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Renal production of recombinant proteins in fish using a novel lipocalin family gene promoter: verification of production concept through generation of transgenic medaka expressing model fluorescent proteins

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Research Article

Keywords: Aquaculture, Bioreactor, Lipocalin-type prostaglandin D2 synthase, Recombinant protein, Urine

Posted Date: September 11th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10834705/v1

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Additional Declarations: No competing interests reported.

Abstract

There is an urgent requirement for proteins and peptides across different industries where recombinant protein production using bioreactors is a common practice. A novel finding about a urinary protein in black rockfish (*Sebastes schlegelii*) inspired the concept of a novel recombinant protein production system in fish as a bioreactor, with the help of the promoter and signal peptide region of the gene encoding this urinary protein, i.e., downstream recombinant proteins can be produced in the kidney and collected/purified from urine or rearing water. To examine the feasibility of this concept of recombinant protein production, transgenic medaka expressing model fluorescent proteins were generated using a dual-promoter donor vector in combination with the I-SceI meganuclease system. The target recombinant red fluorescent protein was detected in the adult male kidney at both the mRNA and protein levels, and its transcription was verified to be androgen-dependent in larvae. Fluorescence was also detected in rearing water after exogenous androgen stimulation. Collectively, our results provide indirect proof of the “urinary recombinant protein production” concept, indicating its great potential.

1. Introduction

Production of proteins and peptides such as therapeutic recombinant proteins and peptides, or industrial enzymes, is urgently needed across different industries. Due to their complex nature, chemical synthesis of proteins is generally unfeasible, so they must instead be produced through biological processes (Overton 2014). Therefore, developing efficient protein bioreactors remains a priority. Currently, bacteria, insects, yeasts or fungi, plants, mammalian cells, and whole animals are the primary hosts for the production of therapeutic proteins, each of which has its own inherent advantages and limitations. A recent finding regarding a specific urinary protein in black rockfish (*Sebastes schlegelii*) has shed light on the development of a new recombinant protein production system using fish as a bioreactor, which could potentially facilitate the conversion of waste urine into a useful substrate.

In some species of viviparous rockfish (genus *Sebastes*), urine accumulation during the copulation season in males has been observed, which has been speculated to be due to pheromone substances in the urine, which can affect courtship (Helvey 1982; Love et al. 2002). In black rockfish, a component of high-molecular-weight urinary proteins (HMWups) was identified as a lipocalin-type prostaglandin D_2 synthase homolog (LPGDSh) for the first time, suggesting that it is a potential protein-type pheromone in this species (Yamaguchi et al. 2023). The gene transcript encoding this urinary protein appears to be expressed predominantly in the testes and kidneys of male rockfish, specifically in the epithelial cells of renal tubules in the kidney, indicating the dominant presence of the protein product in reproductive male urine (Yamaguchi et al. 2023). Furthermore, transcription of the *LPGDSh* gene appears to be upregulated by the stimulation of androgens in the presence of androgen response elements (AREs) in the potential promoter region, where the region between − 2,100 bp and − 1,100 bp upstream from the start codon of *LPGDSh* gene densely recruits ARE-like motifs (Yamaguchi et al. 2025). In this study, a DNA fragment encoding the 3,412 bp upstream and 57 bp downstream flanking the start codon of *LPGDSh* gene

(cloned by Yamaguchi et al. 2025) was designated as the lipocalin promoter (LCNP) with the signal peptide (SP), that is, LCNP/SP.

The concept of producing recombinant proteins and collecting them from the urine of transgenic fish or rearing water is based on the above-mentioned background. The concept is as follows: A donor expression vector, where a fragment of the target protein-encoding sequence is inserted downstream of the *LCNP/SP*, is constructed and incorporated into the genome of the host fish. The recombinant target protein is produced in the kidneys of transgenic host fish under the stimulation of endogenous or exogenous androgen(s) and is secreted into the urine. The recombinant protein was then purified from the urine or rearing water. However, transgenesis in black rockfish is technically challenging because its mode of reproduction, viviparity prevents practical embryo manipulation. Therefore, a surrogate small-model fish, such as minami medaka (*Oryzias latipes*), is required as a proof-of-principle model.

The final goal of this study was to develop a novel recombinant protein production system that harnesses the mechanism of urinary protein production in fish. Toward this end, this study sought to examine the feasibility of such a recombinant protein production concept; specifically, (1) determine whether transgenesis can be successfully achieved and stably inherited from the founder generation following the injection of gene transfer reagents, (2) localize the recombinant protein expression, in particular, whether it occurs in the epithelial cells of renal tubules in the kidney, and (3) detect the model protein in rearing water as indirect evidence of urinary secretion.

2. Material and Methods

The protocols of transgenesis experiments and sample collection in this study were approved by the Animal Care and Use Committee and the Safety Committee on Genetic Recombination Experiments of the National University Corporation Hokkaido University (Authority Number 22–0051 and 2022-017) and conducted under their policies and guidelines.

2.1. Experimental fish and rearing conditions

Mature medaka from an inbred population that had been reproduced for over 60 years at the Faculty of Fisheries Sciences of Hokkaido University (Yamamoto and Yoshioka 1964) were used as broodstock in this study. The rearing conditions and feeding routines are described in our previous report (Namgung et al. 2021), except the rearing water for embryos, which was supplemented with 1% (v/v) 10× Yamamoto's Ringer solution until they were hatched (Yamamoto 1975).

2.2. Vector construction and microinjection

All experiments using commercial kits here or in later sections were conducted according to the manufacturer's protocol unless otherwise stated. The LCNP/SP fragment was first cloned by Yamaguchi (2025), and a fragment (–3142–57 bp, flanking the start codon of the original sequence) was used in this study. Cleavage-resistant mCh (crmCh) was generated in the present study according to Huang et al.

(2014), in which 11 residues at the N-terminus of the original mCherry (GenBank: AAV52164.1) were deleted. The pCAEGFP plasmid containing a CAG promoter (Niwa et al. 1991; RDB03006) was provided by RIKEN BRC through the National BioResource Project of the MEXT, Japan, and the CAG-EGFP fragment, including the poly A signal (147–2771 bp) from this plasmid, was used for the selection of transgenic individuals in this study. The two I-SceI recognition sites (18 bp) were newly created in the vector by mutagenesis using primer sets including the site (Appendix Table 1) and In-Fusion® Snap Assembly Master Mix according to the manufacturer's protocol (Takara Bio Inc., Tokyo, Japan).

Table 1
Primers used in the construction of donor vectors, cDNA cloning and sequencing. The expected product size is shown as including the primers.

Primer name	Sequence 5′ – 3′	Product size	Description
VGP Des	F: ACGGTACCGCGGGCCCGG	4263 bp	Linearization of destination vector prior to In-Fusion for depositing *crmCh* fragment
	R: CGGGCCCAAGTGATCTCC		
LCNP Ins	F: GATCACTTGGGCCCGTGTTCTTCCCAGGATTCATTGTAGA	3499 bp	Amplification of the *LCNP/SP* insert fragment prior to In-Fusion
	R: GGCCCGCGGTACCGTGCAGGTGGCCAGCGCACA		
pCA ISmut	F: AGGGATAACAGGGTAATAGCTGTCCCTCTTCTCTTATGG	5375 bp	Mutagenesis of I-SceI recognition site in pCAEGFP vector
	R: TACCCTGTTATCCCTATTCATGGCAGCCAGCATATGG		
LCNP ISmut	F: TTACCCTGTTATCCCTATCAAGAACCATATGCCGGTACC	7590 bp	Mutagenesis of I-SceI recognition site in LCNP/SP-crmCh vector
	R: GGGATAACAGGGTAATAGCCATACCACATTTGTAGAGG		
pCA Ins	F: TAGGGGGTCAAGAACATCTCCATAAGAGAAGAGGGACAGC	2807 bp	Amplification of the *CAG-EGFP* insert fragment with one I-SceI mutant site prior to In-Fusion
	R: ATCCTGGGAAGAACACATCGATGTCGACATTGATTATTGA		
LCNP Des	F: TGTTCTTCCCAGGATTCATTGTAG	7369 bp	Linearization of the destination LCNP/SP-crmCh vector with one I-SceI mutant site prior to In-Fuison
	R: GTTCTTGACCCCCTACCTTCAG		
ef-1α	F: GCGACAAGATGAGCTGGTTCAAG	81 bp	Amplification of a part of the *ef-1α* gene in reverse-transcription PCR
	R: GAGCTTCAAGGAGTGTTGTTCCAC		
crmCh	F: ATCATCAAGGAGTTCATGCGCTTC	678 bp	Amplification of the 678 bp *crmCh* gene in RT-PCR
	R: CTACTTGTACAGCTCGTCCATGC		

