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Research Paper

Transient and Stable GFP Expression in Germ Cells by the *vasa* Regulatory Sequences from the Red Seabream (*Pagrus major*)

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Abstract

Primordial germ cells (PGCs) are the precursors of gametes responsible for genetic transmission to the next generation. They provide an ideal system for cryopreservation and restoration of biodiversity. Recently, considerable attention has been raised to visualize, isolate and transplant PGCs within and between species. In fish, stable PGC visualization in live embryo and individual has been limited to laboratory fish models such as medaka and zebrafish. One exception is the rainbow trout, which represents the only species with aquaculture importance and has GFP-labeled germ cells throughout development. PGCs can be transiently labeled by embryonic injection of mRNA containing green fluorescence protein gene (GFP) and 3'-untranslated region (3'-UTR) of a maternal germ gene such as *vasa*, *nos1*, etc. Stable PGC labeling can be achieved through production of transgenic animals by some transcriptional regulatory sequences from germ genes, such as the *vasa* promoter and 3'-UTR. In this study, we reported the functional analyses of the red seabream *vasa* (*Pmvas*) regulatory sequences, using medaka as a model system. It was showed that injection of GFP-*Pmvas*3'UTR mRNA was able to label medaka PGCs during embryogenesis. Besides, we have constructed pPmvasGFP transgenic vector, and established a stable transgenic medaka line exhibiting GFP expression in germ cells including PGCs, mitotic and meiotic germ cells of both sexes, under control of the *Pmvas* transcriptional regulatory sequences. It is concluded that the *Pmvas* regulatory sequences examined in this study are sufficient for germ cell expression and labeling.

Key words: *Pagrus major*, PGCs, *vasa*, transgene, GFP

Introduction

Primordial germ cells (PGC) are the precursors of the germ cell lineage, responsible for genetic transmission through generations. The inherent nature endows PGCs with numerous advantages of applications in fish bioengineering, such as cryopreservation and restoration of biodiversity [1]. PGCs can be specifically labeled and isolated for cell culture and transplantation, providing tools for reproduction of endangered species in close relatives [2]. The techniques open a new approach for genetic resource preservation and improve our understanding in germ line development [3]. However, in teleosts, successful surrogate breeding by germline cell transplantation has been limited to the rainbow trout [4, 5] and

zebrafish [6, 7].

PGCs visualization and labeling is the first step to forward the application. They can be transiently labeled by embryonic injection of mRNA containing green fluorescence protein gene (GFP) and 3′-untranslated region (3′UTR) of a maternal germ gene such as *vasa*, *nos1*, etc. The 3′UTR sequences play a critical role in eliminating the mRNA from the somatic cell line via microRNA activity, while stabilize the mRNA in PGCs through the DND (dead end) protein [8]. Besides, the function of the 3′UTR is widely conserved among fish species. For example, the mRNA combining GFP and zebrafish *nos1* 3′UTR has been proved to be able to identify PGCs in a wide range of fish species [9]. Meanwhile, other chimeric mRNA consisting of GFP and *vasa* 3′UTR from Nibe croaker or zebrafish has been used to visualize PGCs in rainbow trout successfully [10], in spite that GFP-O*lvas* 3′UTR mRNA from the medaka is not able to visualize PGCs in either zebrafish or loach [9]. Recently, in cyprinid fish, nontransgenic labeled PGCs (zebrafish *nos1* 3′UTR) have been successfully applied to fluorescence-activated cell sorting (FACS) [11] and produce inter-species germ-line chimeras [7]. Although injection of chimeric mRNA labels PGCs transiently, this nontransgenic technique is especially useful for cryopreservation of PGCs and seed production by surrogate breeding.

Stable PGC labeling has been achieved through the production of transgenic animals by promoter and some regulatory sequences of germ genes, such as the *vasa* promoter and 3′-UTR. At present, transgenic fish lines driven by *vasa* promoter, which express GFP specifically in germ cell lineage, have been reported only in the model fish, such as zebrafish [12] and medaka [13]. The only exception is rainbow trout [14], a species with high aquaculture importance. It has immense benefits for basic research, making FACS, long-term tracing of donor PGCs in recipient individuals, PGCs cryopreservation and screening mutants affecting PGCs feasible. FACS has been successfully applied to isolate labeled PGCs from transgenic rainbow trout [15] and zebrafish [16]. However, it is not easy to apply this approach in commercial fish species for various reasons: intensive labor, time and space consumption of producing transgenic fish; risk of biological contamination caused by releasing of the transgenic fish; consumers′ attitudes toward genetically modified fish.

Red seabream (*Pagrus major*) is an economically important marine species in China. The methods for sperm cryopreservation of the fish have been developed successfully. However, little progress has been achieved in the embryo cryopreservation. The PGCs cryopreservation, which can eventually be used to generate viable individuals by surrogate breeding, provides us an attractive alternative way. However, the technique for labeling the PGCs of red seabream has not been reported. In this study, we used medaka as a model system to investigate the function of the red seabream *vasa* (*Pmvas*) regulatory sequences, which will facilitate further research on labeling, isolation and cryopreservation of red seabream PGCs.

