胞の可視化、単離、追跡技術の開発を行った。

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第 1 章

フローサイトメーターを用いたクロマグロ A 型精原細胞の濃縮

(Flow-cytometric enrichment of Pacific bluefin tuna type A spermatogonia based on light-scattering properties): Theriogenology (2017)

Abstract

We previously established surrogate broodstock in which the donor germ cells transplanted into the peritoneal cavities of xenogeneic recipients were capable of developing into functional eggs and sperm in teleost fish. In this transplantation system, only the undifferentiated germ cells such as type A spermatogonia (ASG) or a portion of the ASG population were capable of being incorporated into the genital ridges of the recipients and undergo gametogenesis. Therefore, the use of enriched ASGs can be expected to achieve efficient donor-cell incorporation. Here, we established a method of isolation and enrichment of the ASG of Pacific bluefin tuna using flow cytometry. Whole testicular cell suspensions were fractionated by forward and side scatter properties, following which ASGs were enriched in a fraction in which the forward scatter signal was relatively high and side scatter signal was relatively low. The diameter of sorted cells using the fraction was identical to the size of ASGs observed in histological analysis, and these cells also expressed the *vasa* gene. In addition, we succeeded in applying this method to several maturation stages of Pacific bluefin tuna. Since this method was based on light-scattering characteristics of ASGs, it can potentially be applied to various teleosts. We expect that this method can contribute to the production of seeds of Pacific bluefin tuna using surrogate broodstock.

Introduction

Tuna farming has recently become popular globally. Major farming sites include the Mediterranean Sea, Australia, and Japan [1, 2]. Generally, Atlantic bluefin tuna (*Thunnus thynnus*) farming in the Mediterranean Sea, referred to as "fattening" uses juvenile-adult bluefin tuna, captured using purse seine. Hence, a considerable number of wild fish are harvested for the tuna farming. Farming of southern bluefin tuna (*T. maccoyii*) in Australia is quite similar to that performed in the Mediterranean Sea [1]. It is noteworthy that these two species have been designated as endangered species by the International Union Conservation of Nature Red List of threatened species [2, 3, and 4]. In Japan, although the full cycle of cultivation and the artificial seed production of Pacific bluefin tuna (*T. orientalis*) has been established [4], tuna farming in Japan has also remained heavily dependent on wild juveniles. Because Pacific bluefin tuna requires 3–5 years to mature and the body weight of an individual can reach >100 kg, the maintenance of Pacific bluefin tuna broodstock requires intensive labor, large facilities, and large capital investment [4]. These obstacles collectively represent one of the main issues hindering artificial seed production of Pacific bluefin tuna.

We previously established a surrogate broodstock technology in salmonids [5–8] in which donor type A spermatogonias (ASGs) are transplanted into the peritoneal cavity of larvae of the xenogeneic recipients, which do not have mature immune systems and are unable to reject xenogeneic cells. The transplanted donor ASGs migrated towards the gonads of the recipients and were eventually incorporated into them. Finally, the incorporated ASGs developed into functional eggs or sperm in the gonads of the xenogeneic recipients depending on the sex of the recipient [6]. To date, we have successfully applied this technology to marine teleosts including Sciaenidae [9–11] and Carangidae [12, 13]. Moreover, Yazawa et al. optimized xenotransplantation of donor-derived germ cells in larvae of chub mackerel (*Scomber japonicas*), belonging to Scombridae [14]. Because chub mackerel can mature within 1–2

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years with the body weight of an individual ranging between 500–1,000 g, seed production of Pacific bluefin tuna is expected to become readily possible in small land-based tanks using chub mackerel as surrogate broodstock in short time periods with low costs [15].

Our previous report showed that only the undifferentiated germ cells, probably a small portion of the ASG population, possess the ability to be incorporated into the genital ridges of the recipients and undergo gametogenesis when whole testicular cell suspension is transplanted into the body cavity [16, 17]. Therefore, for efficient ASGs transplantation, it is important to use testicular cells containing a high proportion of transplantable ASGs as donor cells. However, the proportion of ASGs in a whole mature testis is lower than that in an immature testis because the proportion of ASGs continues to decline in association with the development of a testis, since the proportion of differentiated germ cells increases throughout spermatogenesis. In the case of Pacific bluefin tuna, it is difficult to obtain immature testes from fish farms since the market-sized fish usually possess testes that have already reached a mature stage. In addition, the capture of natural juvenile Pacific bluefin tuna for each experiment is temporally and spatially restricted since they show a strict seasonal migration pattern. In general, harvested tuna are immediately gutted and cleaned at the fish farms, and the testes are removed together with the digestive organs. Although these freshly isolated testes can be an ideal material as a source for donor germ cells, the commercial-sized (2.5–3-year-old and 20–40 kg in body weight) fish carries testes which contain considerable numbers of meiotic and haploid germ cells in addition to undifferentiated transplantable ASGs. Therefore, to accomplish efficient germ cell transplantation, purification, or enrichment of undifferentiated ASGs, which possess high transplantability from whole gonadal cell suspension, is important [18].

In salmonids, we previously established a method to isolate ASGs based on their light scattering characteristics using flow cytometry (FCM), which can analyze cell size and complexity of internal structure by illuminating each cell using a laser beam [18]. The

previous study showed that ASGs were drastically enriched in the population of large sized cells with a simple intracellular structure and that transplantation efficiency was significantly increased by using the enriched cells as donor cells. In fact, when the transplantation of Japanese char (*Salvelinus leucomaenis*) testicular cells into rainbow trout (*Oncorhynchus mykiss*) recipients was conducted to evaluate the translatability of the sorted and unsorted cells, no recipient carrying donor-derived cells were found in the group of unsorted cells; however, a considerable number of recipients carrying donor-derived germ cells were found in the group receiving FCM-sorted cells [18]. Furthermore, we succeeded in the enrichment of transplantable ASGs in another marine fish, the Nibe croaker (*Nibea mitsukurii*) [18]. This result indicates that light scattering properties of ASG are conserved among the various teleosts. It is noteworthy that this methodology does not require any cell surface marker or reporter gene, possibly making it a very practical method to enrich donor ASGs. Therefore, in the present study, we examined applicability of this methodology to Pacific bluefin tuna. First, we conducted FCM analysis to fractionate ASGs by measuring forward scatter (FS) and side scatter (SS) of testicular cells in immature testis. Furthermore, we applied this methodology to the maturing and spermiogenic testes possessing ASGs at relatively low frequencies.

Materials and methods

<u>**Fish**</u>

Immature (1-year-old, 7-kg body weight, gonadal weight 2.9 ± 0.34 g, gonad somatic index $0.039 \pm 0.003\%$) and mature (4-year-old, body weight 30–40 kg, gonadal weight 100–200 g) Pacific bluefin tuna were reared in net pens at Amami Oshima, Kagoshima Prefecture, Japan. Immature fish were sampled in November of 2008 and mature fish were sampled in August of 2008 and 2016. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Tokyo University of Marine Science and Technology.

<u>**Histology**</u>

Immature and mature Pacific bluefin tuna testes were fixed with Bouin's solution at 4°C overnight and embedded with the standard paraffin method. After the embedding, the paraffin block was sliced into 4 µm serial sections. The paraffin sections were stained with hematoxylin and eosin.

<u>**Preparation of testicular cell suspension**</u>

All the testes were minced and incubated with 10 mL of 0.5% trypsin (Worthington Biochemical Corp., Lakewood, NJ) in phosphate-buffered saline (PBS) (pH 8.2) containing 5% fetal bovine serum (FBS) and 1 mM $CaCl_2$ for 1–6 h at 20°C. The reaction time was decided by whether sufficient cells were dispersed. FBS was added to increase the viability of the dissociated cells, which did not disturb the dissociation efficiency under these conditions. Gentle pipetting was applied to physically disperse any remaining intact portions of the testis. Termination of the enzymatic reaction was achieved by the addition of 10 mL of L-15 medium (Invitrogen Co. Inc., Grand Island, NY). The resultant cell suspension was filtered

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through a 42 µm-pore-sized nylon screen to remove aggregated cell clumps. After

dissociation, hemolysis treatment was conducted by low osmotic pressure. The addition of

1 mL distilled water to the dissociated cells and gentle pipetting was applied over a period of

5 s, immediately after which 1 mL of two-fold-enriched PBS (-) was added to the cells and

washed with L-15 medium twice. Resultant cells were stored at 4°C until cell sorting.

Flow-cytometric analysis and the sorting of testicular cells

Flow-cytometric analysis was conducted using an Epics Altra (Beckman-Coulter,

Hialeah, FL) equipped with a 488 nm argon laser. FS and SS signals were collected using a

linear amplifier. The sheath pressure was 7.5 psi and the flow rate did not exceed 800 cells/sec

during either the analysis or the sorting. Dissociated cells were divided into eight fractions

based on FS and SS light scattering properties, and all cell sorting in each fraction was also

performed based only on the same fractions. Sorted cells were observed by microscopy to

analyze their morphologies. After observation, sorted cells were smeared on glass slides. The

smearing experiment was performed as according to Kise et al. [18].

***In situ* hybridization of paraffin section and sorted cells**

In situ hybridization using *vasa* probe was conducted in Pacific bluefin tuna testes and

smeared cells to calculate the proportion of ASG in each fraction. A RNA probe was

synthesized from a 1,094bp cDNA *vasa* fragment (nucleotide 934-2,028; EU253482.1). Probe

synthesis and hybridization were performed according to the protocol by Nagasawa et al. [19].

Experiments of immature and spermiogenic testes were carried out in triplicate (N=3,

one-year-old fish, four-year-old fish). Experiments of maturing testes were carried out in

duplicate (N=2, four-year-old fish). A minimum of 100 cells in triplicate for each specimen

were counted to calculate the proportion of *vasa*-positive cells, and the proportions were

expressed as a percentage of total testicular cells.

Statistical analysis

All data are presented as means ± SEM. To determine whether our samples came from populations which were normally distributed, we used One-Sample Kolmogorov-Smirnov Test for each sample by using IBM SPSS statistics for Windows, Version 19.0. (IBM Corp., Armonk, NY). The results showed that the data were normally distributed (data not shown). GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) was used for further statistical analysis. *F*-test was performed to reveal the variance of populations were equal. Then, the data were analyzed by one-way analysis of variance (ANOVA) for the significant differences between group means. The Tukey-Kramer's multiple comparisons test was used for post-hoc tests. Two-tailed Student's *t*-test was used to determine statistical differences in means for *vasa*-positive cells at each fraction of the 4-year-old fish testis. For all statistical tests, values were considered significantly different at $P < 0.05$.

Results

Histological observation and fractionation of immature testes using FCM

Histological observation of testes isolated from 1-year-old fish harvested during November revealed that the testes contained a large number of ASGs which formed clusters (Fig. 1a, top). The average diameter of these cells was approximately 10 μm. *In situ* hybridization using *vasa* probe in these testes indicated that the observed ASGs accumulated *vasa* transcripts (Fig. 1a, bottom). Moreover, spermatogenesis did not occur and differentiated germ cells were not observed (Fig. 1a). Therefore, in the present study, we referred to the testes of this developmental stage as "immature testes".

FCM analysis was applied to the whole testicular cell suspension prepared from the immature testes (Fig. 1b), following which these cells were plotted by FS and SS light scattering properties based on the plotting of Nibe croaker described by Kise et al. [18] . Furthermore, to isolate ASGs, A–G fractions were set on a plot figure. As a result, similar sized cell populations could be sorted in each fraction (Fig. 1c). *In situ* hybridization using Pacific bluefin tuna *vasa* probe against sorted cells revealed that gates A–E contained *vasa*-positive germ cells (Fig. 2a, b). The *vasa*-positive rate in the unsorted cells was $5.5 \pm 2.5\%$ (Fig. 2b). The *vasa*-positive rates in each cell fraction isolated using gates A–E were $81.5 \pm 1.5\%$, $20.3 \pm 4.8\%$, $22.5 \pm 10.5\%$, $6.0 \pm 6.0\%$ and $7.8 \pm 6.8\%$, respectively (Fig. 2b). The *vasa*-positive rate of gate A was significantly higher than that of unsorted and other gates ($F (7, 16) = 26.24$, $P < 0.0001$).

Histological observation and fractionation of maturing testes using FCM

In the present experiment, 4-year-old fishes harvested during August were used for confirming FCM could purify ASGs out of testicular cells which contained differentiated germ cells. In this group, two types of testes were classified as it contains numerous amount

of sperm or not. When the testes were cut in half through the axial plane, if sperm were not released into the tubular lumen of their testes, we referred to the testes of this developmental stage as "maturing testes". On the other hand, the testes containing numerous amount sperm were referred as "spermiogenic testis" (see 3.3).

Histological observation of the maturing testes revealed that the testes contained various developmental stages of germ cells from ASGs to spermatid (Fig. 3a, top). *In situ* hybridization using Pacific bluefin tuna *vasa* probe in the testes indicated that ASGs and type B spermatogonias (BSGs) were *vasa*-positive (Fig. 3a, bottom).

FCM analysis was applied to the whole testicular cell suspension prepared from the maturing testes, and the same gates as those used in the immature testes experiment were applied to the cell suspension (Fig. 3b). As a result, we could successfully enrich cells which were approximately 10 µm in diameter using gate A (Fig. 3c). Indeed, *in situ* hybridization using Pacific bluefin tuna *vasa* probe in sorted cells revealed that gates A–E contained *vasa*-positive germ cells (Fig. 3d, e). The *vasa-positive* rate in the unsorted cells was $35.6 \pm 4.0\%$. The *vasa*-positive rates in each cell fraction isolated using gates A–E were $77.2 \pm 4.0\%$, $24.9 \pm 4.0\%$, $47.0 \pm 10.6\%$, $25.1 \pm 6.7\%$ and $33.7 \pm 2.9\%$, respectively (Fig. 3e). The *vasa*-positive rates in the cell fraction isolated using gates A was significantly higher than the unsorted sample and other gates except for gate C ($F (7, 8) = 16.60$, $P = 0.0004$, Fig. 3e).

Histological observation and fractionation of spermiogenic testes using FCM

In the present experiment, 4-year-old fishes harvested during August were used in which numerous sperm were released into the tubular lumen of their testes. Therefore, in the present study, we referred to the testes in this maturation stage as "spermiogenic testes". Histological observation of these testes revealed that the lobules were filled with large amounts of sperm (Fig. 4a, top). ASGs were localized at the peripheral region of their lobule, and *in situ* hybridization using Pacific bluefin tuna *vasa* probe revealed that these cells were

vasa-positive (Fig. 4a, bottom). Numerous sperm were observed in dissociated cells since any specialized treatment to eliminate sperm was not applied during the sample preparation (Fig. 4b). However, the contamination of sperm was not observed in sorted cells using gate A (Fig. 4c, d). Moreover, the average diameter of sorted cells using the gate A was approximately 10 µm (Fig. 4d) and the mean *vasa*-positive rate of these cells was $73.3 \pm 3.9\%$, which was significantly higher than that of unsorted cells, $25.5 \pm 3.9\%$ (F (2, 2) = 1.001, P = 0.0010, Fig. 4e, f).

Discussion

In the present study, we successfully purified a cell population highly enriched with ASGs among the whole testicular cells prepared from immature testes. Generally, ASGs possessed cells with the largest diameter among all the germ cells throughout the developmental stages [20, 21]. In addition, Kise et al. (2012) [18] indicated that the ASGs population is enriched in a fraction showing an elevated forward scatter value (indicative of cell size) and low side scatter value (indicative of complexity of internal structure) in several salmonid species and a Sciaenidae species, the Nibe croaker. In the present study, the results showed no contradictions with those of previously described characteristics of ASGs, as mentioned above. Indeed, the average diameter of the isolated cells was approximately 10 µm, identical to the average size of ASGs observed in histological analysis and markedly larger than BSGs. In addition, *in situ* hybridization using *vasa* probe revealed that the cells purified by light scattering property-dependent FCM were *vasa*-positive, which is a known marker for ASGs and BSGs in Pacific bluefin tuna [19]. Considered together, we concluded that the *vasa*-positive germ cells fractionated in gate A were ASGs.