Primer name	Sequence 5’ – 3’	Product size	Description
M13	F: GTAAAACGACGGCCAGT	/	General primers used for sequencing the inserted gene fragment in the pGEM-T Easy vector
	R: CAGGAAACAGCTATGAC		

For all the subcloning in this research, linearization of the destination plasmids, as well as amplification of the insert fragments, were conducted by PCR using PrimeSTAR® MAX DNA Polymerase (Takara Bio Inc.). The primers used for vector construction are listed in Appendix Table 1 and were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan). The sizes of PCR products were verified by electrophoresis in 1% agarose, and the target products were extracted and purified by FastGene™ Gel/PCR Extraction Kit (NIPPON Genetics Co, Ltd., Tokyo, Japan). Then the purified fragments were inserted into the destination vectors using the In-Fusion® subcloning system. The ligated products of the In-Fusion reaction were transformed into chemically induced XL1-Blue competent cells (StrateGene, CA, USA). The plasmid was extracted using the FastGene Plasmid Mini Kit (NIPPON Genetics Co., Ltd.).

A customized mold (AM6540-0104-1; IPN-Factory, Aichi, Japan) was used to create grooves in 2% agarose gel to hold fertilized eggs for microinjection. The injection reagent, including 10 ng/μL plasmid, 0.5% 10× Yamamoto’s Ringer, and 1 μL I-SceI enzyme along with 0.5× rCutSmart™ buffer (New England Biolabs Japan Inc., Tokyo, Japan), was incubated at 30 ℃ for 1 h. Just prior to injection, 1% phenol red was added to stain the solution. Microinjections were performed as described by Namgung et al. (2021).

2.3. Methyltestosterone administration

The 17α-methyltestosterone (MT) was dissolved in ethanol (EtOH) and mixed gently overnight to make the MT stock solution (1 mg/mL MT). For the MT treatment in larvae, 15 μL of this stock solution was added to 50 mL dechlorinated water to make the 300 ng/mL MT treatment solution. In parallel, 15 μL ethanol was added to 50 mL dechlorinated water to prepare the negative control treatment solution. Three groups were created for the MT administration experiment: transgenic (TG) candidate medaka with MT treatment (TG/MT), transgenic candidate medaka with negative control (EtOH vehicle) treatment (TG/EtOH), and wild-type medaka in 50 mL of dechlorinated water as the blank control (WT). The whole treatment process was conducted over a period of 4 days at 25 ℃ with daily water exchange, in which all the treatment water from the previous day was completely replaced with freshly prepared treatment water. For the MT treatment in F0 adults, 3 transgenic medaka and wild-type male medaka each were kept individually in 6-well plates, where every well contained about 12 mL dechlorinated water containing 5 μg/mL MT. They were reared at 28 ℃ for 8 h without water exchange. Feeding was not performed during the treatment period. For kidney cryostat section samples, one individual of F0 male adult medaka was reared in 75 mL of 5 μg/mL MT solution for about 20 h, with complete MT solution exchange taking place at 10 h.

2.4. Expression analysis of *crmCh* transcripts

The tissue samples were homogenized with beads in 1 mL of ISOGEN (NIPPON Genetics Co, Ltd.), following the protocol for “small samples” in the product manual. The reverse transcription (RT) was conducted using Primescript™ II 1st strand cDNA Synthesis Kit (Takara Bio, Inc.) with Oligo dT Primer inside the kit. At this step, two groups were prepared: one was treated with RTase (enzyme for RT) (RT sample), and the other was treated without RTase, serving as a negative control (NRT sample). All other reagents remained the same. The cDNA acquired from the last step, along with the negative control, was amplified using PrimeSTAR® GXL DNA Polymerase (Takara Bio Inc.) premix with “crmCh” and “ef-1α” primers (Appendix Table 1), respectively. The size of the PCR product was confirmed by electrophoresis using a mini-marine electrophoresis unit (AS ONE, Osaka, Japan) and compared with that of the BH 100 bp DNA Ladder RTU (Bio-Helix, New Taipei City, Taiwan). The gel used in electrophoresis was made up of either 1% or 1.5% agarose (Takara Bio Inc.), 0.2 μg/mL ethidium bromide (FUJIFILM Corporation, Tokyo, Japan), and TAE buffer (20 mM acetic acid, 50 mM EDTA, and 40 mM Tris).

For the sequencing of the *crmCh* cDNA, the PCR product was A-tailed using AmpliTaq Gold™ 360 (Thermo Fisher Scientific Inc., Tokyo, Japan) and ligated into a vector with pGEM®-T Easy Vector System (Promega Corporation, Madison, USA) according to the vendor’s protocol. Transformation and purification of the ligated vector were conducted as described in Section 2.2. BigDye® premix (Thermo Fisher Scientific Inc.) was used for the sequencing reaction with “M13” primers (Appendix Table 1) according to the manual of the premix, followed by sequencing with SeqStudio 8 Flex (Thermo Fisher Scientific Inc.).

2.5. Cryostat sectioning

Fresh kidney was immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution at 4 ℃ for 4 h. This was then rinsed with 0.1 M PB solution 3 times and immersed in 10%, 20% and 30% sucrose/0.1 M PB solution in a stepwise manner. The kidneys were embedded in OCT compound (Thermo Fisher Scientific Inc.) and frozen in dry ice. Cryosectioning was conducted using a Leica CM1860 (Leica Biosystems Nussloch GmbH, Tokyo, Japan) and mounted with VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Funakoshi Co., Ltd, Tokyo, Japan).

2.6. Production of recombinant His-crmCh and anti-mCh antiserum

The 678 bp crmCh fragment was inserted into Champion™ pET302/NT-His vector (Thermo Fisher Scientific Inc.) using In-Fusion®, which is in frame with the N-terminal 6×histidine tag to produce recombinant His-crmCh protein. The expression vector was then transformed into Rosetta-gami B competent cells (Sigma-Aldrich Japan K.K., Tokyo, Japan), and the expression was induced with 0.5 mM IPTG at 37 ℃ for 4 h. Then it was lysed with BugBuster® (Sigma-Aldrich Japan K.K.) and purified using ÄKTA start (Cytiva, Tokyo, Japan) equipped with HisTrap™ HP (Cytiva). Briefly, the lysate buffer was exchanged with binding buffer (0.02 M PB, 0.5 M NaCl, and 0.04 M imidazole) by dialysis, bound to the

HisTrap™ and eluted by elution buffer (0.02 M PB, 0.5 M NaCl, and 0.5 M imidazole). The 1 mL fraction with the highest absorbance at 280 nm was selected as the purified sample.

Similarly, the mCh fragment (GenBank: AY678264.1) was cloned into the pET302/NT-His vector to produce recombinant mCherry (r-mCh), which was purified as described above. Anti-mCh rabbit antisera were produced as described by Yamaguchi et al. (2023).