Materials and Methods

Fish breeding and embryo preparation

Fish breeding followed the guidelines on care and use of laboratory animals for scientific purpose, approved by National Advisory Committee in Singapore. The medaka strain: *af* (strain without self-fluorescence) was maintained under an artificial photoperiod of 14 h light to 10 h darkness at 26 ℃. Embryos were collected in the morning after mixing male and female fish which were separated one day before. Embryogenesis and oogenesis were staged according to Iwamatsu [17, 18].

Prepare of GFP-Pmvas 3′UTR and RFP-Olnos3 3′UTR mRNA

Chimeric mRNA containing GFP fused to 3′UTR of red seabream *vasa* gene (Fig. 1A) and RFP combined with 3′UTR of medaka *nos3* gene (Fig. 1B) were synthesized by in vitro transcription. The template plasmid for RFP-*Olnos3* 3′UTR mRNA (which contained RFP and the 3′ UTR of medaka *nanos3* in *PCS2* vector) was kindly supplied by Doctor Li Mingyou; The template plasmid for GFP-*Pmvas* 3′UTR was constructed as following procedures: 3′UTR of the red seabream *vasa* mRNA (Pmvasa, AB378581) was cloned into pGEM-T easy vector by primers and designed as vasa3U-T vector:

vasa3U-F:
5′-GCTGATGATGACGACTGGGATT-3′
vasa3U-R:
5′-AACAAATATTTATTTATTGGTGATC-3′

AatII and *SacII* sites were introduced at the 5′ end and 3′ end of GFP ORF, respectively, by a PCR reaction with primers:

EGFP-F:
5′-TATATTGACGTCCGCCACCATGGTGAGC-3′
EGFP-R:
5′-TATATTCCGCGGTTACTTGTACAGCTCGTC-3′

After double digestion, the fragment was cloned into the corresponding sites of vasa3U-T. The resultant plasmid was linearized by *SalI* digestion for in vitro transcription by Message machine T7 kit (Ambion Inc., Austin, TX).

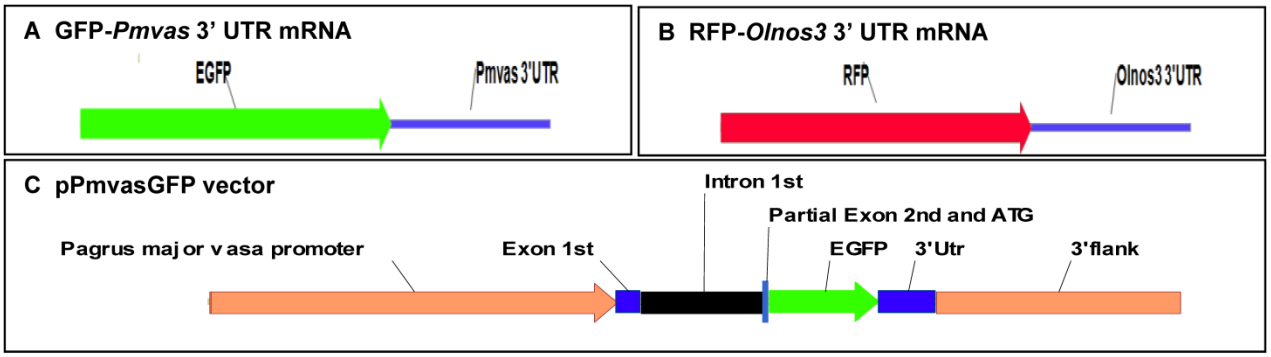

Fig. 1 Structure of chimeric mRNA and transgenic vector for PGCs labeling

Construct of pPmvasGFP vector

The 5′ and 3′ flanking region sequences of the red seabream *vasa* gene were previously isolated by genome walking, basing on the *vasa* mRNA sequence (Pmvasa, AB378581) [19]. The procedures for construction of pPmvasGFP vector (Fig. 1C) were as follows:

The 3′ flanking region downstream the stop codon (TAA) of the red seabream *vasa* gene was amplified from red seabream genome DNA by primers:

vasa3-F:
5′-TCTAGCGGCCGCGATGATGACGACTGGGATTA-3′

vasa3-R:
5′-TCTAGCGGCCGCAGGTCATGATTGCTGATATT-3′

The resultant 1.9 kb fragment was digested with *NotI* and cloned into the corresponding site of pEGFP-4.1 vector and designed as pEGFP-3V.

The 5′ flanking region upstream the start codon (ATG) of the *vasa* gene was amplified by primers:

Vasa5-F:
5′-TAGCAAGCTTCCAACTGTCGCCGCTGAT-3′

Vasa5-R:
5′-GTCAGGATCCTCTTCTTCCCACTCGTCCAT-3′

The resultant 3.8 kb fragment was digested with *BamHI* and *HindIII* and cloned into the corresponding site of pEGFP-3V to produce the pPmvasGFP transgenic vector.