In the second experiment, we used maturing testes containing various developmental stages of germ cells from ASGs to spermatids. Generally, the average diameter of germ cells gradually become smaller as they develop [20, 21]. Therefore, even though we used maturing testes for the cell sorting, it was expected that ASGs could be isolated from a mixed population of various stages of germ cells using FS-SS light scattering properties. As a result, the average diameter of cells sorted by gate A was approximately 10 µm. *In situ* hybridization revealed that *vasa*-positive cells were markedly enriched in gate A. In the case of the experiment with maturing testes, *vasa*-positive rates of gate C and E also showed higher tendency compared with the other fractions. In this study, significant numbers of differentiated BSGs were confirmed in the maturing testes by histological analyses. Nagasawa

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et al. (2009) revealed that BSGs also express a significant amount of *vasa* gene in Pacific bluefin tuna [19]. In addition, it is expected that BSGs would appear in the lower FS value fraction such as gate C and E because the average diameter of BSGs is markedly smaller than that of ASGs. Thus, we concluded that *vasa*-positive cells distributed in gate A appear to be ASGs, and BSGs appeared in the lower FS value fraction, such as gate C and E in maturing testes.

We then performed the same experiment using spermiogenic testes in which the lobules were filled with an enormous amount of sperm, and in which their peripheral regions contained some immature germ cells including ASGs. Although the dissociated testicular cells contained numerous sperm, *vasa*-positive cells were enriched in gate A. Furthermore, the average diameter of sorted cells using gate A was identical to the average size of ASGs observed in the histological analyses. These results revealed that FS-SS-dependent FCM could efficiently enrich ASGs in both immature and maturing testes as well as in spermiogenic testes. Since FCM sequentially sorts cells, an extended operation time is required to collect a sufficient number of target cells if the proportion of target cells is extremely low. In the present experiment, the operating time for spermiogenic testes was much longer than that required for immature testes because spermiogenic testes contain an enormous amount of sperm. Previous research reported that a large amount of haploid cells (including sperm) could be eliminated using density gradient centrifugation [22, 23]. Therefore, if this density gradient centrifugation can be applied to Pacific bluefin tuna before the FCM sorting developed in this study, it would be possible to shorten the operating time required for the enrichment of ASGs from fully mature testes containing many haploid cells and to collect ASGs with a higher purity.

In the present study, we could successfully show that Pacific bluefin tuna ASGs were enriched in gate A from three types of testes, immature, maturing and spermiogenic testes. Previously, we revealed that the same gate could be applied to enrich ASGs in Nibe croaker

[18]. Although both Pacific bluefin tuna and Nibe croaker are members of the Perciformes, there is large genetic distance between these two species (Nibe croaker belongs to Sciaenidae and Pacific bluefin tuna belongs to Scombridae) [24]. Thus, the light scattering properties of ASGs could be widely conserved throughout the evolution of Perciformes. As mentioned above, light scattering properties of ASGs were based on their morphological characteristics, such as cell size, roundness of shape, and complexity of internal cell structure. Generally, these morphological characteristics of ASGs are widely conserved in teleosts. Indeed, similar morphologies have been reported in many species [21] including Japanese eel [25], African catfish [26], gilthead seabream [27], Atlantic cod [28], and zebrafish [29]. This fact suggests that the method developed in the present study is potentially applicable to various teleost species and not only for Perciformes.

To date, various methods for isolation and enrichment for fish ASGs have been reported including differential plating [30, 31], Percoll gradient centrifugation [22], centrifugal elutriation [32], and combinations of these methods [23]. Differential plating can isolate ASGs by the adhesion difference between ASGs and other cells. However, the possibility cannot be denied that cellular characteristics are deteriorated during the *in vitro* culture in spite of the short culture period. On the other hand, Percoll gradient centrifugation is a simple procedure that does not require specialized equipment such as FCM. However, Percoll gradient centrifugation cannot separate different kinds of cells which possess similar cellular densities. Similarly, centrifugal elutriation cannot separate different kinds of cells of similar sizes. FS-SS-dependent FCM is faster and simpler than the conventional methods, even though specialized equipment is required. Thus, it is important to choose an appropriate methodology depending on the purposes of each study and the availability of equipment.

To date, several transgenic fish lines possessing germ cells or ASGs labeled by fluorescent protein genes have been established [33–37]; however, these fish are not suitable for aquaculture applications because their offspring produced by surrogate broodstock carry the

reporter gene. The method established in the present study does not require a transgenic donor, which makes the method useful for aquaculture applications and it can also readily be applied to various species. In mammals, the use of antibodies, which can recognize cell surface antigen, could possibly become one of the most powerful tools to identify and enrich specific cell populations without reporter genes [38–40]. In fish, antibodies which specifically recognize cell surface antigen in ASGs have been discovered [41–43]. However, FCM sorting and magnetic-activated cell sorting (MACS) with these antibodies have not yet become a major method for enriching fish ASGs. Therefore, a spermatogonial population enriched by the method developed in the present study could be an ideal material for use as an antigen for immunization to produce ASG-specific antibodies.

Our previous report showed that when we transplanted whole testicular cells derived from Japanese char into rainbow trout, no germ cells derived from Japanese char colonized the gonads in rainbow trout [18]. It was considered that although both species belong to the same family (Salmonidae), there is a large genetic distance between char and rainbow trout [44]. However, it was indicated that enriched germ cells by FCM could improve their transplantation efficiency [18]. Although mackerel and Pacific bluefin tuna belong to the same family (Scombridae), these species belong to different genera [24]. Therefore, it is expected to improve transplantation efficiency when purified germ cells of Pacific bluefin tuna by light-scattering properties-dependent FCM were transplanted into mackerel recipients. This would be particularly significant for use in a germ cell transplantation experiment in Scombridae in which the survival rate during the larval stage is very low compared with most other species [14, 45]. For this reason, the efficiency of incorporating xenogeneic germ cells into surviving recipient gonads has become one of the key factors for successful production of Pacific bluefin tuna offspring from the recipient species. The method established in the present study is expected to accelerate various studies regarding the basic and applied biology of fish spermatogonia.

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Figure legends

Fig. 1. Light scattering property-dependent fractionation of testicular cells from immature testes. a) Sequential sections, which were constructed from immature testis used for flow cytometry (FCM), were stained with hematoxylin and eosin and hybridized with Pacific bluefin tuna *vasa* antisense probe. The only germ cells present in the testes were ASGs. Arrowheads indicate ASGs. Left bars = 50 µm. Right bars = 10 µm. b) Two-dimensional histogram of the forward scatter (FS)-side scatter (SS) light scattering properties in dissociated cells prepared from immature testes. c) Two-dimensional histogram of FS-SS light scattering properties of cells. Gates A–G were set on the histogram and used for cell sorting. Microscopic observation of sorted cells using gates A–G. Bars = 20 µm.

Fig. 2. Evaluation of sorted cells hybridized with Pacific bluefin tuna *vasa* probe. a) Two-dimensional histogram of forward scatter (FS)-side scatter (SS) light scattering properties of cells and gates A–G were set on the histogram and used for cell sorting. *In situ* hybridization of *vasa* gene against cell smear samples sorted using gates A–G. Arrowheads indicate *vasa*-positive cells. Bars = 10 µm. b) *vasa*-positive rates of sorted cells using gates A–G and unsorted cells in immature testes [mean ± standard error of mean (SEM)]. Means with different letters indicate significant difference ($P < 0.05$).

Fig. 3. Light scattering property-dependent fractionation and enrichment of testicular cells from maturing testes. a) Sequential sections which were constructed from maturing testis used for flow cytometry (FCM) were stained with hematoxylin and eosin and hybridized with Pacific bluefin tuna *vasa* antisense probe. Arrowhead indicates ASG. Arrows indicate BSGs. Differentiated germ cells such as BSGs, spermatocyte (sc) and spermatid (st) were contained. Left bars = 50 µm. Right bars = 10 µm. b) Two-dimensional histogram of forward scatter

(FS)-side scatter (SS) light scattering properties in dissociated cells prepared from maturing testes. The same gates A–G which were used in the immature testes experiment were applied to the maturing testes experiment for cell sorting. c) Microscopic observation of the sorted cells using gate A in maturing testes. Bars = 10µm. d) *In situ* hybridization using Pacific bluefin tuna *vasa* probes was conducted against the sorted cells using gate A in maturing testes. Arrowheads indicate *vasa*-positive cells. Bars = 10 µm. e) *vasa*-positive ratio of sorted cells using gates A–G and unsorted cells in maturing testes [mean ± standard error of mean (SEM)]. Means with different letters indicate significant difference ($P < 0.05$).

Fig. 4. Light scattering property-dependent fractionation and enrichment of testicular cells from spermiogenic testes. a) Sequential sections which were constructed from spermiogenic testes used for flow cytometry (FCM) were stained with hematoxylin and eosin and hybridized with Pacific bluefin tuna *vasa* antisense probe. Arrowheads indicate ASGs. Sz indicate sperm. Enormous amount of sperm were contained. Left bars = 100 µm. Right bars = 20 µm. b) Microscopic observation of dissociated testicular cells in spermiogenic testes. Arrows indicate sperm. Bar = 10 µm c) Two-dimensional histogram of forward scatter (FS)-side scatter (SS) light scattering properties in dissociated-cells prepared from spermiogenic testes. The same gates A–G which were used in the immature testes experiment were applied to spermiogenic-testis experiment for cell sorting. d) Microscopic observation were conducted against the sorted cells using gate A in spermiogenic testes. Bars = 10 µm. e) *In situ* hybridization using Pacific bluefin tuna *vasa* probes were conducted against the sorted cells using gate A in spermiogenic testes. Arrowheads indicate *vasa*-positive cells. Bars = 10 µm. f) *vasa*-positive rate of sorted cells using gate A and unsorted cells in spermiogenic testes [mean ± standard error of mean (SEM)]. Asterisk represents statistical difference ($P < 0.05$).
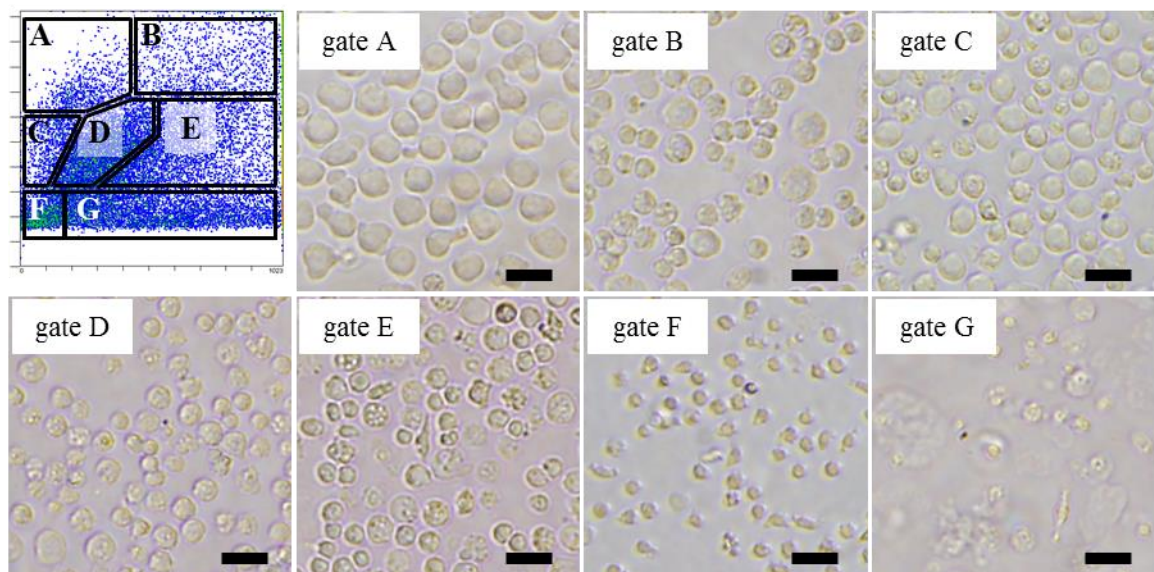
Fig. 1

a)

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Fig. 2
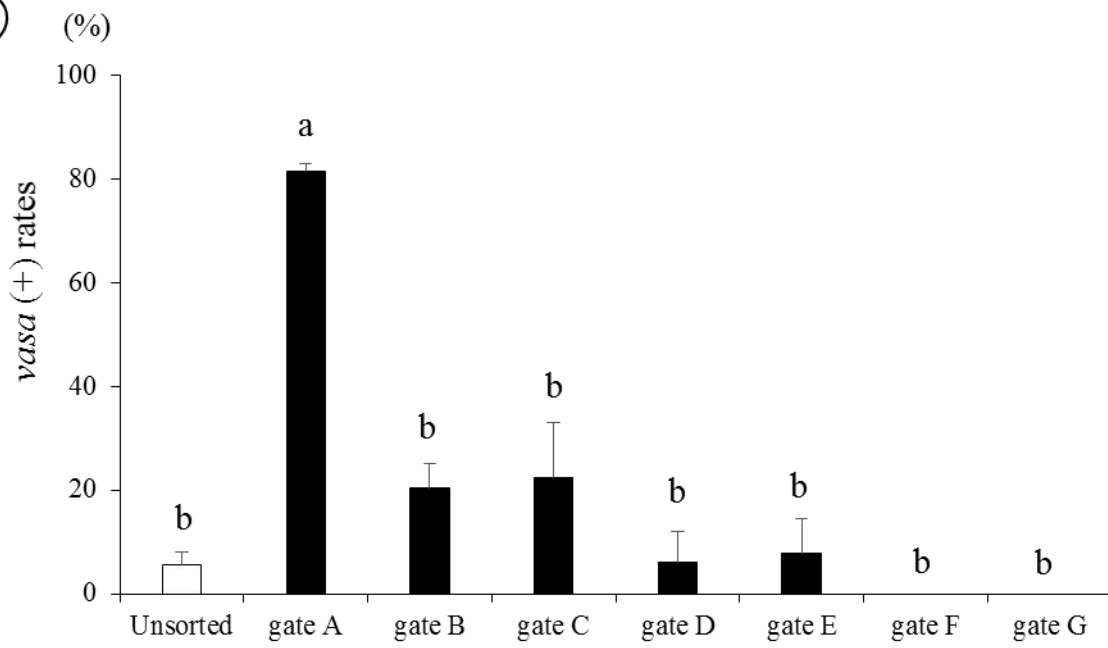
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Fig. 3

a)