2.7. Dissection and fluorescence observation of organs

Dissection was conducted after the medaka were anesthetized using 2-phenoxyethanol. Whole-body fluorescence observation was conducted under the guidance of a Leica AF6000 fluorescence microscope system (Leica Microsystems, Tokyo, Japan), including an M205 FA microscope and EL6000 fluorescence illuminator, equipped with a DFC 310FX camera, and the captured images were processed using the LAS Application Suite. Individual kidneys and cryostat sections were observed using a BZ-X800 microscope system equipped with the corresponding software (KEYENCE, Osaka, Japan). The exposure times for each experiment are described in the corresponding figure legends.

2.8. Fluorescent intensity measurement of rearing water

Rearing water (700 µL per sampling) was sampled by pipetting directly out of the individual culturing well at 4 h and 8 h post MT treatment (hpt). The water samples were applied to the LUMITRAC 96-well white opaque microplate (Greiner Bio-One International GmbH, Tokyo, Japan) as 200 µL per well for 3 repeat applications. The plate was read using SpectraMax iD 3 (Molecular Devices, Tokyo, Japan), where the excitation/emission wavelength was set to 570/610 nm using endpoint measurement. The fluorescent intensity was defined as the average value of three repeated applications. The recombinant His-crmCh prepared in Section 2.6 was diluted in steps to create a concentration (absorbance at 280 nm) vs. fluorescence intensity standard curve using linear regression. The absorbance at 280 nm was measured by NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.); for ease of analysis, an absorbance of 1.0 at 280 nm was regarded as equivalent to 1 mg/ml. Statistical analysis of the measured fluorescence intensity was conducted using JASP (University of Amsterdam, Amsterdam, Netherlands). The standard curve and bar graph were drawn using the raw fluorescence intensity measured by the plate reader, while for the quantification of crmCh concentration, the fluorescence intensity corrected by the corresponding blank buffer was used for the calculation.

2.9. Gel electrophoresis and western blotting

The whole kidney and liver from both WT and F0 individuals were sampled and lysed in RIPA buffer (Poirier et al. 1982; redesigned in the present study as 150 mM NaCl; 50 mM Tris-HCl, pH 8.0; 1% Triton X-100; 0.2% sodium lauryl sulfate), as 20 µL for kidney and 100 µL for liver. Samples were diluted using the same RIPA buffer and mixed with an equal volume of a sample reducing solution (10% sodium dodecyl sulfate:glycerol:2-mercaptethanol = 3:2:1) containing 3% saturated bromophenol blue; the dilution of each sample was conducted to prepare the final mass of approximately 75 µg crude protein to be applied per well in the later gel electrophoresis. Prior to application, the samples were heated in a

water bath at 90ºC for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (WB) were performed according to the methods described by Laemmli (1970) and Towbin et al. (1979). Three days after the MT treatment (described in Section 2.3) of male adults, one TG individual and one WT individual were sampled for their kidneys and livers. After these samples were treated as described above, they were subjected to SDS-PAGE on a 15% acrylamide slab mini gel and stained with Coomassie Brilliant Blue (CBB) R-250 (Bio-Rad, Hercules, CA, USA). Another set of gels was blotted onto polyvinylidene difluoride membranes (Sigma-Aldrich Japan K.K.) using the semi-dry transfer method with an 80mA consistent current for 25 min. The blotted membrane in Tris-buffered saline (TBS, pH 7.5) was incubated with a-mCh (1:1,000 diluted in TBS) overnight at 4 ℃ after blocking with 5% bovine serum albumin (NACALAI TESQUE, Kyoto, Japan) in TBS. After washing with TBS containing 0.05% Tween 20 (TBS-T, 3 times) and TBS (1 time), the membranes were then incubated with 1:10,000 TBS-diluted goat anti-rabbit IgG (H + L) conjugated to horseradish peroxidase (Bio-Rad) at room temperature for 1 h. Signal detection was performed using ECL™ kit (Cytiva) after TBS-T and TBS washing as mentioned above. Molecular weights of proteins on the gels and membranes were estimated by using marker proteins of Precision Plus Protein™ Standards All Blue (10–250 kDa; Bio-Rad) and Precision Plus Protein™ Dual Color Standards (10–250 kDa; Bio-Rad), respectively. Membrane images were taken using WSE-6270 LuminoGraph EM (ATTO, Tokyo, Japan), with an exposure time of 1 min with “HQ” sensing protocol in the associated software.

3. Results

3.1. Establishment of EGFP-CAG:LCNP/SP-crmCh transgenic medaka

The IS-EGFP-CAG:LCNP/SP-crmCh-IS donor vector (Fig. 1) was constructed in the backbone of the pT2AL200R150 vector (Urasaki et al. 2006), in which the LCNP/SP fragment, crmCh coding sequence, and SV40 polyA signal were arranged to create the LCNP expression cassette. Then the expression cassette of pCAEGFP, including CAG promoter, chimeric intron, enhanced green fluorescent protein (EGFP) coding sequence, and β-globin polyA signal, was inserted in a back-to-back position against the LCNP cassette, resulting in a dual-promoter expression cassette flanked by two I-SceI recognition sites. Notably, there is a 36 bp linker-like structure originally from the backbone vector left between SP and crmCh. The details of the features assembled in the donor vector are listed in Table 1.

Table 1
Features of IS-EGFP-CAG:LCNP/SP-crmCh-IS vector

Feature	Location in the vector	Origin	Reference
backbone including ampicillin resistance gene (AmpR)	7346–175 bp	3231–678 bp of pT2AL200R150 (https://ztrap.nig.ac.jp/seq1.html)	(Urasaki et al., 2006)
CMV enhancer, chicken β-actin promoter, chimeric intron, EGFP, β-globin poly(A) signal	292–2916 bp	147–2711 bp of pCAEGFP (catalog # RDB03006)	RIKEN BRC through the National BioResource Project of the MEXT, Japan (unpublished bioresource)
LCNP/SP	2927–6395 bp	1–3469 bp of LC790327 (accession number from DNA Data Bank of Japan)	(Yamaguchi et al., 2025)
crmCh	6432–7109 bp	267–977 bp of pmCherry (GenBank No. MW147276.1) with first 33 bp cut off	(Huang et al., 2014)
SV40 poly(A) signal	7110–7231 bp	950–1084 bp of pT2AL200R150 (https://ztrap.nig.ac.jp/seq1.html)	(Urasaki et al., 2006)
I-SceI recognition site	176–193 bp; 7328–7345 bp	5'-TAGGGATAACAGGGTAAT-3'	(Thermes et al., 2002)

Following injection into 219 fertilized eggs, 82 of the eggs exhibited obvious green fluorescence of EGFP at 3 days post fertilization (dpf), resulting in a fluorescence rate of 37%. Eight individuals (four males and four females) among them survived to become adults (designated here as P0), resulting in overall survival rates of 3.7% and 9.8% in the initial P0 (N = 219) and the individuals selected by green fluorescence (N = 82 at 3 dpf), respectively. The adult P0 founders were then mated with WT individuals to produce F0 founders, where one female and one male were combined in one aquarium as a mating pair (eight mating pairs in total). F0 eggs (N = 136) were collected from two parent pairs, and 22 of these exhibited green fluorescence (16% in total) at 4 dpf (Fig. 2, Panel A shown as an example). Among the eight pairs, fluorescence was only observed in fertilized eggs from two founder pairs (one transgenic female mated with a WT male and one WT female mated with a transgenic male), whereas in the other three pairs, none of the fertilized eggs or non-fertilized eggs showed fluorescence. The rest (three pairs) did not spawn eggs during the mating test. The fluorescence rate of the fertilized eggs obtained from the one adult female transgenic individual was 22%, while that from the one male transgenic individual was 12%. Only embryos showing green fluorescence were raised to the adult stage F0.

Ten F0 individuals (four females and six males) were successfully raised to adulthood. One male was mated with one female as a pair, resulting in the creation of four mating pairs, 3 of which produced fertilized eggs. In total, EGFP fluorescence was observed in 27 out of 36 F1 embryos (75% in total). Surviving larvae were subjected to MT administration and RT-PCR.