Microinjection

The purified vector pPmvasGFP (25 ng/μl in 10mM Tris-Cl PH7.5, 0.02% Phenol Red) was injected into fertilized eggs of *af* strain at 1 cell stage. GFP-*Pmvas* 3′UTR and RFP-*Olnos3* 3′UTR mRNA (100 ng/μl each, DEPC water, 0.02% Phenol Red) were co-injected into fertilized eggs of *af* strain at 1 cell stage, to confirm that the chimeric mRNA containing *Pmvas* 3′UTR label PGCs specifically. The injected embryos were incubated in 1*ERM at 28 ℃ for GFP or RFP observation and photography under fluorescent microscope (Leica MeFIII) at different stages.

Selection of the transgenic line

Sexually mature medaka from injected embryos were mated with nontransgenic fish to obtain F1 fish. Embryos were screened to indentify germline transmitting founder (F0) by both fluorescence microscopy and genomic PCR, using primers for 3′ flanking region (1.9 kb) of the red seabream *vasa* gene. The transgenic offspring (F1) with GFP were raised to maturity and crossed with sibling transgenic F1 to confirm the F2 had a GFP segregation ratio following Mendelian genetics.

Cryosection

In order to confirm that GFP expression cells in gonads of F1 transgenic fish were germ cells, cryosections of transgenic ovaries and tests were performed. The method was described previously [20].

Results

Visualization of medaka PGCs by GFP-Pmvas 3′UTR mRNA

In order to confirm that 3′UTR of *Pagrus major vasa* shares similar function with that of medaka *nanos3* gene, so that can be used to visualize PGCs, GFP-*Pmvas* 3′UTR mRNA was co-injected into fertilized embryos with RFP-*Olnos3* 3′UTR mRNA, which has been used to trace migration of PGCs in medaka [21]. Results showed that the GFP fluorescence didn't differentiate between somatic cells and PGCs until early segmentation period (Fig. 2A). As embryogenesis proceeded, GFP in PGCs became more obvious since the fluorescence of somatic cells reduced gradually, resembling the situation of RFP-*Olnos3* 3′UTR

mRNA injection (Fig. 2B-D). Cells acquiring fluorescence were rounder and had larger size, which were typical characteristics of PGCs (Fig. 2E-H). However, it's interesting that GFP signal distributed uniformly throughout entire cell and the nucleus couldn't be identified, while most RFP signal distributed in cytoplasm and the nucleus could be easily identified. In spite of that, both chimeric mRNA can visualize and reveal the migration route of PGCs in medaka during embryogenesis.