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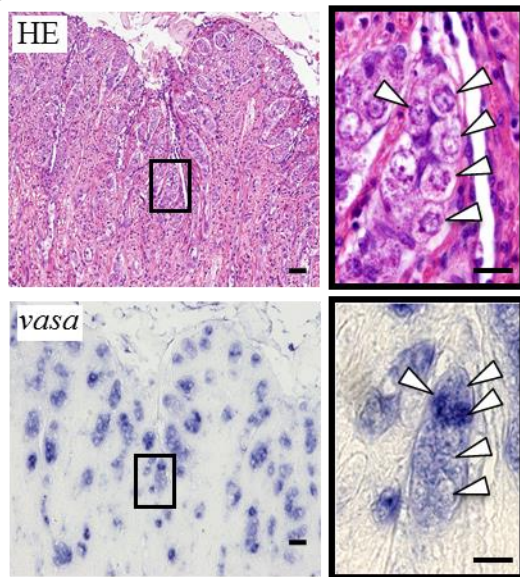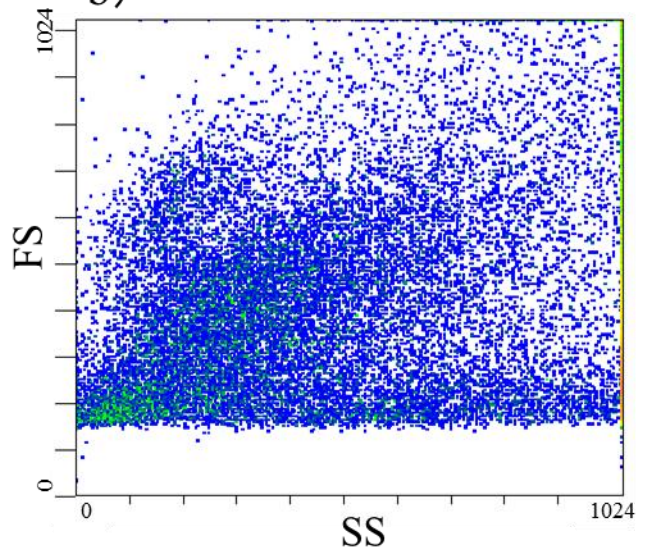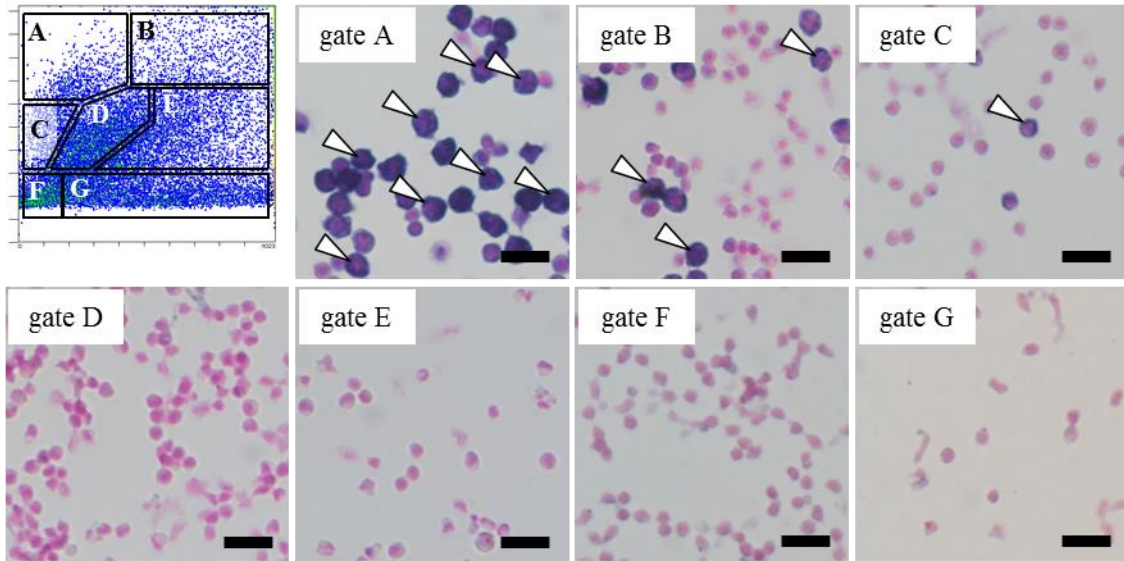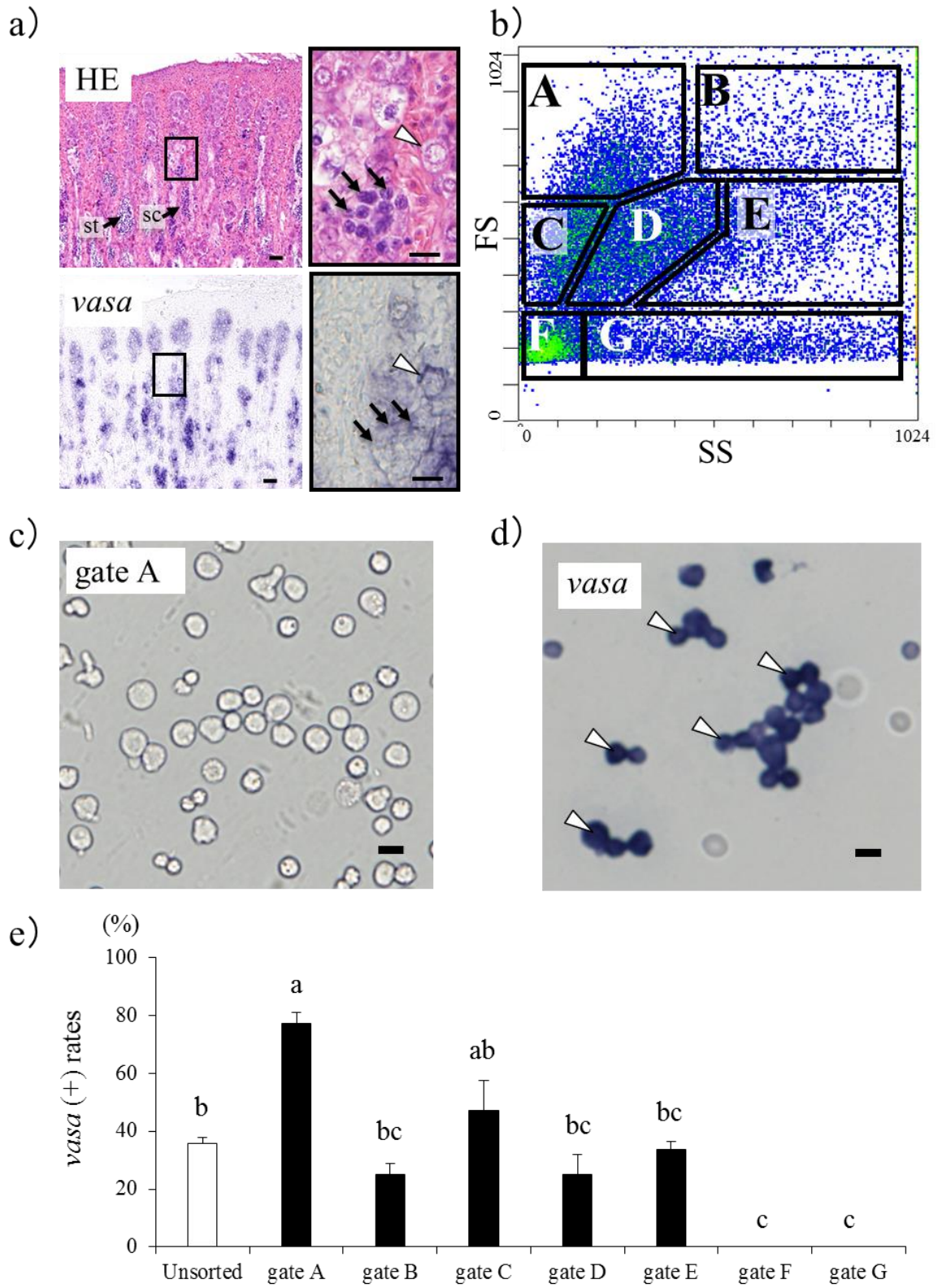
Fig. 4

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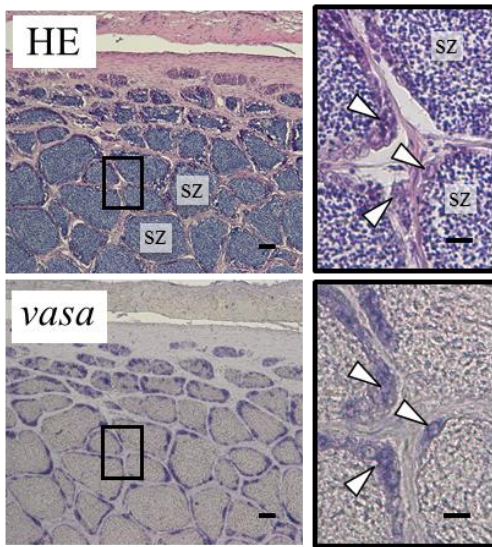
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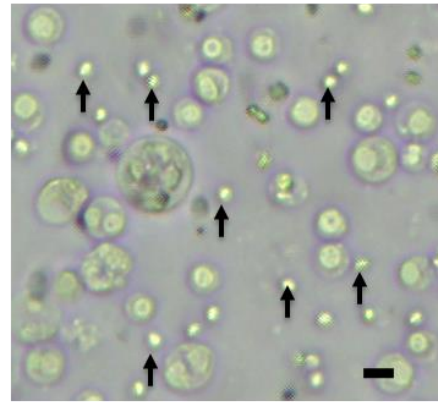
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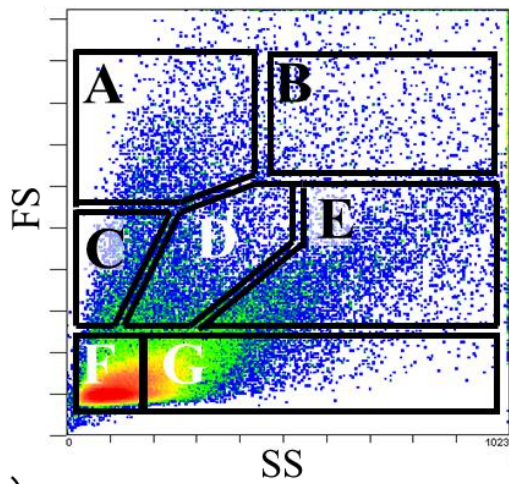
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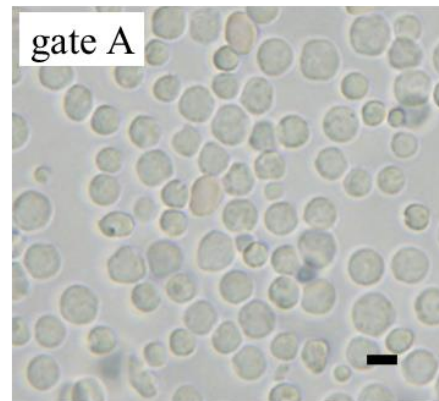
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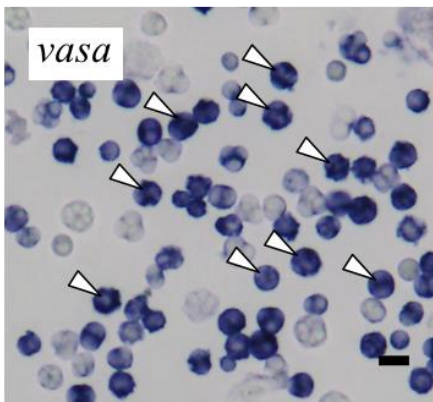
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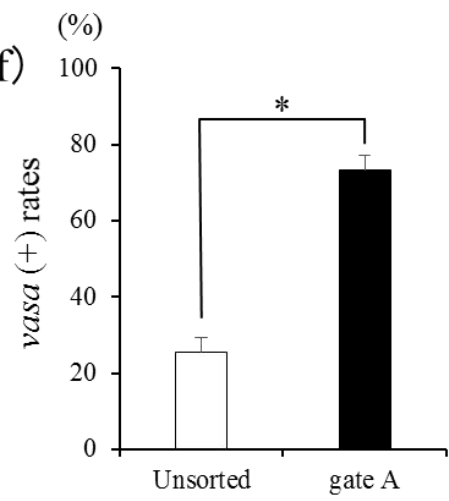
第 2 章

細胞表面抗原を用いたクロマグロ A 型精原細胞の可視化

(Specific visualization of live type A spermatogonia using fluorescence-conjugated antibody in Pacific bluefin tuna)

Abstract

In our continuing attempts to establish surrogate broodstock that can produce Pacific bluefin tuna (PBT) gametes by xenogeneic germ-cell transplantation, we here developed a technique to specifically visualize type A spermatogonia (ASGs) possessing the potency required to colonize recipient gonads. Therefore, specifically visualizing ASGs with sequential analyses of donor cell behavior, including colonization, proliferation and differentiation in the recipient gonads with monoclonal antibodies that can recognize cell surface antigens of ASGs was examined. We generated monoclonal antibodies by inoculating 5×10^6 cells of PBT testicular cells containing ASGs into mice, and then screened them by cell-based ELISA, immunocytochemistry, flow cytometry (FCM) and immunohistochemistry. This method succeeded in selecting two antibodies (No. 152 and No. 180) out of 1152 antibodies. Next, in order to visualize ASGs more easily and simply, we directly labeled these two antibodies with fluorescent dye. Immunocytochemistry with the fluorescence-labeled antibodies could visualize ASG-like cells in a one-step procedure. Furthermore, RT-PCR and *in situ* hybridization against the FCM-sorted fluorescent cells were performed to confirm that the visualized cells were ASGs. In this study, ASGs were confirmed to be highly enriched in the antibody-positive fraction. Finally, visualized cells were transplanted into the peritoneal cavity of nibe croaker larvae to evaluate migratory capability. After transplantation, incorporated fluorescent cells labeled with antibody No. 152 were detected in recipient gonads, suggesting that the visualized ASGs possess migratory and colonization capabilities. Thus, the donor germ cell visualization method developed in this study made conducting PBT germ cell transplantation easier and simpler

Introduction

Bluefin tuna is one of the most valuable aquaculture species and is cultivated in various regions, such as the Mediterranean Sea, southern Australia and Japan [1, 2]. All bluefin tuna, including Atlantic bluefin tuna (*Thunnus thynnus*), southern bluefin tuna (*T. maccoyii*) and Pacific bluefin tuna (*T. orientalis*), have been designated as threatened species and are on the International Union for Conservation of Nature Red List of threatened species [3-5]. It should be noted—capturing juvenile and adult Atlantic bluefin tuna by purse seine and then cultivating them in net cages is referred to as "fattening". Hence, a considerable number of wild fish are harvested for tuna farming in the Mediterranean Sea and, similarly, southern bluefin tuna are farmed in Australia. In Japan, although the full cycle of cultivation and the artificial seed production of Pacific bluefin tuna have been developed [6], tuna farming in Japan has remained heavily dependent on wild seedlings. Therefore, a considerable number of juvenile Pacific bluefin tuna have also been harvested for use as aquaculture seedlings in Japan. Although it is desirable to use artificial seedlings for tuna farming from the point-of-view of sustainability, Pacific bluefin tuna requires 3–5 years to mature and reaches nearly a hundred kilograms [2, 7]. Thus, maintaining Pacific bluefin tuna broodstock is labor-intensive and requires large facilities and capital investment. These obstacles collectively represent one of the main issues hindering the implementation of artificial seedling production in Pacific bluefin tuna.

To overcome these problems, we have been attempting to artificially produce seedlings of Pacific bluefin tuna by xenotransplantation of germ cells into small-bodied recipients. Previously, we succeeded in producing functional gametes using germ cell transplantation from surrogate broodstock [8-11]. In this method, donor germ cells intraperitoneally transplanted into recipients become surrogate broodstock, the transplanted germ cells migrate toward the recipient gonads and are then capable of developing into

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functional eggs and sperm. This technology has been shown to be applicable to xenotransplantation for recipients of different species [8, 10], and it is thus expected that functional tuna gametes can be produced by transplanting bluefin tuna germ cells into a small-bodied surrogate broodstock that is closely related to Pacific bluefin tuna, such as mackerel or other scombrids. If this technique proves to be successful, it will save costs, time and labor in tuna gamete production. To date, we have successfully applied this xenogeneic transplantation technique to various teleosts, including Salmonids, Carangidae and Tetraodontidae [8, 10, 12, and 13].

In our previous studies, we found that in whole testicular cell suspensions, only undifferentiated germ cells, which likely make up only a small portion of the type A spermatogonia (ASG), possess the capability to be incorporated into genital ridges of recipients and undergo gametogenesis [14, 15]. Therefore, evaluating the proportion and numbers of ASGs in donor cell suspensions and tracing the behavior of transplanted ASGs in the peritoneal cavity of recipients are crucial steps toward achieving successful transplantation and subsequent optimization. Using transgenic fish carrying reporter genes, such as green fluorescence protein (*gfp*) that are specifically expressed in germ cells, is one of the most effective ways of tracking ASGs. In the case of rainbow trout, the ASGs are predominantly visualized by green fluorescence, making it possible to evaluate the proportion of ASGs in whole testicular cells and trace the migration, colonization and proliferation of post-transplanted ASGs in recipient gonads by simple fluorescence observation [8-11]. Thus, use of transgenic technology can be a powerful tool for germ cell transplantation in novel species. However, it is difficult to establish transgenic lines in Pacific bluefin tuna because of the difficulty to induce spawning induction and the 1-to-1 mating habit. Therefore, we focused on producing a specific antibody that can recognize cell surface antigens for the visualization of live ASGs.