3.2. Fluorescence of organs and rearing water

One non-MT-treated F0 adult male (i.e., transformant) and one WT male were selected and dissected. Whole visceral tissues (i.e., kidney, liver, spleen, intestine, and testis) were observed for fluorescence *in situ*. The kidneys were dissected, and fluorescence was observed. Compared to WT males, F0 male medaka exhibited strong red fluorescence in the liver and faint fluorescence in the kidney (Fig. 2, panel B). In cryostat sections of the kidney (Fig. 3), red fluorescence was observed across the field, specifically in cells forming circular structures, referring to the epithelial cells of renal tubules.

The fluorescence intensity of the rearing water samples and His-crmCh standards was determined using three technical replicates, where rearing water (700 µL aliquot) from one rearing well in a six-well plate was loaded into three separate wells (200 µL per well) of a 96-well microplate. The coefficient of variation among three technical replicate measurements was less than 5% for all rearing water samples. Within each genotype-time point group (WT-4 h, TG-4 h, WT-8 h, TG-8 h), the coefficient variation among three biological replicates at each sampling point was less than 5%, except the TG samples at 8 h (~ 11.9%). The linear regression of fluorescence intensity vs. pragmatic crmCh concentration was simulated from the data of His-crmCh standards (Fig. 4, panel A); the regression equation is defined as $y = 4.5 \times 10^6 + 1 \times 10^6$ ($R^2 = 0.996$). For the rearing water samples from all sampling points, the fluorescence intensity of the TG water sample was significantly higher than that of the WT at 8 hpt (p = 0.024, N = 6, ANOVA test with Tukey's correction). At 4 hpt, although the rearing water of the TG group showed higher fluorescence intensity than that of the WT group on average, no statistically significant difference was detected between them (Fig. 4, panel B). No significant differences were found between 4 and 8 h in either the WT or TG groups. Taking advantage of the standard curve, the average crmCh production efficiency of F0 adult males per individual was 0.039 µg/h/individual in 12 mL rearing water until 4 hpt, with the highest efficiency of 0.072 µg/h/individual. From 0 to 8 hpt, the average efficiency was 0.049 µg/h/individual in total rearing water, with the highest efficiency of 0.098 µg/h/individual.

3.3. Expression characteristics of the transgene

The expression of *crmCh* transcripts derived from the transformed expression construct was confirmed by RT-PCR in kidney and liver samples of both WT and F0 adult male individuals (Fig. 5, panel A). In the RT-PCR analysis of *ef-1α*, all RT-treated samples exhibited a single band below the 100 bp marker band, corresponding to the expected amplicon size of 81 bp. For the detection of *crmCh* transcripts, the kidney sample from the transgenic individual alone exhibited an obvious single band around the 700 bp marker band, consistent with its expected amplicon size of 687 bp. In contrast, no apparent amplification

products were detected in any of the NRT samples for detecting *ef-1α* or *crmCh*, indicating no amplification of genomic DNA-derived products.

F1 eggs exhibiting green fluorescence, as described in Section 3.1, were selected and maintained to obtain the larvae. F1 transgenic (TG) larvae (15 dpf) grown from embryos exhibiting green fluorescence (Section 3.1) were subjected to a 4-day-long treatment with either MT (TG/MT; N = 4) or EtOH (TG/EtOH; N = 4). Wild-type larvae were treated with MT (WT/MT; N = 3). All TG/MT larvae survived until the end of the treatment, whereas TG/EtOH (N = 3) and WT/MT (N = 3) larvae were alive at the end of the incubation. Two individuals from TG/MT, along with one individual from each of WT/MT or TG/EtOH, were sampled and used in RT-PCR amplification of *crmCh* and *ef-1α* transcripts in the kidney samples of these three treatment groups (Fig. 5, panel B). Reverse-transcribed cDNAs obtained from 1 WT/MT, 1 TG/EtOH, and 2 TG/MT larvae exhibited obvious ~ 100 bp bands following PCR amplification of *ef-1α*; the expected size of *ef-1α* amplicon is 81 bp. In the PCR amplification of crmCh in the same samples, both TG groups, but not WT/MT, exhibited a single ~ 700 bp crmCh band, though bands corresponding to the TG/EtOH individual was difficult to visually distinguish; the expected size of the *crmCh* amplicon is 678 bp. When the ~ 700 bp band in the TG/MT individual was extracted and sequenced following the cDNA cloning, the obtained 678 bp sequence was 100% identical to the known sequence of the *crmCh* template.

3.4. Western blotting of kidney and liver samples

In CBB staining, no specific band corresponding to the expected size of crmCh (~ 25.5 kDa) was observed on the gel in either the liver or kidney samples from TG fish (Fig. 6, panel A). After western blotting (Fig. 6, panel B), no band was detected in the WT samples, whereas the positive control His-crmCh (expected ~ 26.9 kDa) showed a single band. In the TG individual, both the kidney and liver samples exhibited a ~ 25 kDa band corresponding to the expected size, while an extra band with a larger size (~ 27 kDa) appeared in the kidney sample, which was consistent with the expected size of the linker-crmCh product (~ 26.8 kDa).

4. Discussion

4.1. Production of EGFP-CAG:LCNP/SP-crmCh transgenic medaka

The first and most essential step in this study was the generation of transgenic medaka capable of producing model fluorescent proteins under the regulatory control of LCNP/SP *in vivo*. To accomplish this, the meganuclease system is an efficient transgenesis system that creates double-strand breaks (DSBs) during the process of intron homing in the host genome and insertion of target DNA fragments involving the host DSB repair system, owing to the creation of recombinogenic ends in both the host genome and the inserts (Grabher and Wittbrodt 2007). In this study, utilizing I-SceI meganuclease, 75% of F1 medaka embryos were offspring of F0 intercrosses. Notably, offspring from the F0 intercross

achieved the theoretical transgenic ratio of Mendel's Law of Segregation, indicating that the expected ratio of homozygous transgenic:heterozygous transgenic: wild-type was 1:2:1 in F1 offspring.

Genotyping using a piece of fin samples has been commonly used to identify transgenic individuals; such methods include fin dissection, PCR amplification targeting an exogenous gene, gel electrophoresis, visualization of PCR products, etc. If a check needs to be done for transgenic broodstock selection, the fish must be raised to adulthood and kept alive after surgery until mating. Thus, this conventional genetic screening method is costly and labor-intensive, and surgery in early life stages is associated with a high risk of mortality. The dual promoter expression system has been shown to ensure tissue-specific regulation of two reporter genes in medaka when two different promoters are set back-to-back (Shimizu and Shimizu 2013). The CAG promoter can achieve high transactivation in mammals without any obvious tissue specificity (Niwa et al. 1991). These promoters have also been shown to be effective in five fish cell lines (Leisy et al. 2003). Therefore, the application of such a system is valuable for verifying the integration of exogenous genes into the host genome at an early life stage in medaka without physical harm. This is achieved by monitoring the autonomous expression of fluorescent proteins driven by the CAG promoter, which serves as an effective indicator of successful genomic insertion. When LCNP is used simultaneously with the CAG promoter in this dual promoter system, two types of fluorescent proteins, EGFP following the CAG promoter and crmCh following LCNP, can be independently produced in the same or different tissues (cells) depending on the characteristics of these promoters. Transgenic embryos or larvae exhibiting EGFP fluorescence can be selected as potential transgenic adults or broodstock candidates that produce crmCh under stimulation by endogenous or exogenous androgen(s). Although this fluorescence-based selection can be conservative because EGFP expression can vary among individuals, the present study successfully generated P0 transgenic medaka adults, as well as F0 and F1, confirming the success of transgenesis across generations. Taken together, these results indicate that the essential prerequisites for establishing a stable transgenic line—serving as the foundation for developing an *in vivo* recombinant protein production system in fish—have been successfully fulfilled. Whether these EGFP transformants produced crmCh is discussed below.