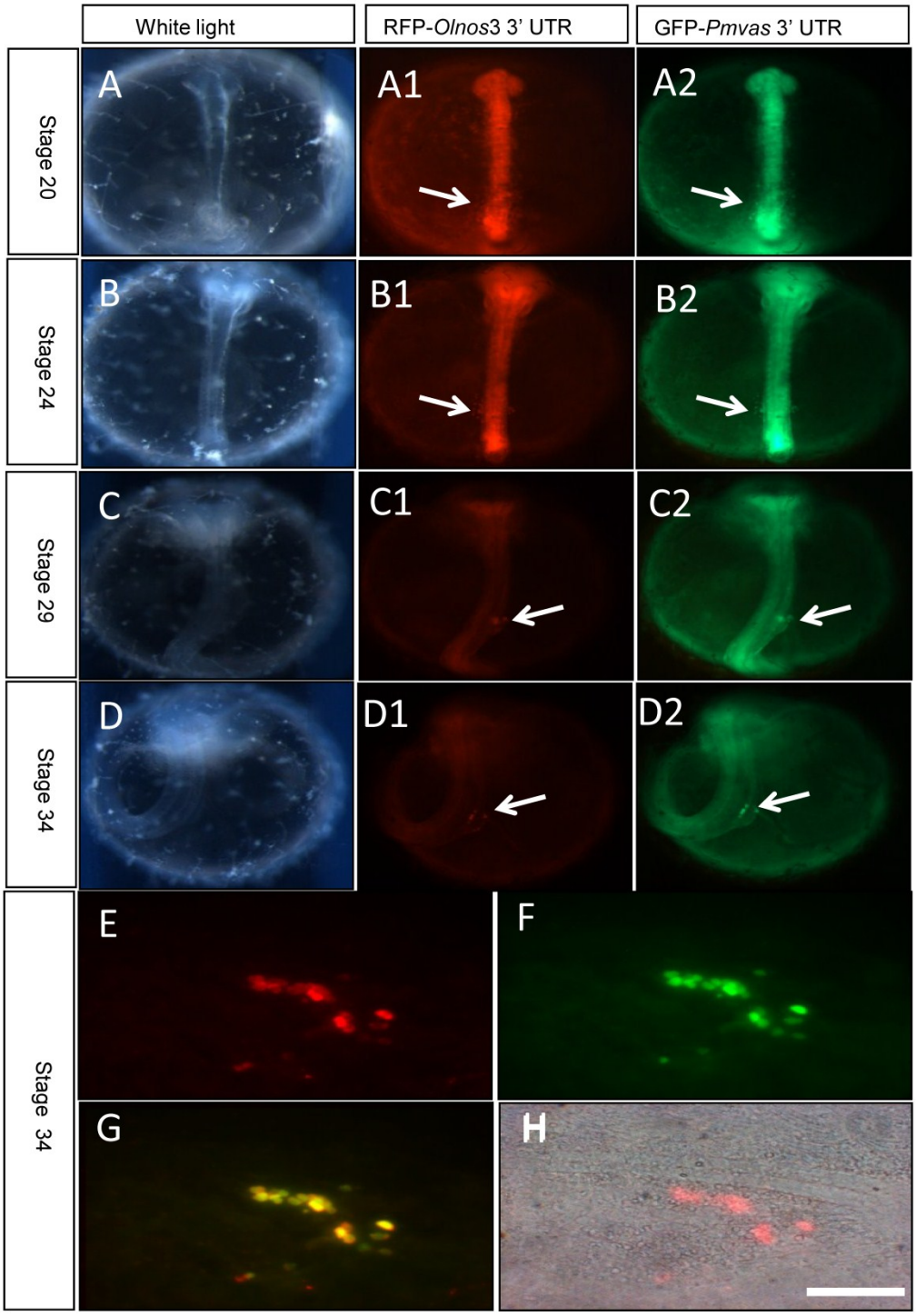

Fig. 2 Transient PGCs labeling by co-injection of RFP-*Olnos3* 3'UTR (Red) and GFP-*Pmvas* 3'UTR (Green) mRNA. PGCs were indicated by white arrows. A-D) embryos of stage 20, stage 24, stage 29 and stage34, respectively, E-H) the gonad region of stage 34 was squashed into cell lever and observed under fluorescence microscopy, Scale bar, 50 μm.

Establishment of transgenic medaka line with germ cells expressing GFP

GFP fluorescence was observed in 78% of 257 injected embryos. Among those, 54 embryos (21%) carrying GFP expression in PGCs (Table 1). The injected embryos with GFP expression were raised to maturity and crossed with nontransgenic strain to screen the germ-line transmitting founder (F0) using fluorescence microscopy combined with genomic PCR of the F1 progeny (Fig. 3). 3 out of 26 fish from injected embryos were confirmed to be germ-line transmitting founder (F0), with appearance ratio of GFP expression in F1 embryos ranging from 9.8% to 13.4% (Table 2). The F2 offspring represented approximately 75% of GFP expression segregation ratio, from an F1 transgenic/ F1 transgenic cross (Table 3). The results demonstrated that pPmvasGFP was stably transmitted to offspring following the Mendelian genetics. Therefore, pPmvasGFP transgenic medaka line was successfully established.

Table 1 Result of microinjection of pPmvasGFP vector in medaka

Strain	No. of injected embryos	GFP expression	GFP positive in PGCs	No. of hatching embryos	Sexual maturity founder
af	257	200 (78%)	54 (21%)	110 (43%)	83

Table 2 Result of transgenic founder screening

No. of Screened fish	No. of germline transmitting founder	Ration of GFP expression in F1
26	3 (12%)	
	#1	37/296(12.5%)
	#2	46/467(9.8%)
	#3	31/231(13.4%)
	Mean	114/994(11.5%)

Table 3 GFP expression ratio in offspring (F2) from F1 transgenic cross

No. of embryos	GFP positive embryos	GFP negative embryos
129	96 (74.4%)	33 (25.6%)

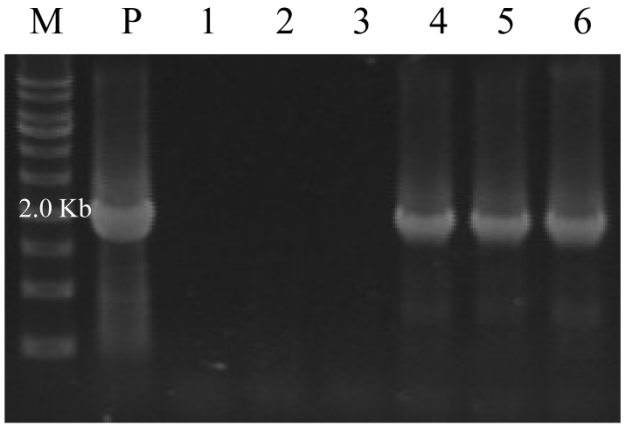

Fig. 3 Identification of germline transmitting progeny by genomic PCR. M) DNA marker; P) positive control from pPmvasGFP vector; 1-3) F1 embryos without GFP expression; 4-6) F1 embryos with GFP expression.