In rodents, cell surface markers are extensively used to characterize specific lineages

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of live cells. In the case of hematopoietic stem cell research, numerous cell surface markers that can be used for the fractionation and enrichment of hematopoietic stem cells have been identified, including CD34, CD33, c-kit and Thy1 [16-20]. Indeed, research on the characterization of hematopoietic stem cells has progressed markedly through use of these markers. Further, the use of cell surface markers CD9, ITGB1, ITGA6, GFRA1, EPCAM, NCAM1, THY1, CDH1, and MCAM and the fractionation of spermatogonial stem cells has supported the progression of spermatogonial stem cell research [21, 22]. Although cell surface markers are powerful tools for identifying and fractionating specific cell populations, there are few reports on cell surface markers in teleosts. Previously, Ly75/CD205 was identified as a cell surface marker of ASGs in rainbow trout [23] and Pacific bluefin tuna [24]. However, there are no reports to date describing the labeling and fractionating of live ASGs by Ly75/CD205.

Recently, Hayashi and colleagues (unpublished data) produced antibodies that can recognize cell surface antigens of ASGs in rainbow trout. However, these antibodies could not recognize ASGs of bluefin tuna and, in addition, their epitope has not yet been identified. Therefore, it is difficult to isolate orthologous cell surface antigens from Pacific bluefin tuna. Using antibodies that can recognize cell surface antigens of ASGs in bluefin tuna and that are conjugated with fluorescent dye would make it possible to visualize ASGs by simply mixing with testicular cell suspensions. However, no antibodies that can recognize cell surface antigens of ASGs in any kinds of bluefin tuna have been identified to date. Here, we produced novel monoclonal antibodies specific to cell surface antigens of ASGs in Pacific bluefin tuna and used these antibodies to visualize and trace ASGs.

Materials and methods

<u>Ethics</u>

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Tokyo University of Marine Science and Technology.

<u>Fish</u>

Two- to four-year-old Pacific bluefin tuna (body weight, 20-40 kg) were reared in net pens at Kushimoto, Wakayama Prefecture, Japan and Takashima, Saga Prefecture, Japan.

Blue mackerel (*Scomber australasicus*) estimated to be 2 years old that were caught by hook and line at Tateyama Bay, Chiba Prefecture, Japan in December 2014 were kept in land-based tanks at Tateyama Station, Field Science Center of Tokyo University of Marine Science and Technology, until use in this study. The average body length, body weight and testicular weight of the blue mackerel were 33.9 ± 0.84 cm, 442.5 ± 29.8 g and 1.53 ± 0.21 g (means $\pm$ SEM), respectively.

Broodstock of 1-year-old nibe croakers (*Nibea mitsukurii*) were maintained at Tateyama Station in 1000-l circular fiber-reinforced plastic tanks with culture conditions that maintain year-round spawning: water temperature, 24-25°C and a photoperiod of 16 h light. Fertilized eggs were collected by natural spawning; no exogenous hormones were administered. Fertilized eggs and larvae were held in cylindrical 100-l rearing tanks (440 mm in diameter $\times$ 700 mm in depth) at 24-25°C with a photoperiod of 16 h light.

<u>Preparation of testicular cell suspension</u>

In this study, Pacific bluefin tuna and blue mackerel testes possessing all stages of germ cells (from ASGs to spermatozoa) were dissected from one to three individuals, pooled, and processed as follows. The adipose tissue attached to the testes was removed by forceps,

scissors and surgical knife under a binocular microscope (model SZX-12; Olympus, Tokyo, Japan). Approximately 5-8 g of testes were minced by Weckel scissors and then, the minced testicular tissues were washed three times with L-15 medium (pH 7.8; Thermo Fisher Scientific, Waltham, MA) supplemented with 25 mM HEPES (Merck KGaA, Darmstadt, Germany), and antibiotics (50 µg/ml of ampicillin, 50 µg/ml of penicillin, and 50 µg/ml of streptomycin; all antibiotics were from Wako, Osaka, Japan). After washing, the minced testicular tissue was dissociated for 2 h at 25°C in 35-40 ml of one of these dissociation buffers: buffer A, L-15 medium containing 2.5 mg/ml collagenase from *Clostridium histolyticum* (Merck KGaA) and 150 U/ml deoxyribonuclease I (Merck KGaA) or buffer B, L-15 medium containing 2.0 mg/ml collagenase H (Roche, Basel, Switzerland), 1.65 mg/ml disperse II (Godo Shusei Co. Ltd., Tokyo, Japan), 5% fetal bovine serum (FBS; Thermo Fisher Scientific) and 150 U/ml deoxyribonuclease I. Buffer A was used for preparation of testicular cell suspensions for isolation of immunogen, screening of cell-based ELISA, and immunocytochemistry. Buffer B was used for preparation of testicular cell suspensions for RT-PCR, *in situ* hybridization and germ cell transplantation. FBS was added to buffer B to increase the viability of the testicular cells, which did not disturb the dissociation efficiency under these conditions. During the incubation, gentle pipetting was conducted to physically disperse any remaining intact portions of testes. The enzymatic reactions were terminated by adding excess amounts of L-15 medium. After enzymatic digestion, dissociated cells were washed three times by L-15 medium. The resultant cell suspensions were filtered through a 42-µm pore size nylon screen (Tokyo Screen Co. Ltd., Tokyo, Japan) to eliminate non-dissociated cell clumps and then stored on ice until use.

Enrichment of ASGs used for immunogens

To prepare ASGs for use as immunogens, ASGs were enriched out of whole testicular cell suspensions using Percoll density gradient centrifugation followed by

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light-scattering-dependent flow cytometry (FCM). First, spermatozoa and blood corpuscles were isolated from whole testicular cell suspensions by density gradient centrifugation using a Percoll gradient (Percoll Plus; GE Healthcare UK, Ltd. Princeton, NJ) built up in a 15 ml centrifuge tube: 2 ml of 30% percoll/PBS, followed by 2 ml of 20% percoll/phosphorus buffer saline (PBS) and approximately 1.2 to 1.5×10^7 cells/2 ml of L-15 medium. Then, the tube was centrifuged at $800 \times$ g for 30 min at 4°C. Following centrifugation, three different cell layers containing testicular cells were visible. Cells at the interface of the 20% and 30% Percoll layer were gently aspirated and washed three times in excess L-15 medium.

Next, to obtain enriched ASGs of Pacific bluefin tuna, FCM sorting was performed against the testicular cells fractionated by Percoll centrifugation. We previously established a method to enrich Pacific bluefin tuna ASGs out of whole testicular cell suspension using the light-scattering properties of FCM [25]. Briefly, ASGs of Pacific bluefin tuna were enriched in the "gate A" fraction (forward-scatter high and side-scatter low) based on analysis of testicular cells analyzed with light-scattering properties as indicators. The testicular cells enriched in gate A were sorted and collected in 15 ml tubes using an Epics Altra flow cytometer (Beckman-Coulter, Miami, FL) equipped with a 488 nm argon laser. Forward and side scatter were collected using a linear amplifier. The sheath pressure was 7.5 psi, and the flow rate did not exceed 1,800 cells/s during either analysis or sorting. The collected cells were suspended in 10 ml of L-15 medium containing 10% FBS.

Generation of monoclonal antibodies using live ASGs

To generate monoclonal antibodies, enriched ASGs prepared by FCM sorting were inoculated into five 4-week-old, female BALB/c mice. Then, approximately 100,000 cells were injected into each mouse, after injected the 100 µl emulsion containing same volume of PBS and Freund's Adjuvant, Complete (Merck KGaA).then inoculated into approximately 100,000 cells diluted in 100 µl of PBS into one mouse for each injection, as summarized in

Table 1. For the last inoculation, testes of blue mackerel, which is closely related to bluefin tuna, were used instead. Eight days after the last inoculation, lymph nodes were removed and lymph node cells were recovered. Next, to generate hybridomas, the lymph node cells were fused with mouse myeloma cells of the P3U1 line in the presence of 50% PEG4000 (Merck-Millipore, Billerica, MA)/RPMI medium (Merck KGaA). The fused cells were plated on six 96-well plates and selected by culturing with Hybridoma-SFM (Thermo Fisher Scientific) supplemented with 15% FBS (Hyclone), 1% HT Supplement (Thermo Fisher Scientific), 0.2 µg/ml Aminopterin (Merck KGaA), 1% BM-condimed (Merck KGaA) and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin) in a 37ºC incubator with 5% CO_2. After 3 days, medium was changed to Hybridoma-SFM supplemented with 15% FBS (Hyclone), 1% HT Supplement (Thermo Fisher Scientific), and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). The supernatants from these wells were subjected to screening for antibodies secretion and for reactivity to cell surface of ASGs.

Selected hybridoma cells were expanded, and subclones were established by limiting dilution. Hybridoma cells were cultured with Hybridoma-SFM supplemented with 10% FBS, and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin) in a 37ºC incubator with 5% CO_2.

Screening monoclonal antibodies that can recognize cell surface antigen of ASGs

To screen antibodies that can specifically recognize cell surface antigens of ASGs, antibody screening was performed by cell-based ELISA, immunocytochemistry and immunohistochemistry. First, to identify antibodies having strong affinity for the immunogens, cell-based ELISA was conducted against testicular cells of Pacific bluefin tuna isolated by Percoll centrifugation. First, approximately 1×10^5 cells/100 µl of PBS containing 0.5% BSA and 2 mM EDTA were dispensed in each well of twelve 96-well plates, then supernatant was removed. Next, 50 µl of primary antibodies isolated from hybridomas were added to each

well, and the cells were incubated for 30 min at room temperature. Following the immunoreaction, cells were washed three times with PBS containing 0.5% BSA and 2 mM EDTA. Next, 50 µl of anti-IgG (H+L chain) (Mouse) pAb-HRP (MBL, Aichi, Japan) was diluted 1:10,000 fold in HEPES buffer (MBL), added to each well as a secondary antibody, and then incubated for 30 min at room temperature. Following immunoreaction, the cells were washed three times with PBS containing 0.5% BSA and 2 mM EDTA; then, 50 µl of TMB peroxidase substrate (MBL) was added to each well, and the plates were incubated for 10 min at room temperature. Color development was stopped by adding 50 µl of 1.5 N phosphoric acid. The absorbance in each well at 450 nm was measured, and wells of antibodies with absorbance >0.095 were selected for further processing.

Further, to screen antibodies that can recognize cell surface antigens of ASGs, immunocytochemistry and FMC analysis against live testicular cell suspensions were conducted. Approximately 10^5 dissociated cells were incubated with 50 µl of selected primary antibodies that were undiluted and diluted 1:10 and 1:100 fold in wash buffer (PBS containing 2 mM EDTA, 0.5% BSA and 30 U/ml deoxyribonuclease I) at 4°C for 30 min, and then cells were rinsed twice with wash buffer. After rinsing, cells were incubated for 30 min at 4°C with 35 µl of Alexa flour 488 Goat Anti-mouse IgG IgM (H+L) (Thermo Fisher Scientific) diluted 1:200 fold in wash buffer as a secondary antibody. Then, the cells were rinsed twice with wash buffer. After rinsing, the stained cells were suspended in 150 µl of L-15 medium and observed under a fluorescence microscope (model 1X71N-PH; Olympus, Tokyo, Japan), and cells showing fluorescent signals from the antibodies on the cell membrane were selected. Next, the selected antibodies were analyzed by fluorescence- and light-scattering-dependent FCM. Previous studies showed that ASGs were distributed in gate A when whole testicular cells were analyzed by light-scattering properties [25]. Therefore, cells with ASG-specific antibodies should be concentrated in gate A. Thus, the enrichment ratio of ASGs was calculated as (antibody-positive cells detected in gate A) /(total cells detected in gate A).

Enrichment ratios >1 indicate that the ASGs can be enriched with antibodies.

Finally, to screen antibodies that are specific to ASGs, immunohistochemistry was conducted against the tissue sections. First, the testes of 4-year-old Pacific bluefin tuna containing all stages of germ cells (from ASGs to spermatozoa) were fixed in 4% paraformaldehyde/PBS for 12-14 h at 4°C and then embedded in paraffin by standard methods. After embedding, the paraffin block was sliced into 4 µm serial sections. The paraffin sections were dewaxed and rehydrated by passage through a xylene-ethanol series. After rehydration, antigen retrieval treatment was conducted with Histo VT One solution (Nacalai Tesque, Kyoto, Japan) at 90°C for 20 min. To prevent non-specific antibody binding, sections were blocked by Block-Ace (DS Farmer Biomedical, Osaka, Japan) at room temperature for 30 min. After blocking, the sections were incubated for 16 h at 4°C with the selected primary antibodies, which were diluted 10- to 100-fold in Can Get Signal immunostain solution B (Toyobo Co., Osaka, Japan), and then the sections were washed three times for 5 min each with PBST (PBS containing 0.1% Tween 20 (Merck KGaA)). After washing, the sections were incubated for 1 h at room temperature with Alexa Flour 488 Goat anti-Mouse IgG (H+L) (Thermo Fisher Scientific) which was diluted to 1:200 with Can Get Signal immunostain solution A (Toyobo Co.) as the secondary antibody solution. Following immunoreactions, the sections were washed three times with PBST for 5 min each and then observed under the fluorescence microscope (model BX53; Olympus). Following observation, the slides were counterstained with hematoxylin and eosin and then dehydrated by passing through an ethanol-xylene series. After dehydration, the slides were mounted using Entellan new (Merck KGaA).

Protein A purification and fluorescence labeling against antibodies

Alexa Flour 488 carboxylic acid, succinimidyl ester (Thermo Fisher Scientific) was directly conjugated to the antibodies. Cultured supernatant of hybridoma was purified using

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Hitrap protein A HP (GE Healthcare) according to manufacturer's instructions. Next, purified

IgG was dialyzed in 0.1 M NaHCO$_3$ for 16 h at 4°C. Following dialysis, purified antibodies

and Alexa flour 488 diluted in DMSO, were mixed and held at room temperature for 1 h. Next,

to remove residual Alexa flour 488 from the antibody solution, gel filtration chromatography

was conducted. The mixed antibody solutions were filtered on a Bio-Gel P-4 Gel (Bio-Rad,

Hercules, CA), and the purified antibodies were stored at 4°C as Alexa flour 488-conjugated

antibodies.

Immunocytochemistry with antibodies directly labeled with fluorescent dye

To visualize ASGs more easily and simply, immunocytochemistry with the Alexa

flour 488-conjugated antibodies were conducted. Alexa flour 488-conjugated antibodies were

diluted 1:10 and 1:100 in L-15 medium, and 100 µl of the antibody was incubated with

approximately 2×10^6 dissociated cells for 30 min at 4°C. Following the immunoreaction,

testicular cells were washed twice with L-15 medium. The stained cells were observed under

the fluorescence microscope (model BX53), and the diameters of antibody-positive and

-negative cells were measured using microscope digital camera (model DP72; Olympus) and

imaging software (cellSense standard; Olympus).

Expression analyses of FCM-sorted cells

To identify whether the live cells labeled with antibodies were ASG or not, gene

expression analyses against the FCM-sorted cells was conducted. First, RT-PCR was used to

analyze the gene expression profiles of antibody-positive and -negative cells. To do this,

testicular cells prepared from testes containing all stages of germ cells (from ASGs to

spermatozoa) from 3-year-old Pacific bluefin tuna were labeled with antibodies conjugated

with Alexa flour 488, and then antibody-positive and -negative cells were sorted by

fluorescence-dependent FCM. Further, three types of cDNA were synthesized and used as

templates for PCR: cDNA from unsorted cells, cDNA from antibody-positive cells, and cDNA from antibody-negative cells fractionated by FCM sorting. Total RNA from 4 to 5×10^5 sorted cells were extracted using Isogen (Nippon Gene, Tokyo, Japan). Reverse transcription was performed using Ready-To-Go You-Prime First Strand-Beads (GE Healthcare) with the oligo(dT) primer according to manufacturer instructions. Next, PCR was performed with *TaKaRa Ex Taq* (Takara Bio Inc., Shiga, Japan) and the primers and PCR conditions are shown in Table 2. PCR products were separated by electrophoresis on a 2% agarose gel.