4.2. Characterization of expression and localization of recombinant crmCh fluorescence protein

In copulative male black rockfish, LPGDSh transcripts were predominantly detected in the kidney and testis, and localization of the LPGDSh protein was observed in the epithelial cells of the renal tubules in the trunk kidney as well as in the Sertoli cells and lumen of the seminal lobule of the testis (Yamaguchi et al. 2023). During fluorescence imaging (i.e., crmCh) of the entire visceral region in an F0 adult male in the present study, relatively stronger fluorescence was observed in the liver, in addition to very faint fluorescence in the kidney, but not in the testis; such patterns were different from our expectations for mature males described above. With regard to the common existence of lymphoid tissue in fish kidneys, Oguri (1961) described that medaka have no portion to be called head kidney composed of hemopoietic lymphoid tissue, and the entire kidney is made up of renal structures (e.g., renal tubule, renal collecting

duct, etc.), suggesting that the location of the red fluorescence in this study is likely to be renal tubular structures instead of hemopoietic lymphoid tissues. To verify this hypothesis, cryostat kidney sections were prepared. Fluorescence was observed across the sections, specifically in the epithelial cells forming the renal tubules, implying that crmCh may be synthesized in the epithelial cells. Although fluorescence was expected throughout the entire kidney based on Oguri's description (1961) and the initial recombinant protein production concept, the relatively low signal observed in the kidney can be explained by the fact that the recombinant crmCh is immediately secreted into the urine and released out of the kidney immediately after production, considering that freshwater species excrete urine in large quantities to maintain their body osmotic balance and medaka do not exhibit an obvious urinary bladder structure.

The liver and kidney samples were further analyzed to detect *crmCh* transcripts and crmCh protein. The results indicated that although crmCh products were detected in both tissues of TG individuals, only the kidney was positive for transcript expression. This implies that crmCh proteins may be produced in the kidney and transported to the liver in some manner. In western blotting, two bands (~ 25.5 kDa and ~ 27 kDa) were detected in the kidney sample but only a single band (~ 25.5 kDa) in the liver. Considering the sizes of possible protein products encoded by the vector, if SP failed to be cleaved, the whole size was expected to be ~ 28.9 kDa; as the largest size of the immunoreactive band in western blotting was ~ 26.8 kDa, implying that the cleavage of SP may be processed normally. If looking into the specific sequence, there is a ~ 1.3 kDa leftover peptide resulting from the vector backbone between the SP and crmCh; the size can be ~ 26.8 kDa if the larger band represents the structure as 'leftover-crmCh.' More specifically, the remaining peptide (TVPRARDPPVAT) between SP and crmCh contains potential residues resembling a proprotein convertase recognition motif. Proprotein convertases, such as furin, are localized in the trans-Golgi network and cleave substrates at motifs typically as R-X-(R/K)-R↓ (↓ refers to the cleavage site), though non-canonical sequences can be processed with reduced efficiency (Thomas 2002; Duckert et al. 2004). Therefore, the leftover peptide was potentially cleaved at RARD↓ with less efficiency, resulting in the presence of both cleaved crmCh (~ 25.5 kDa) and uncleaved 'leftover-crmCh' (~ 26.8 kDa). The liver is a major organ for the clearance and uptake of circulating proteins via receptor-mediated endocytosis in vertebrates (Sørensen et al. 2012). However, there is no known pathway that directly transports proteins from the kidney to the liver, and we hypothesize the following pathway: Recombinant crmCh is secreted towards both the lumen and blood vessels, and because crmCh is recognized as an exogenous protein by the immune system, it may be transported to the liver for clearance. However, the reasons for this preferential accumulation in the liver are unclear and must be further verified. Moreover, it remains unknown as to which size of crmCh (~ 25.5 kDa versus ~ 26.8 kDa) was secreted into urine.

To test whether crmCh accumulates in rearing water following its secretion into urine, fluorescence was measured in the rearing water. The fluorescence intensity of the TG group was significantly higher than that of the WT group at 8 hpt, indirectly indicating the potential presence of crmCh in the rearing water and urine of the TG fish. Collectively, the findings regarding the renal expression of transcripts and

proteins as well as the specific presence of crmCh fluorescence in the rearing water of TG fish were consistent with the conceptual framework proposed in the present study.

To evaluate androgen sensitivity, which is a key component of the protein production system, an androgen exposure experiment was conducted on F1 medaka larvae. In juvenile black rockfish, following MT administration, both LPGDSh transcript and protein levels increased in the renal tissue in conjunction with an increase in the protein level in the urine when compared with the control; within a certain range, the transactivation activities of the 5' -flanking region of the LPGDSh gene in the presence of androgen receptors (Ars) increased relevant to the concentration of androgen (Yamaguchi et al. 2025). These findings indicate that both androgen and Ars are crucial for the activation of LCNP and significant for the high expression of the downstream recombinant protein. A previous study demonstrated that with the promoter of *spiggin* genes (i.e., kidney-derived androgen-sensitive nesting proteins) in three-spine stickleback, transgenic medaka whose fluorescent protein production is sensitive to androgen was created, and strong fluorescence was observed in the kidney after MT treatment; dosages as low as 1.5 µg MT /L of rearing water have succeeded in inducing GFP expression in the kidney of newly hatched TG medaka under the control of spiggin promoter (Sébillot et al. 2014). In the MT treatment performed in the present study, 300 µg/L was used for the larvae MT treatment; thus, it was expected to be sufficient to induce significant expression when compared with the vehicle (ethanol) control group. As expected, crmCh mRNA was expressed only in TG F1 medaka larvae. However, because only one individual from each control group was selected, further experiments should be performed to confirm these findings.

4.3. Potential of the fish urinary bioreactor concept

Bacterial systems can achieve very efficient production, but often encounter challenges related to protein misfolding and secretion when used to produce large proteins (> 30 kDa), whereas yeast or fungal protein production systems can achieve more effective protein folding and processing. In insect cells or insect-based expression systems, both relatively high levels of protein expression and the ability to produce functional eukaryotic proteins can be achieved, although undesirable post-translational modifications can still occur. In plants, complex protein production and low growth costs are possible, and there is a low risk of human pathogen contamination. However, none of these systems can generate human-type glycosylation patterns that include immunologically significant carbohydrate structures. If using either mammalian cells or whole organisms, it is possible to achieve both high yields and correct post-translational modifications, but this approach involves long production timescales, high-cost and complex culturing processes, and it raises the risk of human pathogens (reviewed in: Verma et al. 1998; Demain and Vaishnav 2009; Ghaderi et al. 2012; Overton 2014; Gomes et al. 2019). As vertebrates, fish are expected to produce functional recombinant vertebrate proteins, particularly with regard to post-translational modifications and possible human-type glycosylation. In addition, when applied to human medicine, fish share close structural homology with humans but they are expected to have a lower risk of zoonotic diseases compared with other land animals, such as mad cow disease or avian flu. Fish products are widely accepted, particularly when considering religious strictures. Combined with the

sustainability of this production system, there is great hope that such fish production systems will serve as practical and useful bioreactor systems in the future.

Although fish bioreactors are not popular in the recombinant protein production industry, some research has been conducted on the production of recombinant proteins in fish. In transgenic zebrafish (*Danio rerio*), recombinant fluorescent protein production, equivalent to 4.8–27.2 mg/g wet muscle tissue, was achieved using the mylz2 promoter (Gong et al. 2003), while functional recombinant tilapia Igf-1 and Igf-2 protein of 0.49 and 0.58 mg, respectively, were produced from 650 fertilized eggs (Hu et al. 2011). Given that the common urine flow rate of freshwater teleosts is 1–6 mL/h/kg body weight (Takvam et al. 2021), a 0.5-gram adult medaka is expected to excrete urine of 0.5–3 μL/h; combining an approximate calculation of recombinant crmCh production mentioned in Section 3.3, one medaka can produce 0.196 mg recombinant crmCh per mL of urine. Although this production yield seems to be modest, if given a larger body weight, for example, 1 kg, the maximum production is expected to be 1.18 mg/h, which seems to be moderate compared with other fish systems. One of the major strengths of the present concept lies in the continuity of protein production. Freshwater fish, including medaka, continuously produce urine; therefore, the transgenic fish generated in this study are expected to secrete recombinant proteins into urine or rearing water over a long period as long as they remain alive, which has considerable potential for mass production.