GFP expression pattern in transgenic line

Examination of transgenic embryos by fluorescence microscopy showed that the expression pattern of the pPmvasGFP transgenic fish was similar to the previous reported transgenic line by Tanaka [13]. The female transmitting progeny exhibited GFP fluorescence in every cell before gastrulation (Fig. 4A). After later gastrulation stage (stage 16), GFP fluorescence became intensive in germ ring and embryonic shield (Fig. 4B). Zygotic expression of GFP in male transmitting progeny was not visible until early segmentation. In segmentation period, the GFP fluorescence of embryonic body became stronger. However, obvious PGCs with GFP expression couldn't be detected (Fig. 4C) in the stage. By stage 29, two PGCs clusters formed aligning on both sides of the trunk around the 10^{th} to 12^{th} somites (Fig. 4D). The GFP fluorescent cells migrated dorsally from the surface of yolk mass and formed a single clump at the dorsal region of the intestine by stage 34 (Fig. 4E). Then, the clump moved bilaterally and formed two rows of cells dorsolateral to the digestive tract until stage 38 (Fig. 4F). After hatching, GFP positive cells could still be obviously detected on the dorsolateral side of the gut (Fig. 5A-B). Two months after hatching, GFP continued to be present in gonad region of transgenic medaka (Fig. 5C-F). In particular, GFP showed strong expression in gonad, little or none expression in gill, heart, spleen and kidney (Fig. 6A2-B2). However, weak expression of GFP was also detected in brain, gut and liver (Fig. 6A1-B1). In ovary, GFP was found abundant in oogonia and early stage oocytes, and reduced or diluted in large oocytes (Fig. 7A-B). However, in testis, GFP

seemed to be expressed abundantly in spermatogonia, while little in later stages of spermatogenesis (Fig. 7C-D). It suggested that the adult pPmvasGFP expression might be germ cell specific in both sexes of medaka, and it might be more specific in testes.