Next, to calculate the proportion of ASGs contained in antibody-positive fractions, *in situ* hybridization using *vasa* and *dead end* probe were conducted against the antibody-positive cells. Approximately 1 to 2×10^5 of antibody-positive cells were sorted by FCM sorting, and then sorted cells were smeared on glass slides. The smearing procedure was performed according to Yazawa et al. [26] and Nakajima et al. [27]. RNA probes were synthesized from a 1,094 bp cDNA *vasa* fragment (nucleotide 934-2,028; EU253482.1) [28] and a 1,150 bp cDNA *dead end* fragment (nucleotide 43-1,192; KF128758) [26]. Probe synthesis and hybridization were performed according to the protocol by Nagasawa et al. [28]. All experiments were carried out in triplicate. A minimum of 100 cells in triplicate for each specimen were counted to calculate the proportion of *vasa*-positive and *dead end*-positive cells, and the proportions were expressed as a percentage of total testicular cells.

Germ cell transplantation

To investigate whether the antibody-labeled cells show transplantability and can be traced in the peritoneal cavity or recipients after transplantation, germ cell transplantation was performed. The cells fractionated by Percoll centrifugation are labeled with antibodies conjugated with Alexa flour 488 and a type of fluorescent dye, PKH26 (Merck KGaA). Antibody-labeled and PKH26-labeled cells were transplanted into the peritoneal cavity of nibe croaker larvae. At least 10,000 cells were injected into the peritoneal cavity of each

recipient. Transplantation into the peritoneal cavity of nibe croaker larvae was performed as described by Takeuchi et al. [29] and Yoshikawa et al. [30]. For visualization of donor cells, labeling with PKH26 was performed as described by Takeuchi et al. [29]. The transplantation(TP) success rate of donor-derived germ cells in the recipient genital ridges was calculated by the following formula: TP success rate (%) = number of fish incorporating fluorescent cells in genital ridges at 14 days post-transplantation (dpt)/number of fish observed×100. All experiments were carried out in triplicate, and fluorescence observation was carried out in at least 10 recipients.

Statistical analysis

All data are presented as mean ± standard error of the mean values. Excel-Toukei 2012 software (Social Survey Research Information Co., Ltd, Tokyo, Japan) was used for all statistical analysis. In this study, The Dunnett's test was used for multiple comparisons of the mean *vasa* and *dead end* antibody positive rates in fractions by comparisons with the control in that of unsorted fraction. The Dunnett's test was also used for multiple comparisons of the mean value of transplantation efficiency (TP success rate and incorporated cell numbers) of antibody-labeled cells by comparisons with the control in that of PKH26-labeled cells. Further, two-tailed Student's *t*-test was used to evaluate whether the mean diameters of antibody-positive and -negative cells were significantly different (*F*-test was performed to reveal the variance of populations were equal). For all statistical tests, values were considered to be significantly different at $P < 0.05$.

Results

Generation of monoclonal antibodies by inoculating live ASGs

Direct immunization of live ASGs enriched with light-scattering-dependent FCM produced antibodies that recognized cell surface antigens of ASGs, and the products of cell fusion between lymph node cells and myeloma cells was conducted at 8 days post-immunization were seeded on twelve 96-well plates. At 2 weeks, hybridoma colonies had proliferated in all wells, and we obtained 1,152 hybridoma clones that potentially include clones that produce monoclonal antibodies capable of specifically recognizing cell surface antigens of ASGs.

Screening antibodies capable of recognizing cell surface antigens of ASGs

The following four screenings were conducted on the 1,152 hybridoma clones to identify antibodies capable of recognizing cell surface antigens of ASGs. First, we selected antibodies showing strong titer by cell-based ELISA, and 384 antibodies producing high absorbance values were selected as having the potential to specifically recognize cell surface antigens (Tables 3-1 and 3-2).

Next, we used the 384 antibodies identified as having the potential to specifically recognize cell surface antigens in immunocytochemistry with fluorescence observations of live testicular cells. Fluorescence signals on cell membranes were observed for 40 out of 384 antibodies (Fig. 1), indicating that 40 antibodies can recognize molecules localized in the cell membrane.

Further, to screen for antibodies that can enrich live ASGs, we performed flow cytometry analysis against the 40 antibodies confirmed to recognize cell surface antigens. Previous data showed that ASGs are distributed in gate A based on analysis of the light-scattering properties of whole testicular cells [25]. For antibodies that specifically

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recognize ASGs, the distribution of antibody-positive cells in light-scattering properties are concentrated in gate A. Therefore, we assessed the capability of each antibody to enrich ASGs in gate A after FCM analysis, and we selected 19 antibodies with enrichment ratio larger than 1 (Table 4).

Finally, to screen antibodies that can specifically recognize ASGs, we performed immunohistochemistry. Although 19 antibodies are identified as enriching ASGs, these did not necessarily specifically recognize ASGs. Since it is difficult to distinguish germ cell stages following dissociation, immunohistochemistry was conducted with 19 antibodies against mature testes possessing various stages of germ cells (from ASGs to sperm). Immunohistochemistry identified antibodies No. 152 and No. 180 as showing specific signals in ASGs, and no signals were observed in other developed germ cells and somatic cells (Fig. 2 A-D and A'-D'), indicating that these antibodies had the capability to specifically recognize the cell surface antigen of ASGs.

Visualization of live ASGs using fluorescence-conjugated antibodies

To visualize live ASGs using antibodies No. 152 and No. 180, the antibodies were directly labeled with Alexa flour 488 and used in immunocytochemistry. A small portion of cells were visualized (Fig. 3 A, B, A', and B'). For antibody No. 152, the diameter of fluorescent and non-fluorescent cells was 9.29 ± 0.33 and 5.78 ± 0.46 µm, respectively. For antibody No. 180, the diameter of fluorescent and non-fluorescent cells was 9.87 ± 0.41 and 6.28 ± 0.39 µm, respectively. The diameter of fluorescent cells was significantly larger than that of non-fluorescent cells. Since cell size of ASGs is known to be larger than other types of testicular cells, these results indicate that the antibodies conjugated with Alexa flour 488 could specifically visualize ASGs.

Next, to investigate whether visualized cells are ASGs or not, RT-PCR was performed against the antibody-positive and -negative cells. Live testicular cells were labeled

with antibodies No. 152 and No. 180 conjugated with Alexa flour 488, then antibody-positive and -negative cells were sorted by fluorescence-dependent FCM. To conduct RT-PCR, mature testes possessing various stages of germ cells (from ASGs to sperm) were used as initial cells to investigate whether the antibodies can specifically recognize ASGs among all maturation stages of germ cells. Next, RT-PCR performed against the cDNA of antibody-positive, antibody-negative and unsorted fractions using some gonadal cell markers identified *dead end*, which is an ASGs marker, as being expressed in the antibody-positive fractions but not in antibody-negative fractions for both antibodies No. 152 and No. 180 (Fig. 3 C and D). On the other hand, *gsdf*, which is a Sertoli cell marker, was not expressed in the antibody-positive fraction and was expressed in the antibody-negative fractions for both antibodies No. 152 and No. 180 (Fig. 3 C and D). *Sycp3*, which is a meiotic marker, was expressed in all fractions but with higher expression levels in the antibody-negative fractions for both antibodies No. 152 and No. 180 than in other fractions. These results indicate that the visualized cells were enriched with ASGs and did not contain gonadal somatic cells and differentiated germ cells.

Further, the proportion of ASGs in cells identified by *vasa* and *dead end* probes in cells identified by antibodies No. 152 and No. 180 by *in situ* hybridization against smeared cells and sorted by fluorescence-dependent FCM showed a *vasa*-positive rate in the unsorted cells of 63.1 ± 4.4%. The *vasa*-positive rates in each cell fraction isolated using antibodies No. 152 and No. 180 were 94.7 ± 1.3% and 90.5 ± 0.9%, respectively (Fig. 4 A-C and G). To investigate whether the antibodies can specifically enrich ASGs at all germ cell maturation stages, mature testes possessing germ cells at various stages (from ASGs to sperm), were used as initial cells. Thus, the *vasa*-positive rate of the antibody-positive fractions was significantly higher than that of the unsorted fraction. Further, the *dead end*-positive rate in unsorted cells was 16.6 ± 1.3%, while that in in each cell fraction isolated using antibodies No. 152 and No. 180 was 77.3 ± 6.4% and 70.5 ± 4.5%, respectively (Fig. 4 D-F and H). The *dead end*-positive rate of the antibody-positive fraction was significantly higher than that of the

unsorted fraction. These results indicate that visualized cells were highly enriched with ASGs and that antibodies No. 152 and No. 180 conjugated with Alexa flour 488 can specifically visualize ASGs.

Tracing post-transplanted cells in the peritoneal cavity of recipients

Finally, to investigate whether visualized cells possess transplantability and can be traced after transplantation, germ cell transplantation was performed using visualized cells. Dissociated cells were labeled with antibodies No. 152 and No. 180 and PKH26 followed by transplantation into the peritoneal cavity of nibe croaker larvae. After transplantation, the TP-success rate and incorporated cell number were evaluated by fluorescence observation at 14 dpt.

Dissociated cells were stained with fluorescence dye or Alexa flour 488-conjugated antibodies. Although all testicular cells were labeled with PKH26, antibodies No. 152 and No. 180 labeled a small portion of cells predicted to be ASGs (Fig. 5 A-C and A'-C'). Transplanting the antibodies-labeled cells into the peritoneal cavity of recipients resulted in the spread of fluorescence in whole body cavities (Fig. 5 D-G).

At 14 dpt, the visualized cells labeled with antibody No. 152 were incorporated into the genital ridge of a recipient larva (Fig. 6A). It is noteworthy that the donor germ cells showed clear green fluorescence even after 14 dpt (Fig. 6A, arrowheads). However, no cells were incorporated into the genital ridges of recipients that received donor cells labeled with antibody No. 180 (Fig. 6B). The visualized cells labeled with PKH26 were also incorporated into the genital ridge of the recipient larva (Fig. 6C).

Further, to investigate whether antibody No. 152 inhibits the migration and incorporation capability of transplanted cells, TP success rate and incorporated cell numbers were quantified by fluorescence observation. The TP success rate of donor cells visualized by antibody No. 152 and PKH26 was $63.3 \pm 8.8\%$ and $33.3 \pm 13.3\%$, respectively (Fig. 6D). The

number of donor cells visualized using antibody No. 152 and PKH26 incorporated into the genital ridge of each recipient was 3.63 ± 2.09 and 1.9 ± 1.1, respectively (Fig. 6E). There were no significant differences for TP success rate and incorporated cell numbers between antibody No. 152- and PKH26-stained cells.

Discussion

In order to label live cells using antibodies, proteins or sugars located on the cell surface membrane must be recognizable as epitopes. In this study, we directly inoculated live ASGs into mice to produce antibodies that can recognize cell surface antigens of ASGs. However, immunogens may contain molecules that were leaked out from the cytoplasm derived from dead ASGs in addition to molecules located on the cell surface. Therefore, we performed screening for antibodies that can specifically recognize cell surface antigens of ASGs in two stages: first, screening for antibodies capable of recognizing cell surface antigens by immunocytochemistry using live testicular cells and second, screening for antibodies that can enrich ASGs by FCM analysis. Although the antibodies can enrich ASGs, they do not necessarily specifically recognize ASGs. Therefore, immunohistochemistry with the selected antibodies was conducted in order to screen for antibodies that can specifically recognize ASGs. As a result, we revealed that antibodies No. 152 and No. 180 can specifically recognize ASGs in histological analysis. Taken together, we conclude that antibodies No. 152 and No. 180 can specifically recognize cell surface antigens of both live and fixed ASGs.

Although numerous antibodies capable of recognizing cell surface antigens of germ cells have been produced in mammals [21], there are few such antibodies have been identified in any kind of fish species. We previously identified lymphocyte antigen 75 (Ly75/ CD205) as a germ cell-specific cell surface marker by combination of expressed sequence tag analysis of purified ASGs, *in silico* prediction of membrane proteins and expression analyses [23]. However, immunocytochemistry with Ly75-specific antibodies was conducted against the live testicular cells, no signals were observed on ASGs dissociated by protease [31], probably due to digestion and degeneration of epitopes by protease during cell preparation. Indeed, analysis of floating cells such as blood corpuscles showed that it is not necessary to dissociate tissue masses using proteases in order to obtain cell suspensions, and subsequently, large numbers of

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cell surface markers such as cluster of differentiation (CD) markers were developed in the field of hematology, and combination of CD markers drastically improved the efficiencies of purification and identification of hematopoietic stem cell populations [16, 17]. However, germ cells prepared from gonadal tissues were associated with the risk of epitope digestion by protease treatment during tissue dissociation. Therefore, in this study, we attempted to use cells that were already treated with a protease, as an immunogen, in order to produce antibodies capable of recognizing the cell surface of protease-treated live germ cells. Germ cells used as immunogens were prepared by the methods established by Kise et al. [32] and Ichida et al. [25]. This technique can enrich fish ASGs by flow-cytometric cell sorting based on the light-scattering properties of each cell. In the case of Pacific bluefin tuna testes, it is possible to enrich *vasa*-positive cells to approximately 80% of purity by the latter technique [25]. Although this purity is not high enough to apply the resulting cells as immunogens to produce polyclonal antibodies, the antibodies with capability of specifically recognizing the cell surface membrane of ASGs could be identified by making monoclonal antibodies and further screening of resulting hybridomas. In this experiment, we succeeded in identifying two antibodies that recognize cell surface antigens of ASGs out of 1152 antibodies by a combination of cell-based ELISA, immunocytochemistry, FCM analyses and immunohistochemistry. In these screening processes, no transgenic technology, such as *vasa-gfp* strain or cell-specific markers, were used. These are particularly important advantages when considering the application of these technologies to commercially important species or endangered species that lack transgenic strains or specific molecular markers necessary for identifying particular cell lineages.

In this study, we succeeded in establishing a simple technique for visualizing bluefin tuna ASGs using a monoclonal antibody directly labeled with a fluorescent dye, Alexa flour 488, to monoclonal antibodies. In this visualization procedure, it is only necessary to incubate whole testicular cells with antibody conjugated with fluorescent dye and rinse the cells to

eliminate extra antibodies. This procedure requires less than 1 hour and remarkably does not require any special equipment. Cell populations labeled with these antibodies possessed larger diameter cells than those of the unlabeled cells, and, moreover, more than 90% of cells were *vasa*-positive and more than 70% of cells were *dead end*-positive. These results indicate that these antibodies conjugated with Alexa flour 488 can visualize predominantly bluefin tuna spermatogonia, especially ASGs.

In our previous studies, we have been attempting to establish a cultured ASG line with stem cell activity [33, 34]. Therefore, if an *in vitro* culture method that enables spermatogonia to expand is established, it may become possible to produce Pacific bluefin tuna seedlings by transplanting spermatogonia cultured *in vitro* into recipient fish without using live Pacific bluefin tuna as donors. In *in vitro* culturing, it is important to conduct optimization of culture conditions that allow the spermatogonial population to expand and be maintained for long periods while maintaining their original cellular characteristics. In germ cell cultures, there is a possibility that overgrowth of gonadal somatic cells present in the initial cell population may eventually outcompeted cultured ASGs, which have slower cell cycles than gonadal somatic cells. Therefore, it is important to distinguish ASGs, differentiated germ cells, and gonadal somatic cells in petri dishes when conducting these experiments. To identify and count ASGs in petri dishes, *in situ* hybridization using some molecular markers against the fixed cells must be conducted [25, 26]. These methods require many experimental manipulations and a long experimental period to obtain results. Therefore, the ASG visualization methods developed in this study can simplify the identification of ASGs in *in vitro* culture conditions.