Fisheries, as well as aquaculture, are commonly recognized to be focused on food production, and high profit is relatively difficult to achieve, particularly for individual farmers. It will be beneficial for aquaculture concerns to incorporate the culture of highly valuable species for pharmaceutical purposes, particularly in inland aquaculture with closed recirculating systems. Because the concept proof was verified to be practical for the very first step in a small model species (i.e., medaka) in the present study, further application to species with more urine production and larger body sizes, such as tilapia (genus *Oreochromis*), is expected to estimate the efficiency and practicality of this production system. If production is abundant and the protein is functional, it is expected to contribute significantly not only to the aquaculture industry but also to the medical industry. Inland aquaculture in Japan is mainly conducted by large companies because it is heavily labor- and fund-intensive, resulting in few independent farmers being capable of maintaining sustainable inland aquaculture businesses. Such a high-value fish recombinant protein production system has the potential to attract more independent farmers to participate in the business and thus promote inland aquaculture in the future.

4.4. Conclusion

In a transgenic trial combining an I-SceI meganuclease with a dual-promoter system, embryos exhibiting green fluorescence were obtained, enabling efficient early stage selection of candidate transgenic individuals and the successful production of transgenic offspring. Both the transcription and translation of crmCh were confirmed in the kidneys of transgenic F0 male adults, and fluorescence was observed in the epithelial cells of the renal tubules. Predominant accumulation of recombinant proteins, rather than transcripts, was detected in the liver, indicating that crmCh was transferred into the liver. Fluorescence was also detected in the rearing water of transgenic F0 adult males after MT administration, indicating

urine secretion. Collectively, these results support the potential for the renal production of recombinant proteins, thereby fulfilling one of the necessary conditions for the final goal of this study. However, the lack of direct evidence of urinary secretion and preferred accumulation toward the liver could not be validated, indicating that further investigations are required to advance this system toward practical application. In F1 larvae, androgen-dependent expression of recombinant proteins under the control of LCNP was observed, indicative of the androgen sensitivity of this system. These findings, as well as the transgenic tools and animals obtained through this research, as described above, provide a basis for the future realization of a novel recombinant protein production system in fish.

Declarations

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

All authors contributed to the study conception and design. Material preparation was performed by YX Y, Y Y and K N, and the following experimental studies, data collection and analysis were performed by YX Y. The first draft of the manuscript was written by YX Y and all authors revised previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgement

We acknowledge the help from all the members from “Medaka Group” in our laboratory for the daily maintenance of the experimental fish. We express our gratitude to J. Miyazaki, Ph.D., Oosaka University, for developing the CAG promoter. We also thank Dr. M. Shimizu, Graduate School of Fisheries Sciences, Hokkaido University, for the helpful discussions. We would like to thank Editage (www.editage.jp) for English language editing. We thank the editors and anonymous reviewers for their critical review of this manuscript.

Data Availability

Data will be made available on request.

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Figures

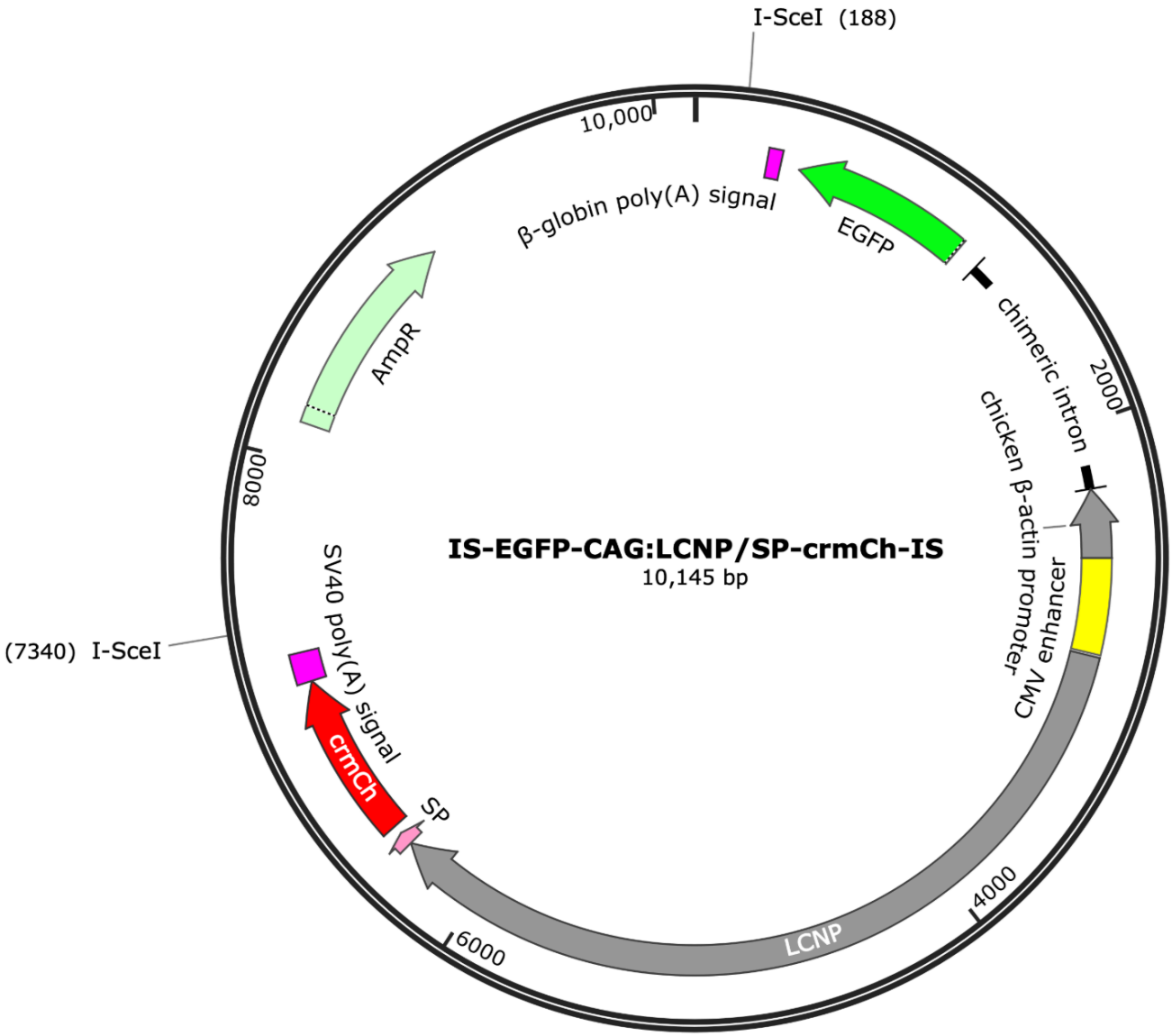

Figure 1

Vector map of IS-EGFP-CAG:LCNP/SP-crmCh-IS. Details of features are described in Table 1.