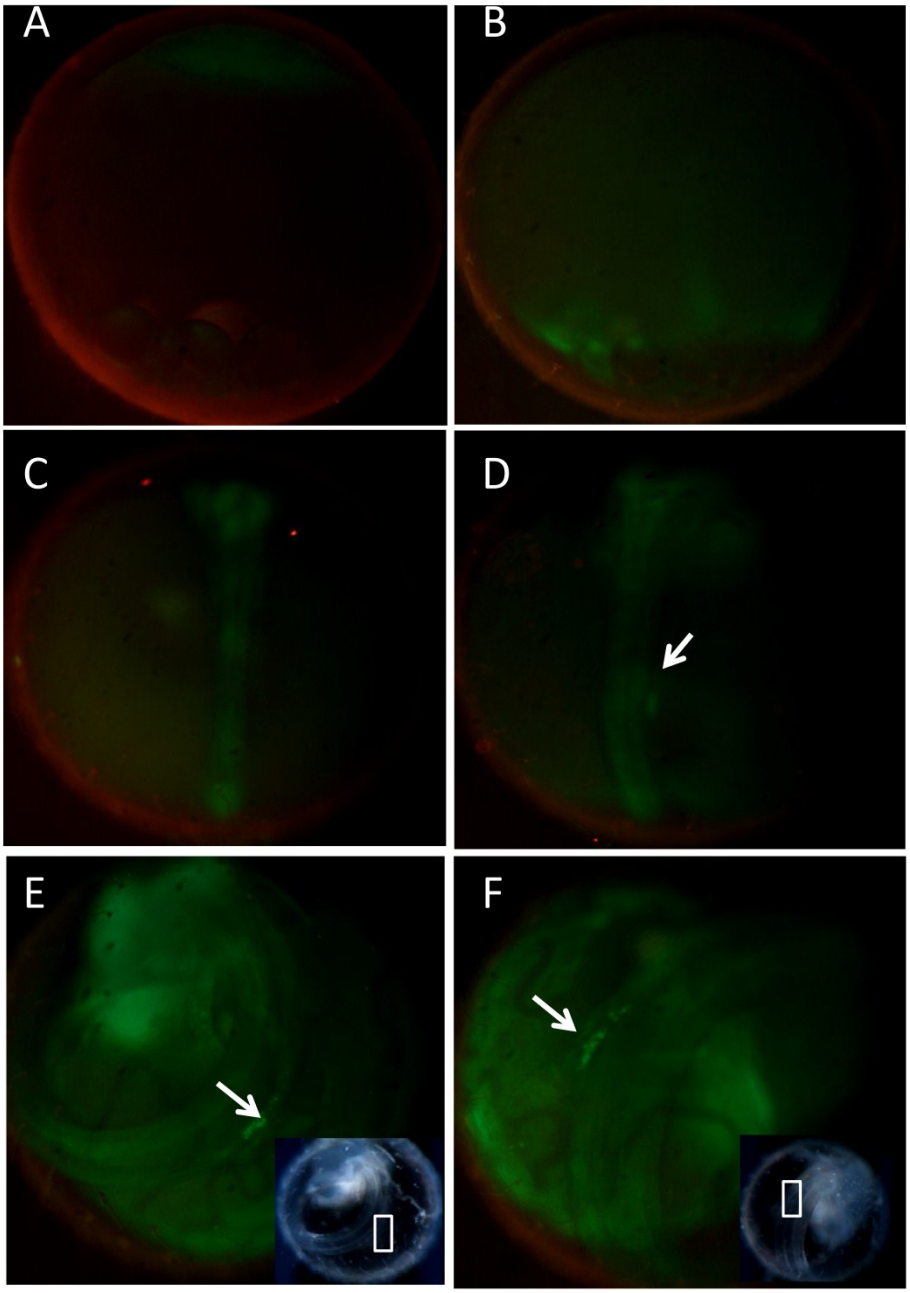

Fig. 4 Germline Expression of GFP in transgenic medaka before hatching. GFP was monitored by fluorescence microscopy. A-F showed the expression pattern of the transgenic offspring following female transmission. The PGCs were indicated by white arrows. White square box represented the presumptive gonad region under white light. A) stage 10; B) stage 16; C) Stage 24; D) stage 29; E) stage 34; F) stage 38.

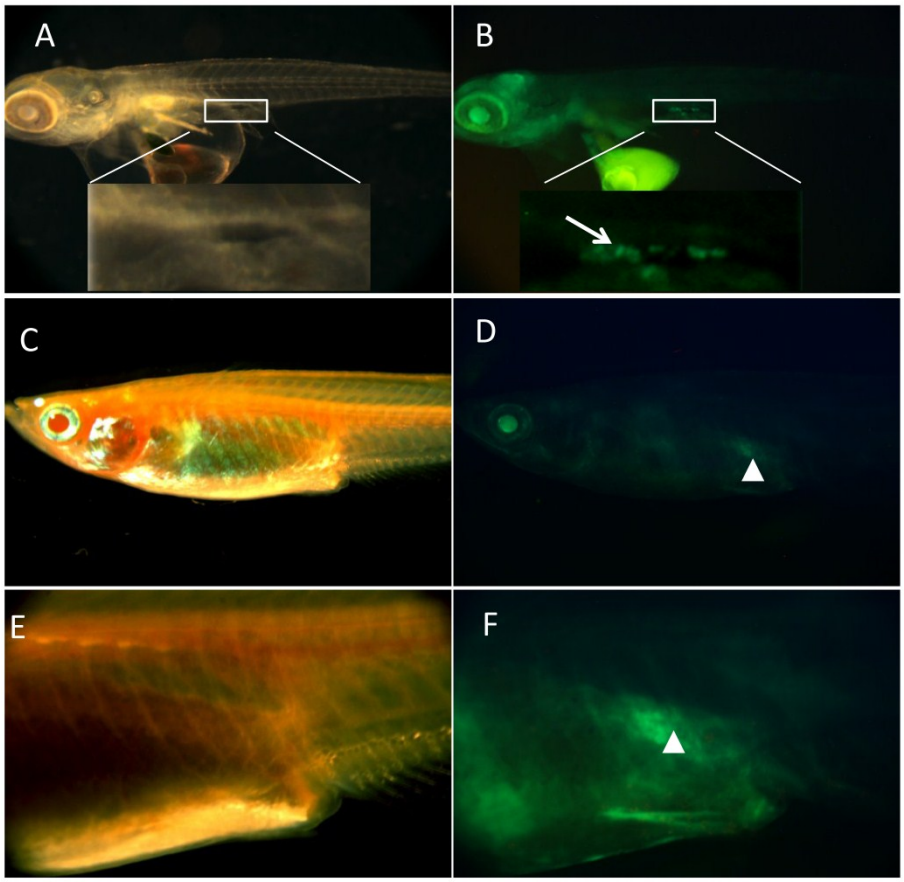

Fig. 5 Germline Expression of GFP in transgenic medaka after hatching. A-B) medaka of two days after hatching, with PGCs expression GFP, located at dorsal side of intestine; C-D) medaka of two months after hatching, with gonad expressing GFP; E-F) magnification of corresponding area of C and D, respectively. Tissue with germ cells was encompassed by white square box. PGCs were indicated by white arrow. Gonad with GFP was indicated by white arrowhead.