Antibody labeling can be a powerful tool for germ cell transplantation. In this study, we examined whether it is possible to trace the behavior of post-transplanted cells by fluorescence observation in the recipient body cavity. To do so, two issues had to be overcome. First, whether fluorescence signal and antibody binding can be maintained until the

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post-transplanted cells complete migration towards the recipient gonads. Generally, antibody binding is considered to potentially influence the function of target molecules or target cells [35, 36]. Therefore, the second obstacle was whether the antibody binding inhibits the migration and incorporation capability of the transplanted cells of the donor cells after transplantation into the recipient body cavity. Transplantation studies with germ cells labeled with the antibodies revealed that although no cells were incorporated into the genital ridge of recipients that received donor cells labeled with antibody No. 180, the visualized cells labeled with antibody No. 152 were incorporated into the genital ridge of recipient larvae without a decline of function. The transplantability of antibody No. 152-labeled cells compared favorably with that of PKH26-labeled cells as a positive control. This result indicates that labeling with antibody No. 152 maintained binding to ASGs even during the 14 days post transplantation and that antibody binding did not inhibit migration and incorporation capability of the transplanted ASGs. Previously, donor cell labeling was performed by vital fluorescent dyes such as PKH26 to trace colonized germ cells in recipient gonads [12, 13, 29, 30, 37-40]. However, donor-derived germ cells and somatic cells cannot be distinguished in the recipients by fluorescence observation alone because PKH26 stains both gonadal somatic cells and all germ cell stages. In the technique developed in this study, fluorescence-visualized cells are mostly in the ASG populations, making it easy to evaluate simply by fluorescent observation whether the donor ASGs derived from bluefin tuna are incorporated into recipient gonads or not. Despite the simplicity of this cell tracing technology, this is the first report of its use. This cell visualization technique will be particularly important when applied to non-model, endangered or domestic animals for which it is difficult to apply transgenic techniques. As described above, we succeeded in establishing a technique to visualize ASGs in bluefin tuna using newly isolated antibodies without any transgenic technologies. The methodology of visualizing ASGs developed in this study will be a powerful tool for simplifying the transplantation and manipulation of germ cells in Pacific bluefin tuna.

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Figure legends

Fig. 1. Cell-based ELISA screening with monoclonal antibodies of testicular cells with detection of antibodies by green fluorescence. Numbers in the upper left corners of panels correspond to antibody numbers in Table 3. Bars = 20 µm.

Fig. 2. Immunohistochemistry with monoclonal antibodies in testis of mature Pacific bluefin tuna for signals from antibody No. 152 (A', A", B' and B") and No. 180 (C', C", D' and D"). Bright field (A, B, C, and D), fluorescent (A', B', C', and D') and merged (A", B", C", and D") views of paraffin sections of mature testis. (B-B") High magnification of insets in A-A". (D-D") High magnification of insets in C-C". Antibody signals were found on the type A spermatogonia (ASGs; arrowheads). BSG, SC and ST indicate type B spermatogonia, spermatocytes and spermatids, respectively. Scale bars of low and high magnification indicate 20 µm (A-A" and C-C") and 10 µm (B-B" and D-D"), respectively.

Fig. 3. Visualization of Pacific bluefin tuna ASGs using Alexa flour 488-conjugated antibodies. Bright field (left) and fluorescent (right) views of the testicular cells were labeled with antibodies No. 152 (A and A') and No. 180 (B and B') directly conjugated with Alexa flour 488. Green fluorescence indicates the signals caused by antibodies. Bars = 20 µm. (C) RT-PCR analyses of *dead end* (ASG marker), *gsdf* (Sertoli-cell marker), *sycp3* (meiotic marker) and *actb* as an internal control using unsorted testicular cells and cells isolated using fluorescence-dependent flow cytometry with the Alexa flour 488-conjugated antibodies. (D) US, 152+, 152-, 180+, and 180- indicate unsorted, antibody No. 152-positive, antibody No. 152-negative, antibody No. 180-positive and antibody No. 180-negative fractions, respectively. DW indicates distilled water as the negative control containing no cDNA template.

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Fig. 4. Proportions of *vasa-* and *dead end*-positive cells contained in antibody-positive fractions detected by *in situ* hybridization with *vasa* (A-C) and *dead end* (D-F) probes against cell smears sorted using Alexa flour 488-conjugated antibodies. Smears of unsorted testicular cells were also applied to *in situ* hybridization as a control (A and D). Arrowheads indicate *vasa-* and *dead end*-positive cells. Bars = 10 µm. (G) *vasa*-positive rates of unsorted cells and sorted cells using antibodies No. 152 and No. 180. (H) *dead end*-positive rates of unsorted cells and sorted cells using antibodies No. 152 and No. 180. Values in G and H are expressed as mean ± SEM (error bars). Asterisks indicate significant differences between the unsorted and antibody-positive fractions by Dunnett's test ($P < 0.05$).

Fig. 5. Fluorescent views of transplanted cells labeled with Alexa flour 488-conjugated antibodies and PKH26. Dissociated testicular cells labeled with antibodies No. 152 (A and A'), No. 180 antibody (B and B') and PKH26 (C and C'). Scale bars indicate 20 µm. Fluorescent views of transplanted (D-F) and non-transplanted (G) larvae immediately after microinjection. Arrowhead indicates transplanted donor testicular cells labeled with antibody No. 152 (D), antibody No. 180 (E) and PKH26 (F). Bars = 500 µm.

Fig. 6. Transplantation of donor germ cells visualized with fluorescence-conjugated antibodies. Fluorescent views of genital ridges of recipient fish at 14 dpt for antibody No. 152-labeled (A), antibody No. 180-labeled (B) and PKH26-labeled (C) cells. Arrows (A-C) and arrowheads (A and C) indicate recipient genital ridges and donor-derived germ cells, respectively. Bars = 20 µm. (D) The ratios of recipient fish that possess visualized cells within gonads at 14 dpt (shown as transplantation (TP)-success rate). (E) Number of the visualized cells incorporated into recipient genital ridges. Values in D and E are expressed as mean ± SEM (error bars). There is no significant difference between PKH26-labeled cells and

antibody No. 152-labeled cells by Dunnett's test.