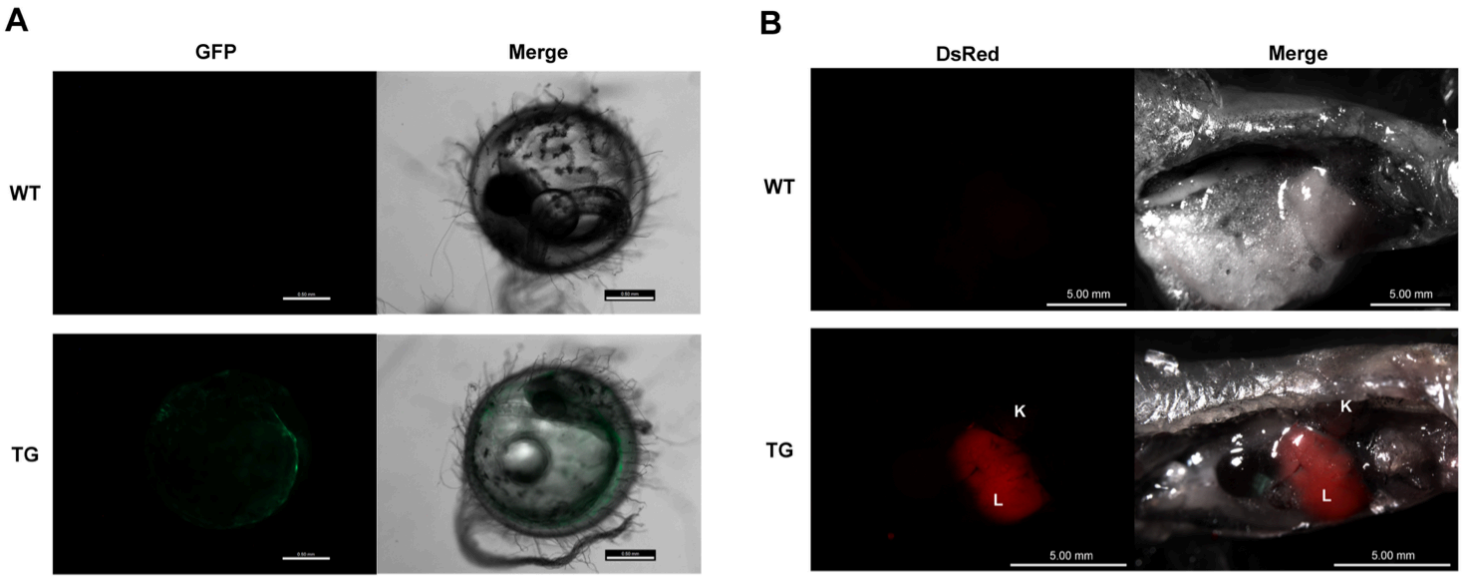

Figure 2

Observation for green fluorescent protein (GFP) in F0 embryos at 15 dpf (A), as well as red fluorescent protein (crmCh) in abdominal organs (B). Fluorescence in wild-type (WT) and transgenic (TG) adult F0 males was compared. Fluorescence images of WT embryo and F1 TG embryo were taken under GFP filter with an exposure time of 1 sec; GFP image was merged with bright field (Merge). Fluorescence images of abdominal organs (K: kidney, L: liver) and dissected kidney were taken under a red filter (DsRed) with an exposure time of 1 sec and merged with bright field (Merge).

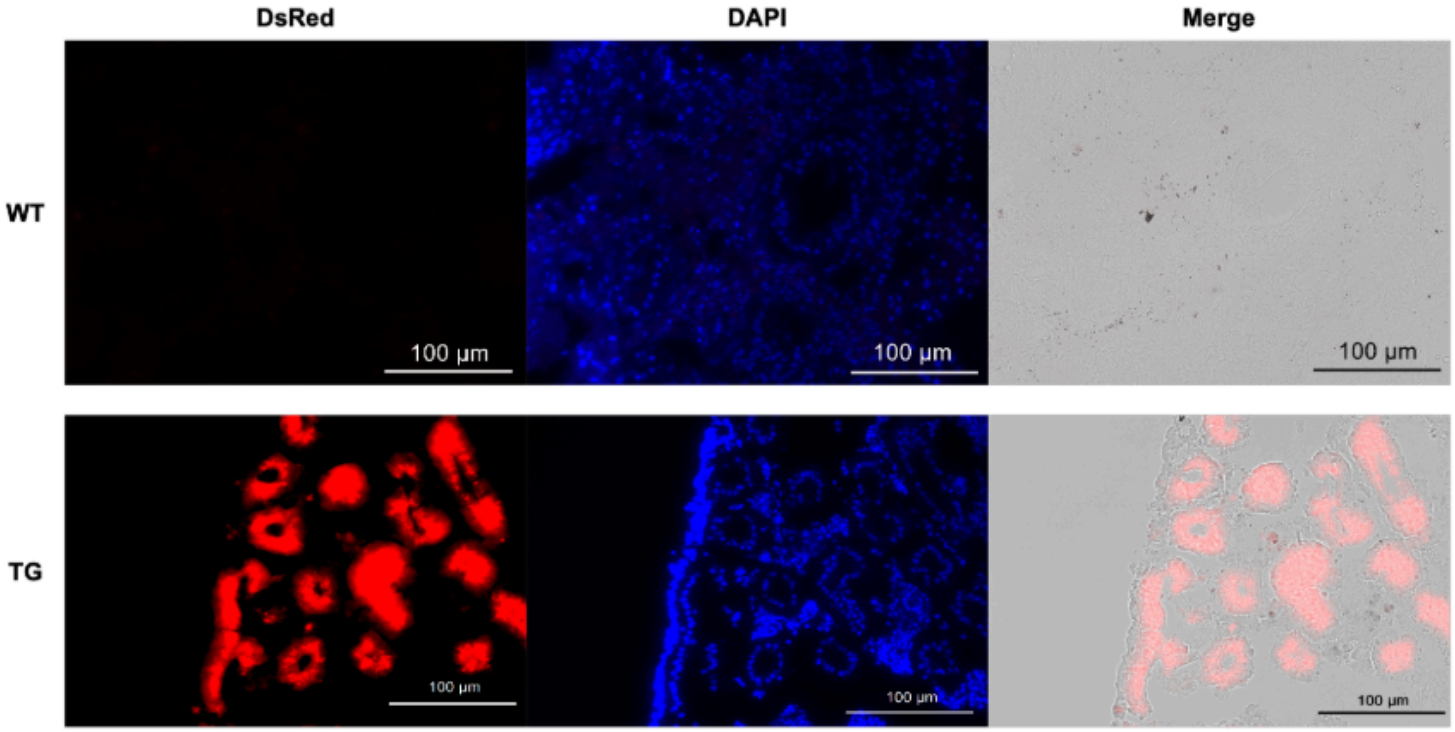

Figure 3

Observation of cryostat-sectioned kidney. Fluorescence images of wild type (WT) adult male and transgenic (TG) F0 adult male were taken under red filter (DsRed) with exposure time of 1/12 sec, underblue filter (DAPI) and DsRed merged with bright field.