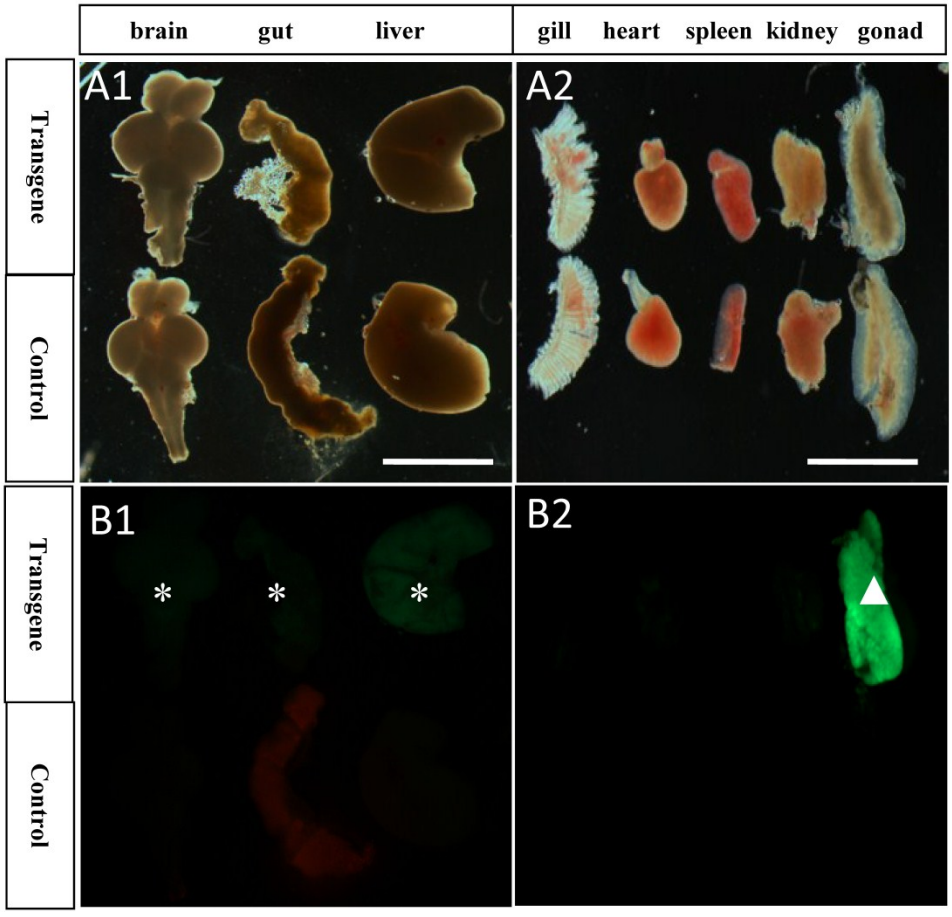

Fig. 6 GFP expression pattern in different tissues of transgenic and control medaka. A1-B1) GFP showed some weak expression in brain, gut and liver of transgenic tissues, while no expression in the control; A2-B2) GFP showed strong expression in gonad, little or no expression in gill, heart, spleen and kidney, while no expression in the control. Asterisks represent tissues with weak GFP expression; White arrowhead represents tissue with strong GFP expression; Scale bar, 2 mm.