Fig. 1

Fig. 2

Fig. 3

A No. 152   A' No. 152

B No. 180   B' No. 180

C   US   152+   152-   DW

dead end

gsdf

sycp3

actb

D   US   180+   180-   DW

dead end

gsdf

sycp3

actb
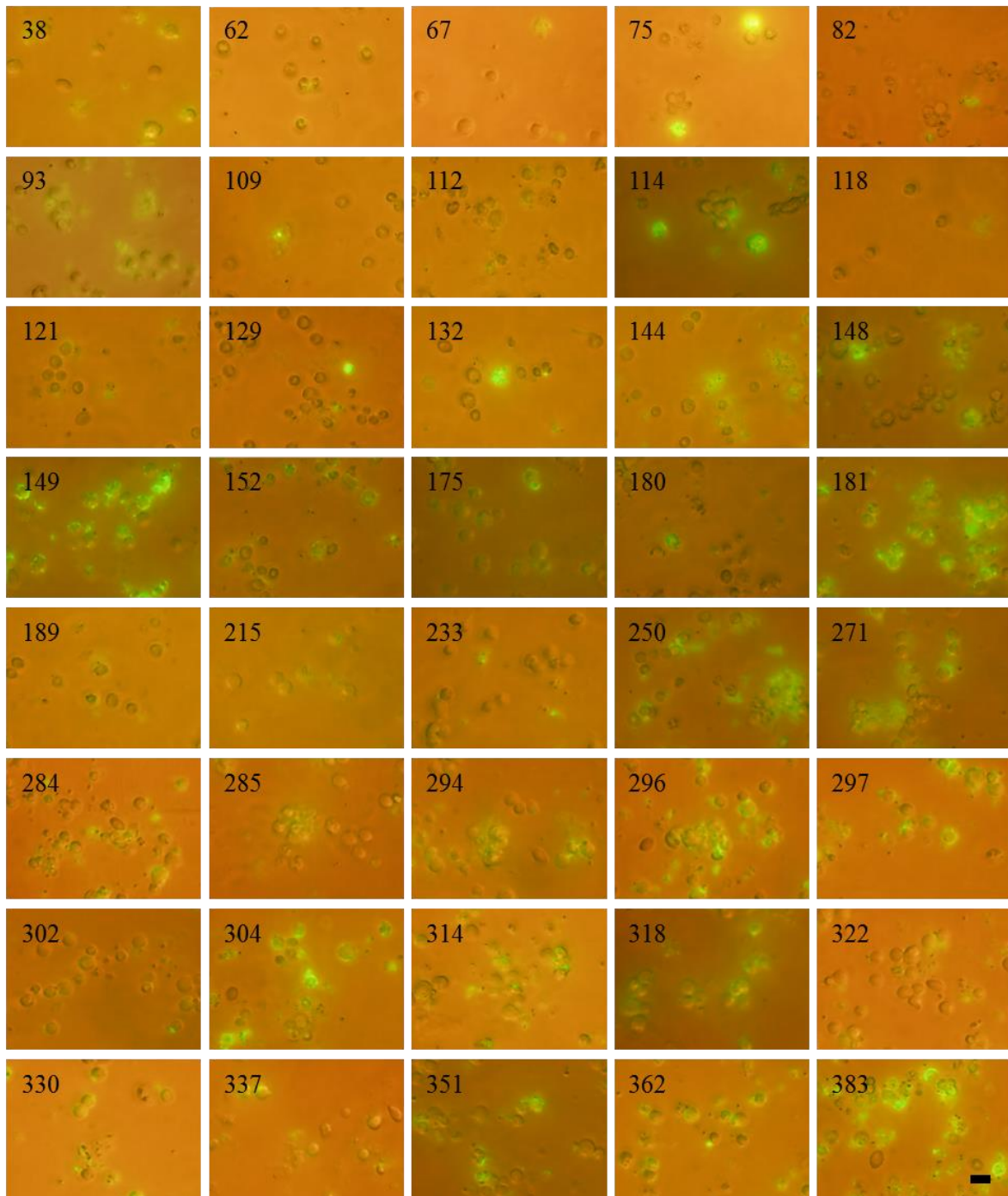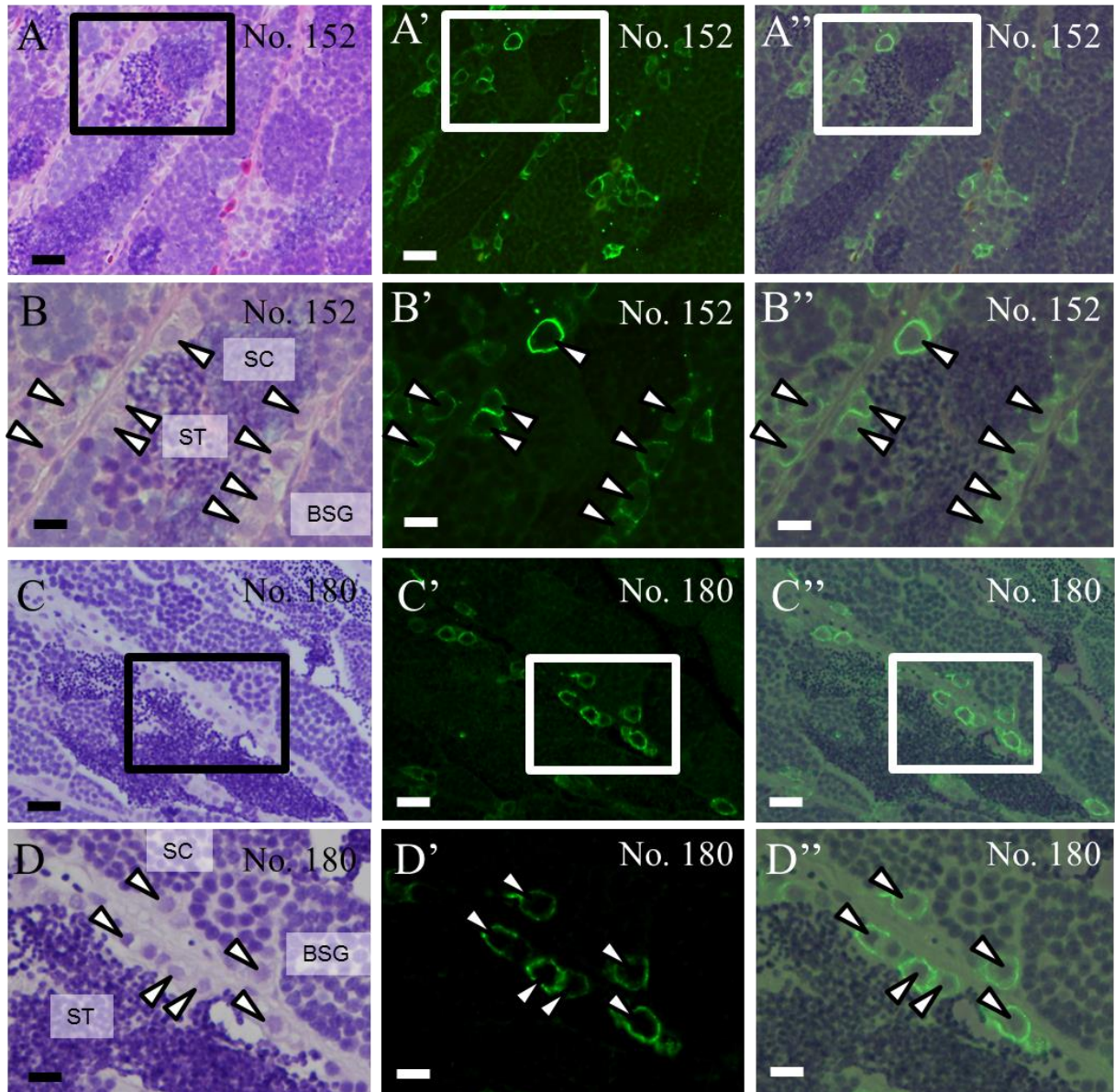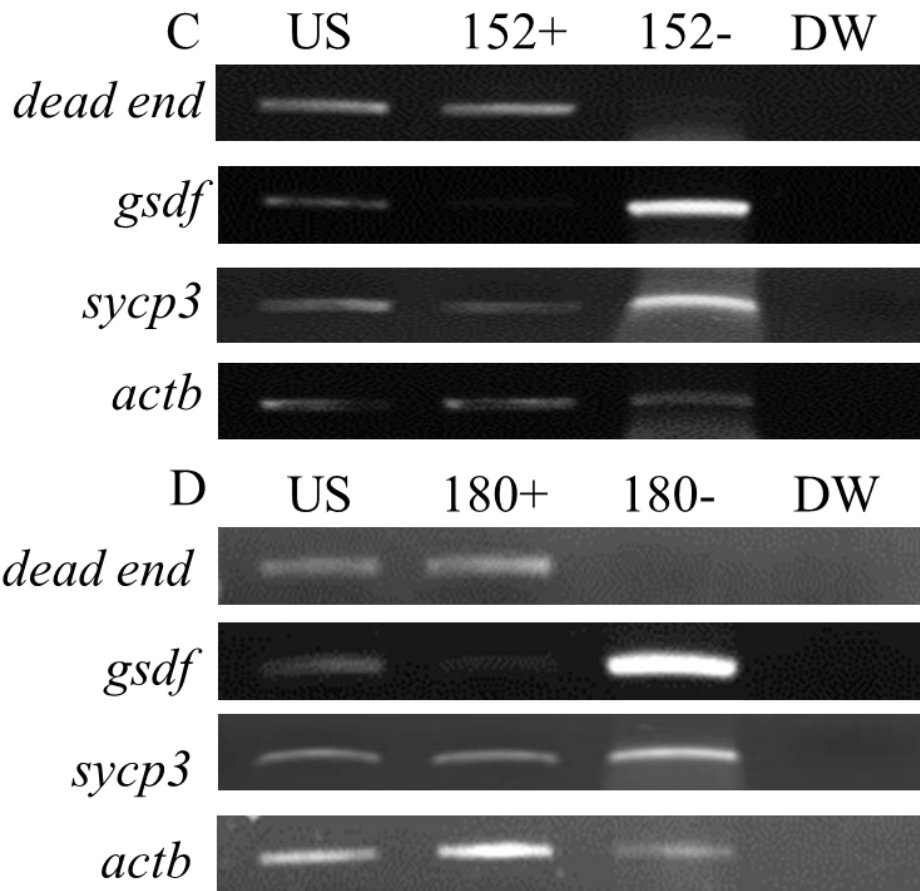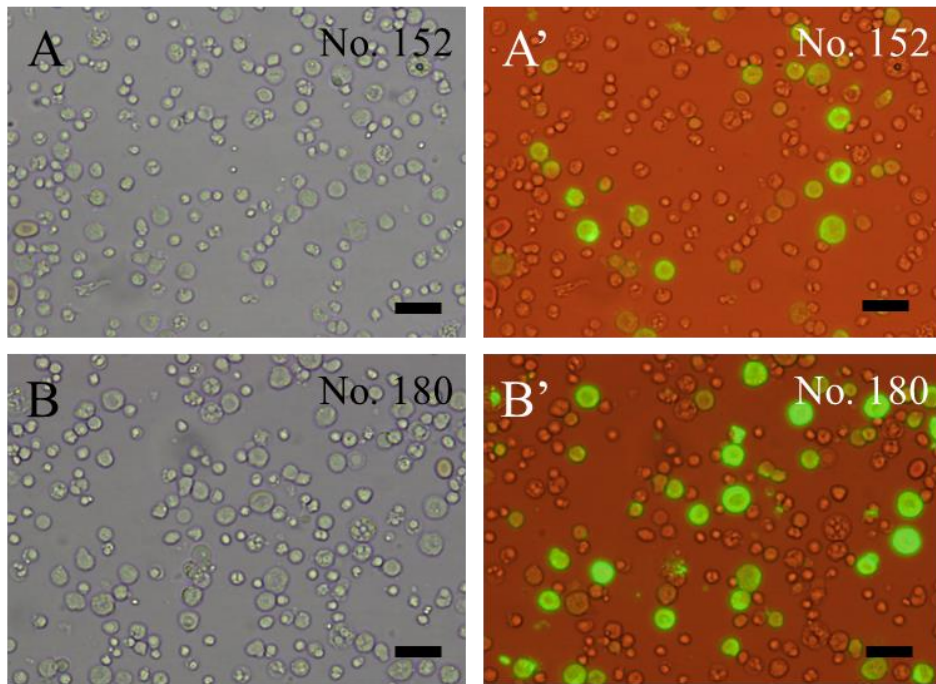
Fig. 4
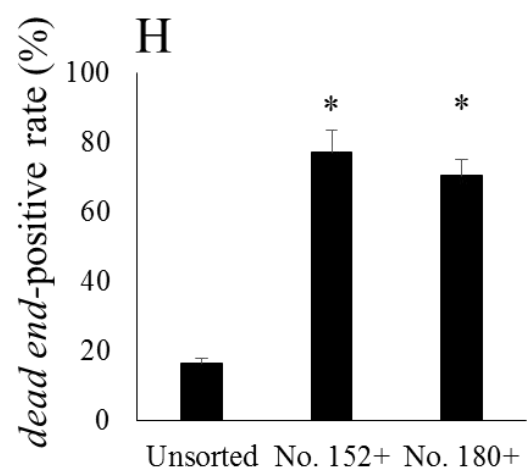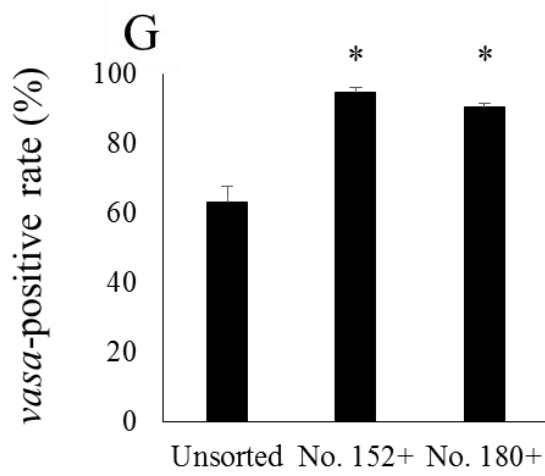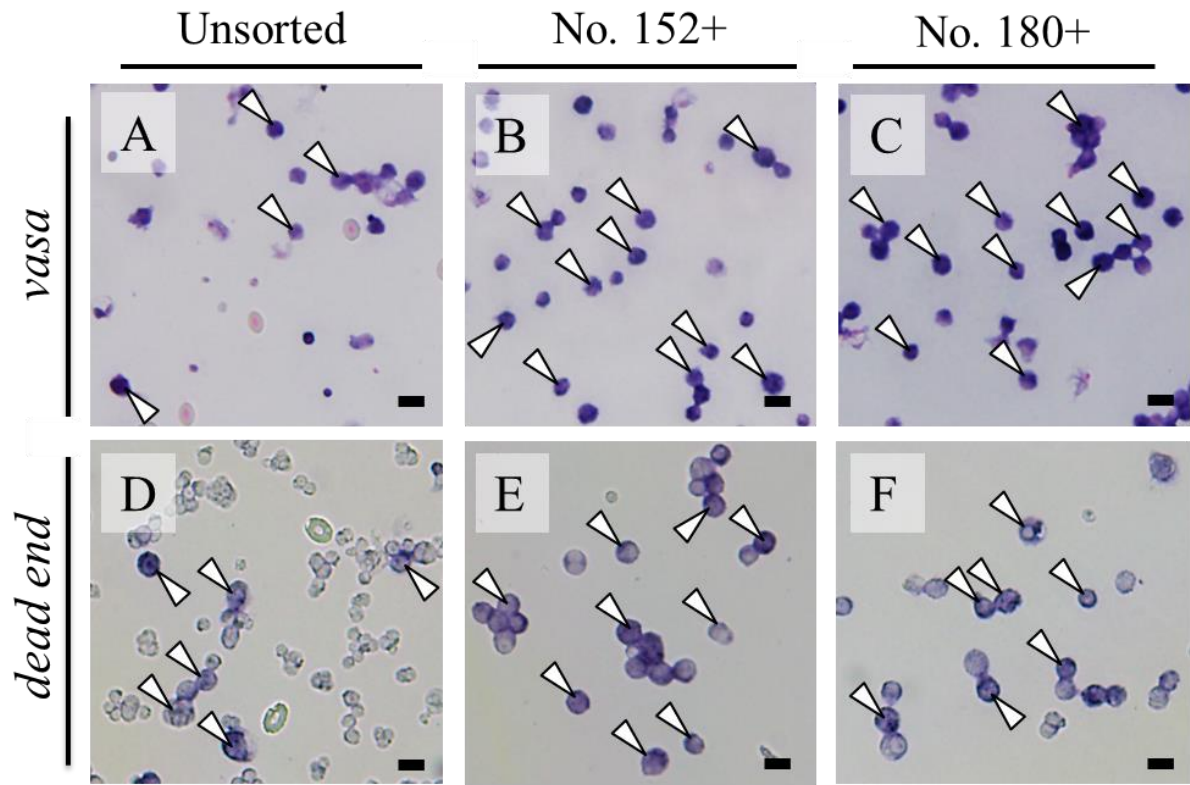
Fig. 5
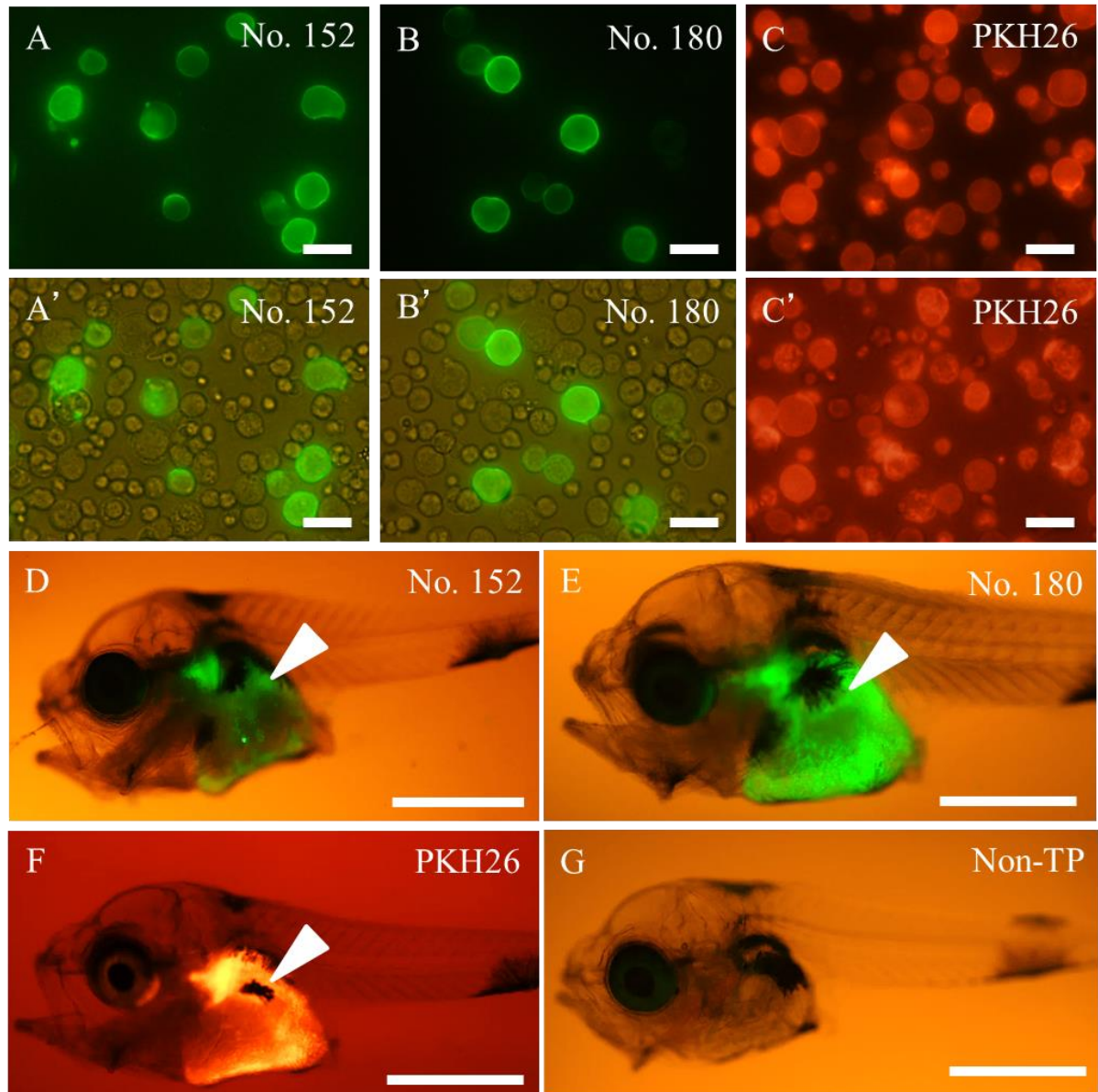
Fig. 6
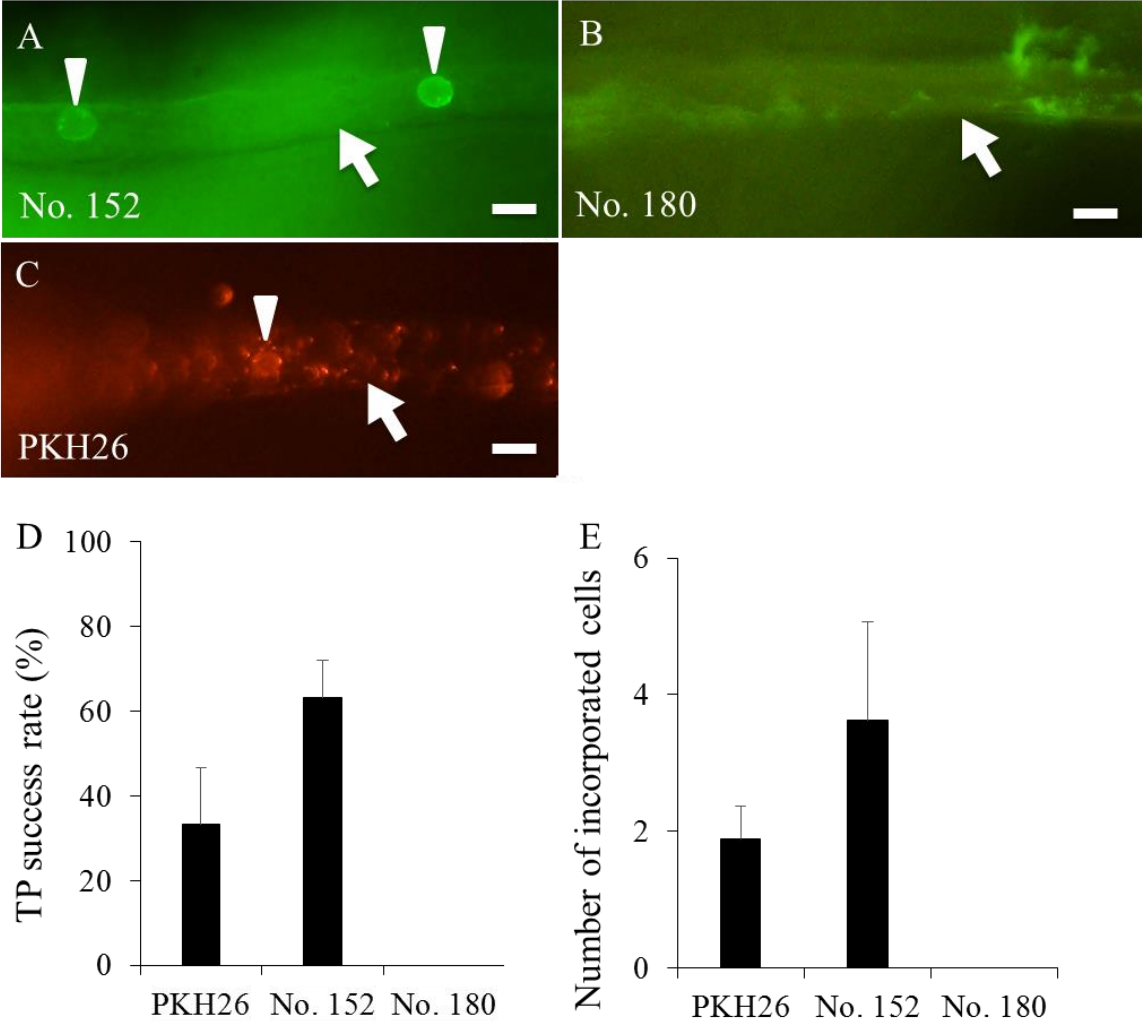
Table 1. Inoculation schedule in 4-week-old, female BALB/c mice

Date	Fish used as donor	Inoculation cell count
2015/11/29	Bluefin tuna	2.54×10^6
2015/12/6	Bluefin tuna	5.0×10^6
2015/12/12	Bluefin tuna	5.0×10^6
2015/12/18	Bluefin tuna	4.85×10^6
2016/1/6	Blue mackerel	5.0×10^6

Table 2. Primer sequences and PCR conditions

Gene	Forward primer sequence	Reverse primer sequence	Denaturation Temp. / Time	Annealing Temp. / Time	Extension Temp. / Time	Cycles
b-actin	AAGAGAGGTATCCTGA CCCTGAA	TCAACCAGTGCAACATA ACA	95°C / 30 s	60°C / 30 s	72°C / 30 s	35
dead end (*in situ* hybridization)	ACAACAACTGTGGAGA TGA	ACTGCTTGTTGTAAACA GA	95°C / 30 s	55°C / 30 s	72°C / 60 s	30
dead end (RT-PCR)	CAACAACTGTGGAGAT GATGGAAAA	GCAGCATGGTGGGATGA AAA	95°C / 30 s	60°C / 30 s	72°C / 30 s	35
gsdf	ATGTCCTTTACGTTCAT TATTACGA	AGGATATAACAAGGGTG CTGAATT	95°C / 30 s	60°C / 30 s	72°C / 30 s	35
sycp3	TTGCAGCAGTGGGAGA CTGA	TCAACGGCAGCTATTTA ACC	95°C / 30 s	56°C / 30 s	72°C / 30 s	35

Table 3-1. Results of cell-based ELISA with monoclonal antibodies raised against bluefin tuna ASGs