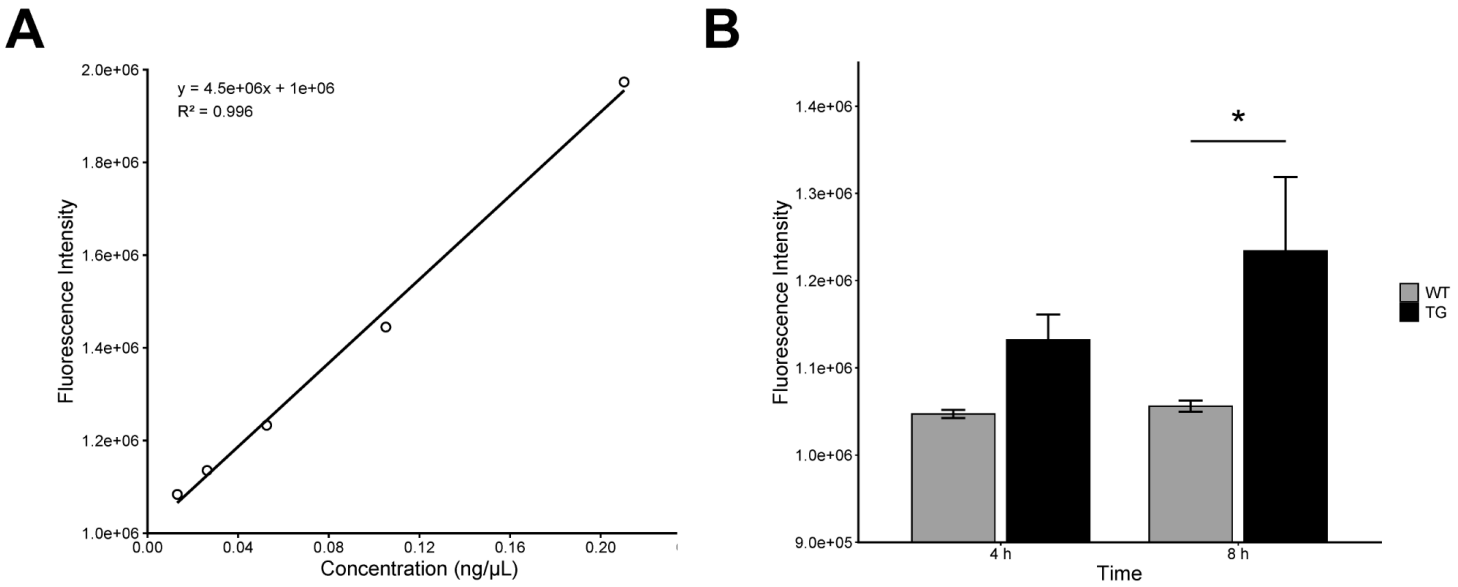

Figure 4

Linear regression of crmCh fluorescence intensity and concentration (A) and fluorescence intensity of rearing water at 4 and 8 hours post MT treatment (B). Significant difference was tested among wild type (WT) and F0 transgenic (TG) adult male medaka groups at both sampling points ($p < 0.05$, N = 6, two-way ANOVA). Error bar refers to the standard error.

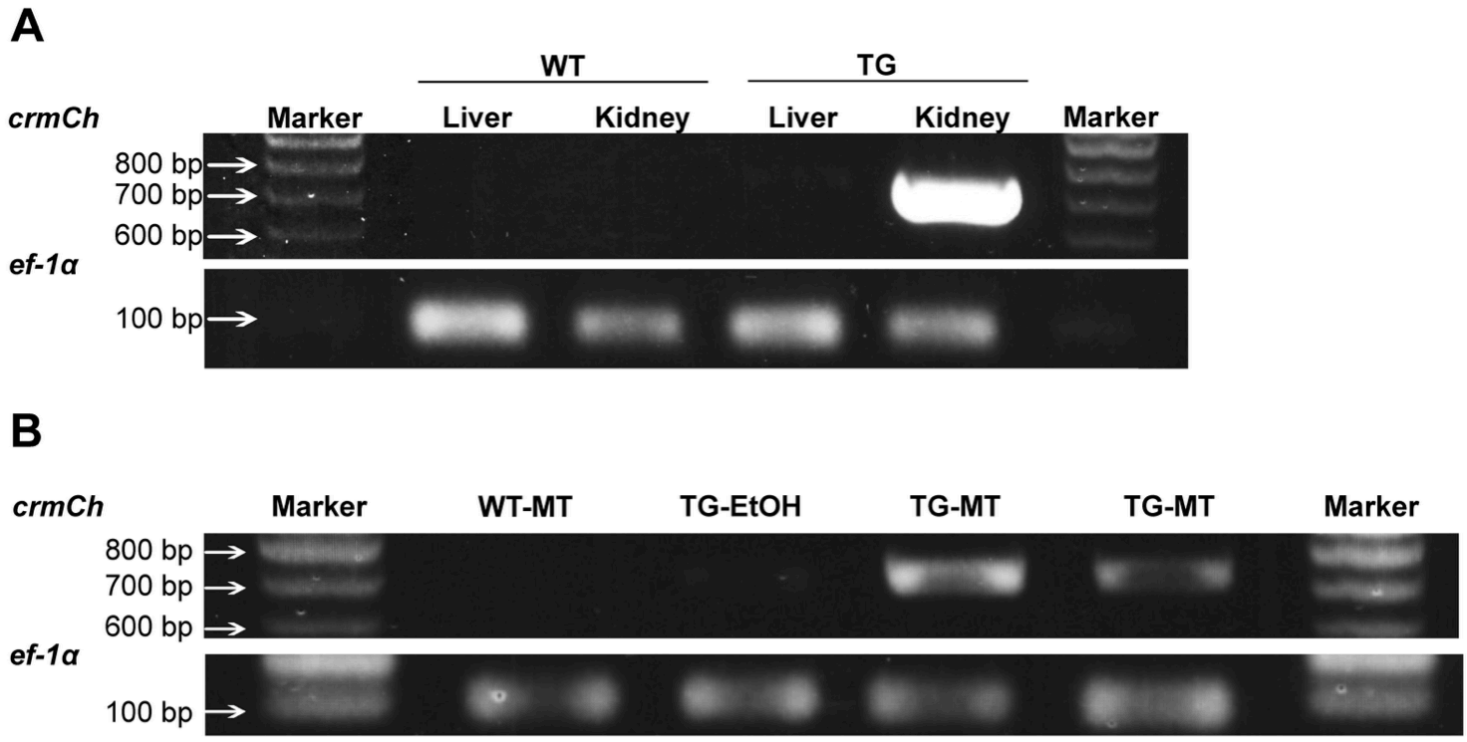

Figure 5

1% (A) and 1.5% (B) agarose gel electrophoresis of PCR products amplified using either *crmCh* or *ef-1α* specific primers and reverse-transcribed cDNA templates. Reverse-transcribed cDNA derived from either adult male liver and kidney samples(A) orwhole body of larvae (B) were used in PCR amplification. In adult males, total RNA samples were extracted from tissues (liver and kidney) of wild type (WT) and F0 transgenic (TG) medaka without methyl-testosterone (MT) treatment. In larvae, whole body of wild-type after testosterone treatment (WT-MT), as well as of F1 transgenic larvae after ethanol medium (TG-EtOH), or after MT (TG-MT) treatments, were used for total RNA extraction. Arrows and numbers represented sizes of marker bands.

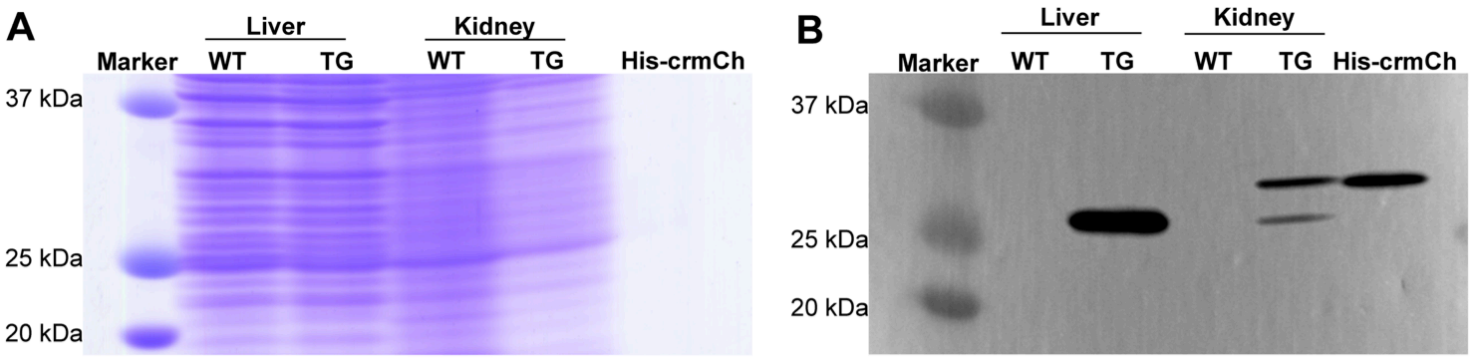

Figure 6

SDS-PAGE using 15% polyacrylamide gel of kidney and liver samples from adult males(A) and corresponding ECL™ western blotting using anti-mCh as primary antibody (B). Proteins extracted from liver and kidney samples of wild type (WT) and transgenic (TG) medaka, along with recombinant His-crmCh, were applied at a constant amount of 75 µg/well. ECL image was taken with exposure time of1 min under 'HD' camera protocol.

Supplementary Files

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