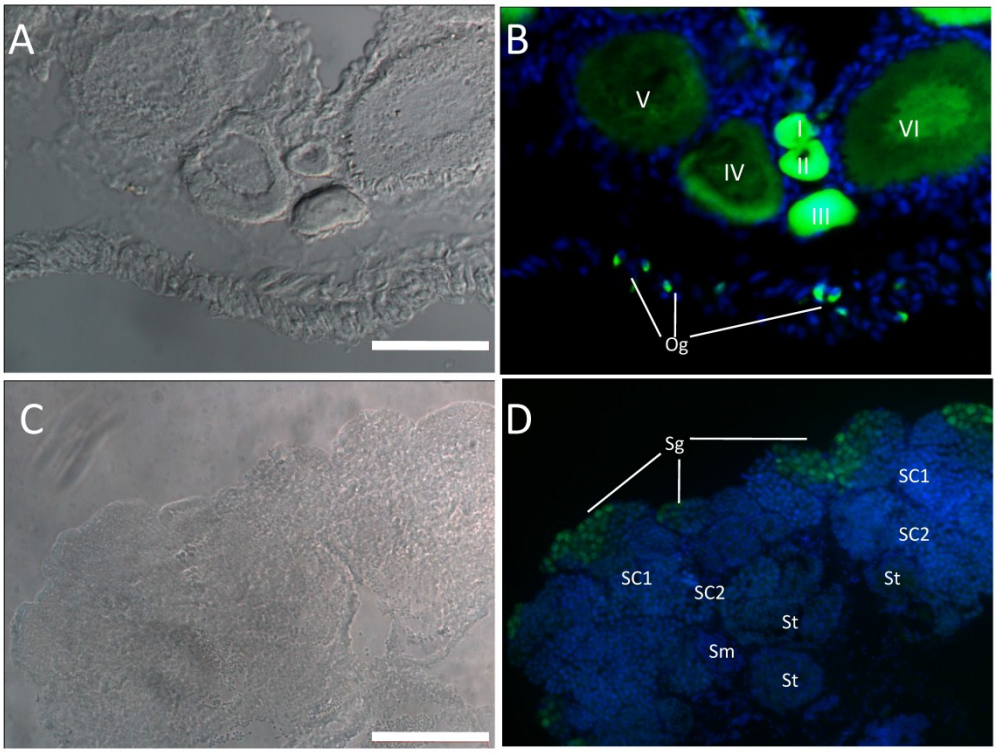

Fig. 7 GFP expression pattern in germ cells of gonads. Cryosections of ovary (A-B) and testis (C-D) were used to detect endogenous GFP. Nuclei was stained by DAPI (Blue). In ovary, GFP expression was abundant in early stages of oogenesis (oogonia, oocytes of I-III stage) and reduced in late stages oocytes (IV-VI); In testis, Abundant GFP expression was detected in spermatogonia, while little expression was detected in other stages of spermatogenesis. Og, oogonia; I-VI, different stages of oocytes; Sg, spermatogonia; Sc1 and Sc2, primary and secondary spermatocytes; St, spermatids; Sm, sperm; Scale bar, 100 μm.

Discussion

In this study, we demonstrated that medaka PGCs could be visualized by microinjection of GFP-*Pmvas* 3′ UTR chimeric mRNA. Besides, we also successfully established a stable transgenic medaka line expressing GFP in germ cells including PGCs, mitotic and meiotic germ cells of both sexes, under control of the transcriptional regulatory sequences from a highly diverged species, the red seabream (*Pagrus major*).

It is known that the 3′ UTR of *vasa* plays critical role for stabilization of chimeric RNA in PGCs but not in somatic cells [22]. In rainbow trout, a construct containing the SV40 polyadenylation signal, instead of the 3′UTR of the *vasa* gene, failed to label PGCs with GFP, suggesting that the region was essential for visualizing PGCs in the species [10]. Our results also indicated that the 3′UTR of the red seabream *vasa* gene could stabilize the GFP chimeric mRNA in PGCs of medaka embryos, similar to the 3′UTR of medaka *nanos3* gene. Therefore, we inferred that the red seabream shared a similar mechanism with medaka that specific stabilization of RNA in PGCs by *vasa* 3′UTR, although we couldn't rule out the other mechanism of *vasa* 3′UTR's RNA localization function [23]. To distinguish the possibility, whole mount in situ hybridization of the *vasa* gene in oocytes and embryos of the red seabream will be performed in the further studies.

It has been reported that PGCs of fishes could be visualized by chimeric RNAs fused to *vasa* 3′ UTR from highly diverged taxonomic groups. For example, PGCs of rainbow trout (belonging to Salmoniformes) could be visualized by mRNA containing *vasa* 3′UTR from both Nibe croaker (a marine fish belonging to Perciformes) and zebrafish (belonging to Cypriniformes) [10]. However, GFP chimeric mRNA containing medaka (belonging to Beloniformes) *vasa* 3′UTR did not identify PGCs in either zebrafish or loach embryos, although it did enable visualization of the PGCs in medaka embryos. Similarly, in the study, medaka PGCs can be visualized by chimeric mRNA containing *vasa* 3′UTR sequences from the red seabream (belonging to Perciformes). It has been demonstrated that euteleosts (including medaka, trout, .et al) but not ostariophysans (including zebrafish, carp, .et al) *vasa* 3′UTRs have lost the basal function of RNA localization [23]. Considering all of those, we speculated that *vasa* 3′UTRs possessing the

basal function of RNA localization could label a larger range of fish species than those losing the function.

In transgenic F1 fish, GFP labeling PGCs couldn't be detected until they clustered bilaterally at stage 29. However, GFP showed nonspecific expression in brain, gut and liver regions, different from the exclusive expression pattern in germ cells of the previous report by Tanaka [12]. This phenomenon may ascribe to phylogenetic distance between medaka and red seabream, as well as lack of some transcription regulatory sequences in the pPmvasGFP construct. In gonads, GFP seemed to be present in early stages of gametogenesis. GFP was abundant in oogonia and early stage oocytes, and reduced or diluted in large oocytes, mimicking the expression pattern of endogenous *vasa* in situ hybridization results [24-26]. Interestingly, in testes, GFP seemed to be present abundantly in spermatogonia, while little in other stages of spermatogenesis, different from the expression pattern of endogenous *vasa* mRNA: abundant in spermatogonia, gradually reduced in spermatocytes and absent in sperm [24-26]. By this aspect, the transgenic line showed more specific expression pattern in gonads and might facilitate the cell culture of spermatogonia.

In conclusion, we have proved that *vasa* transcriptional regulatory sequences from the red seabream examined in this study are sufficient for germ cells expression and labeling. This work in the model fish indicated that medaka and red seabream shared similar mechanism that specific stabilization of RNA in PGCs by *vasa* 3'UTR. Both pPmvasGFP construct and GFP-*Pmvas* 3'UTR mRNA can be applied to visualize PGCs of the red seabream, which will facilitate further research on PGCs isolation, cryopreservation, and surrogate breeding of the species.

Acknowledgement

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Competing Interests

The authors have declared that no competing interest exists.

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