Antibody number	Absorbance	Antibody numbe	Absorbance	Antibody number	Absorbance	Antibody number	Absorbance	Antibody number	Absorbance	Antibody number	Absorbance
No. 1	0.101	No. 33	0.095	No. 65	0.158	No. 97	0.096	No. 129	0.238	No. 161	0.271
No. 2	0.116	No. 34	0.122	No. 66	0.194	No. 98	0.101	No. 130	0.583	No. 162	0.103
No. 3	0.096	No. 35	0.171	No. 67	0.1	No. 99	0.183	No. 131	0.115	No. 163	0.193
No. 4	0.116	No. 36	0.152	No. 68	0.165	No. 100	0.144	No. 132	0.276	No. 164	0.154
No. 5	0.252	No. 37	0.183	No. 69	0.103	No. 101	0.104	No. 133	0.111	No. 165	0.147
No. 6	0.156	No. 38	0.214	No. 70	0.28	No. 102	0.1	No. 134	0.124	No. 166	0.243
No. 7	0.216	No. 39	0.127	No. 71	0.189	No. 103	0.177	No. 135	0.122	No. 167	0.166
No. 8	0.265	No. 40	0.299	No. 72	0.287	No. 104	0.098	No. 136	0.113	No. 168	0.126
No. 9	0.107	No. 41	0.114	No. 73	0.102	No. 105	0.588	No. 137	0.207	No. 169	0.165
No. 10	0.13	No. 42	0.137	No. 74	0.839	No. 106	0.219	No. 138	0.144	No. 170	0.171
No. 11	0.107	No. 43	0.514	No. 75	0.96	No. 107	0.132	No. 139	0.185	No. 171	0.249
No. 12	0.634	No. 44	0.122	No. 76	0.919	No. 108	0.113	No. 140	0.316	No. 172	0.136
No. 13	0.127	No. 45	0.125	No. 77	0.282	No. 109	0.577	No. 141	0.107	No. 173	0.179
No. 14	0.501	No. 46	0.12	No. 78	0.104	No. 110	0.623	No. 142	0.129	No. 174	0.099
No. 15	0.103	No. 47	0.542	No. 79	0.099	No. 111	0.099	No. 143	0.119	No. 175	0.201
No. 16	0.121	No. 48	0.399	No. 80	0.777	No. 112	0.537	No. 144	0.256	No. 176	0.248
No. 17	0.126	No. 49	0.21	No. 81	0.236	No. 113	0.148	No. 145	0.138	No. 177	0.104
No. 18	0.11	No. 50	0.095	No. 82	0.149	No. 114	0.394	No. 146	0.135	No. 178	0.107
No. 19	0.101	No. 51	0.113	No. 83	0.095	No. 115	0.734	No. 147	0.279	No. 179	0.131
No. 20	0.639	No. 52	0.209	No. 84	0.096	No. 116	0.117	No. 148	0.316	No. 180	0.947
No. 21	0.14	No. 53	0.181	No. 85	0.201	No. 117	0.105	No. 149	0.277	No. 181	0.15
No. 22	0.13	No. 54	0.266	No. 86	0.293	No. 118	0.225	No. 150	0.099	No. 182	0.13
No. 23	0.138	No. 55	0.196	No. 87	0.488	No. 119	0.1	No. 151	0.095	No. 183	0.112
No. 24	0.249	No. 56	0.115	No. 88	0.355	No. 120	0.101	No. 152	0.31	No. 184	0.165
No. 25	0.129	No. 57	0.095	No. 89	0.188	No. 121	0.155	No. 153	0.134	No. 185	0.123
No. 26	0.611	No. 58	0.163	No. 90	0.112	No. 122	0.109	No. 154	0.103	No. 186	0.124
No. 27	0.107	No. 59	0.121	No. 91	0.101	No. 123	0.113	No. 155	0.21	No. 187	0.109
No. 28	0.106	No. 60	0.135	No. 92	0.1	No. 124	0.302	No. 156	0.152	No. 188	0.108
No. 29	0.234	No. 61	0.218	No. 93	0.433	No. 125	0.096	No. 157	0.312	No. 189	0.313
No. 30	0.384	No. 62	0.162	No. 94	0.117	No. 126	0.184	No. 158	0.112	No. 190	0.114
No. 31	0.095	No. 63	0.103	No. 95	0.517	No. 127	0.104	No. 159	0.103	No. 191	0.726
No. 32	0.104	No. 64	0.115	No. 96	0.833	No. 128	0.095	No. 160	0.116	No. 192	0.104

Table 3-2. Results of cell ELISA with monoclonal antibodies raised against bluefin tuna ASGs

Antibody number	Absorbance	Antibody number	Absorbance	Antibody number	Absorbance	Antibody number	Absorbance	Antibody number	Absorbance	Antibody number	Absorbance
No. 193	0.172	No. 225	0.141	No. 257	0.163	No. 289	0.338	No. 321	0.188	No. 353	0.174
No. 194	0.315	No. 226	0.109	No. 258	0.124	No. 290	0.156	No. 322	0.692	No. 354	0.159
No. 195	0.102	No. 227	0.136	No. 259	0.105	No. 291	0.177	No. 323	0.211	No. 355	0.448
No. 196	0.099	No. 228	0.096	No. 260	0.292	No. 292	0.105	No. 324	0.109	No. 356	0.123
No. 197	0.338	No. 229	0.105	No. 261	0.154	No. 293	0.099	No. 325	0.115	No. 357	0.418
No. 198	0.098	No. 230	0.118	No. 262	0.102	No. 294	0.096	No. 326	0.111	No. 358	0.166
No. 199	0.216	No. 231	0.403	No. 263	0.132	No. 295	0.175	No. 327	0.112	No. 359	0.135
No. 200	0.15	No. 232	0.128	No. 264	0.1	No. 296	0.393	No. 328	0.165	No. 360	0.166
No. 201	0.353	No. 233	0.243	No. 265	0.195	No. 297	0.512	No. 329	0.098	No. 361	0.11
No. 202	0.126	No. 234	0.106	No. 266	0.154	No. 298	0.097	No. 330	0.832	No. 362	0.83
No. 203	0.123	No. 235	0.132	No. 267	0.15	No. 299	0.159	No. 331	0.136	No. 363	0.111
No. 204	0.259	No. 236	0.256	No. 268	0.204	No. 300	0.236	No. 332	0.123	No. 364	0.155
No. 205	0.113	No. 237	0.123	No. 269	0.429	No. 301	0.113	No. 333	0.195	No. 365	0.102
No. 206	0.112	No. 238	0.126	No. 270	0.14	No. 302	0.326	No. 334	0.216	No. 366	0.106
No. 207	0.103	No. 239	0.174	No. 271	0.298	No. 303	0.1	No. 335	0.207	No. 367	0.466
No. 208	0.12	No. 240	0.14	No. 272	0.096	No. 304	0.568	No. 336	0.294	No. 368	0.096
No. 209	0.116	No. 241	0.119	No. 273	0.184	No. 305	0.123	No. 337	1.038	No. 369	0.162
No. 210	0.566	No. 242	0.123	No. 274	0.394	No. 306	0.118	No. 338	0.366	No. 370	0.132
No. 211	0.189	No. 243	0.2	No. 275	0.576	No. 307	0.387	No. 339	0.291	No. 371	0.121
No. 212	0.127	No. 244	0.125	No. 276	0.136	No. 308	0.095	No. 340	0.111	No. 372	0.129
No. 213	0.098	No. 245	0.109	No. 277	0.124	No. 309	0.099	No. 341	0.24	No. 373	0.132
No. 214	0.097	No. 246	0.125	No. 278	0.185	No. 310	0.107	No. 342	0.121	No. 374	0.095
No. 215	0.475	No. 247	0.156	No. 279	0.127	No. 311	0.243	No. 343	0.347	No. 375	0.336
No. 216	0.109	No. 248	0.107	No. 280	0.13	No. 312	0.111	No. 344	0.171	No. 376	0.251
No. 217	0.145	No. 249	0.108	No. 281	0.161	No. 313	0.107	No. 345	0.159	No. 377	1.129
No. 218	0.098	No. 250	0.403	No. 282	0.158	No. 314	0.689	No. 346	0.146	No. 378	0.399
No. 219	0.109	No. 251	0.102	No. 283	0.185	No. 315	0.209	No. 347	0.095	No. 379	0.145
No. 220	0.117	No. 252	0.134	No. 284	0.464	No. 316	0.973	No. 348	0.097	No. 380	0.183
No. 221	0.128	No. 253	0.115	No. 285	0.217	No. 317	0.25	No. 349	0.114	No. 381	0.156
No. 222	0.138	No. 254	0.102	No. 286	0.118	No. 318	1.341	No. 350	0.182	No. 382	0.154
No. 223	0.141	No. 255	0.122	No. 287	0.119	No. 319	0.199	No. 351	0.153	No. 383	0.745
No. 224	0.122	No. 256	0.145	No. 288	0.175	No. 320	0.104	No. 352	0.107	No. 384	0.177

Table 4. Enrichment ratio of antibody-positive type A spermatogonia by antibody number

Antibody number	Enrichment ratio	Antibody number	Enrichment ratio	Antibody number	Enrichment ratio
No. 67	1.91	No. 152	1.22	No. 233	0.13
No. 383	1.84	No. 148	1.20	No. 129	0.12
No. 149	1.82	No. 215	1.17	No. 118	0.10
No. 318	1.69	No. 189	1.11	No. 175	0.08
No. 297	1.66	No. 180	1.09	No. 181	0.05
No. 337	1.65	No. 93	0.71	No. 109	0.05
No. 362	1.60	No. 38	0.69	No. 114	0.04
No. 112	1.58	No. 330	0.63	No. 285	0.03
No. 296	1.46	No. 80	0.49	No. 294	0.02
No. 351	1.44	No. 132	0.47	No. 302	0.02
No. 314	1.34	No. 250	0.38	No. 121	0.01
No. 322	1.27	No. 271	0.36	No. 144	—
No. 62	1.25	No. 75	0.34		
No. 284	1.24	No. 304	0.24		

Enrichment ratio = proportion of cells detected in gate A in antibody-positive fraction/the proportion of cells detected in gate A in total-cell fraction.

総括

　本研究では遺伝子導入技術を用いずに、クロマグロの生殖細胞系列の可視化、単離、濃縮、追跡といった様々な細胞操作法の樹立を行った。まず第1章では、クロマグロ生殖細胞集団をフローサイトメーターで光学的特徴を指標に解析することで、精原細胞集団が分布する分画を同定し、その分画を単離することで精原細胞の濃縮技術の開発を行った。本技術は木瀬らがサケ科魚類を用いて樹立した精原細胞の濃縮技術をそのままクロマグロに応用したものである（Kise at al., 2012）。しかしながら、本技術は精原細胞の光学的特徴を指標として精原細胞を濃縮する技術であるため、たとえ分類群の大きく異なる魚種でも精原細胞の光学的、形態的な特徴が保存されていれば、様々な魚種において応用可能であると考えられた。実際に本技術をクロマグロに応用したところ、様々な成熟段階の精巣からでも精原細胞を高純度で濃縮することが可能であった。これまでにクロマグロ精原細胞を特異的に単離、濃縮可能な方法は報告されていなかった。そのため、本技術は濃縮したクロマグロ精原細胞を、移植、発現解析、抗体作製時の材料などに用いることを可能にするため、今後クロマグロ生殖細胞の研究における極めて重要な基礎技術となると考えられる。

　続いて第2章では第1章で樹立した濃縮技術で調整したクロマグロ精原細胞を免疫抗原に用いることで、クロマグロ精原細胞の細胞表面抗原を特異的に認識可能な抗体の作製に成功した。クロマグロは大型かつ遊泳性の強い魚類であることから、産卵誘発や1対1交配が極めて困難である。さらに、初期減耗の激しい魚種でもあるため（Sawada et al., 2005; Masuma et al., 2011）、遺伝子導入系統やゲノム編集系統を樹立することが非常に困難である。そのため、クロマグロ精原細胞の可視化、単離、追跡を実現するには、細胞表面抗原を特異的に認識可能な抗体を作製し、その抗体に対して蛍光物質を標識するという方法論が、最も現実的かつ効果的な方法であると考えた。実際に本研究で樹立したNo152抗体に蛍光標識を行ったことで、遺伝子導入魚

を用いることなくクロマグロ精原細胞の可視化、単離、追跡といった様々な細胞操作を行うことが可能となった。さらに、本技術の実験操作は、解離した生殖細胞懸濁液に蛍光標識した抗体を加え静置したのち、上清を洗浄するという操作のみであり、精原細胞を可視化するのに必要とする時間は 30-60 分程度である。これまでクロマグロ精原細胞を可視化するためには、細胞を固定したのち、*vasa* 遺伝子や *dead end* 遺伝子などの RNA プローブを用いて検出を行うのが唯一の方法であった（Nagasawa et al., 2008; Yazawa et al., 2013）。この方法では細胞を 1 度固定しなければ、精原細胞を可視化できないことに加え、結果を得るために 2-7 日程度の時間を必要とした。通常、精原細胞移植や培養は魚体から生殖腺を摘出後、細胞が生存している 1-2 日以内に実験を行わなければならない。そのため、生殖腺細胞を移植や培養実験に供する段階で、生殖腺細胞中に含まれる精原細胞を識別することが時間的に間に合わず、実験終了のさらに数日後にその判別が可能となるといった状況であった。今後は本技術により、移植実験における適切なドナー精巣の評価や培養実験における精原細胞の増殖率の算出をリアルタイムで行うことが可能となったため、移植、培養実験における各種条件検討が飛躍的に進むと期待される。

また本研究において、可視化した精原細胞を高純度で単離することにも成功した。海産魚の仔魚はその体サイズが非常に小さいことに起因して、1 個体あたりに移植可能な細胞数も限りがある。そのため精原細胞の存在比が低い生殖腺をドナーとして使用しなければならない場合、生殖腺細胞中から精原細胞を単離、濃縮しなければ、十分な移植効率を得られないケースが存在する。そのため No. 152 抗体を用いて単離したクロマグロ精原細胞をドナー細胞に用いることで、精原細胞の低い生殖腺における移植効率の改善が可能となると期待される。

さらに本研究で可視化した精原細胞をそのまま孵化仔魚腹腔内へと移植することで、移植後に宿主生殖腺内での挙動を追跡する技術の樹立に成功した。これにより宿主生殖腺への精原細胞の生着を蛍光観察のみで判断できるようになった。これまで非

遺伝子導入魚の移植細胞の挙動を追跡するのは、PKH26 という蛍光色素で細胞膜を染色することが唯一の信頼性の高い方法であった（Takeuchi et al., 2009; Lacerda et al., 2010; Yazawa et al., 2010; Morita et al., 2012; Majhi et al., 2014; Morita et al., 2015; Yoshikawa et al., 2017; Hamasaki et al., 2017)。しかしながら PKH26 は全ての細胞の細胞膜を染色してしまうため、宿主生殖腺へと取り込まれた PKH26 陽性細胞が確実に精原細胞集団であるかを蛍光観察のみで判断することができなかった。一方、本技術は精原細胞のみを特異的に可視化するため、蛍光観察のみで、精原細胞が宿主生殖腺へ取り込まれていることを確実に確認することが可能となった。また本技術は抗体で可視化した細胞をそのまま移植するという非常に簡便な技術でありながら、他の動物種、細胞種で報告のない全く新しい技術であり、今後遺伝子導入系統の存在しない動物種で生殖細胞の移植技術を構築する際に非常に有効なツールとなると期待される。一方本追跡技術は、可視化した細胞を数か月から数年という期間追跡することは原理上難しいと考えられる。そこで、現在は本研究で樹立した抗体群の中で、クロマグロ生殖細胞系列は認識するが、宿主となるその他サバ科魚類の生殖細胞系列を認識しない抗体をスクリーニングすることで、移植細胞の長期間の追跡する技術の開発にも取り組んでいる(窪川ら, 未発表)。これにより、本一連の追跡技術により移植した細胞が宿主生殖腺の中で増殖、分化をするのかを確認しながら研究を進めてゆくことが可能となるであろう。

　以上、遺伝子導入技術を用いずに、クロマグロ精原細胞の可視化、単離、濃縮、追跡といった細胞操作を、あたかも遺伝子導入魚を用いた場合と同じように実施することが可能となった。今後は本研究で樹立した技術を駆使することによるクロマグロ代理親魚技法の樹立と発展が期待される。

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