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ORIGINAL ARTICLE

Biology

Development of an efficient bioreactor system for delivering foreign proteins secreted from liver into eggs with a vitellogenin signal in medaka *Oryzias latipes*

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Abstract

In this study, we developed a novel bioreactor system to deliver and accumulate foreign proteins in eggs using medaka fish *Oryzias latipes* with the aid of a partial sequence of vitellogenin (Vtg). In teleost fish, Vtg, the hepatically generated precursor of egg yolk proteins, is secreted into the bloodstream and then taken up into eggs. We predicted in silico a probable region (Vtg signal) of Vtg that mediates transportation of proteins from the liver into eggs. Then, we established two transgenic lines expressing the fused proteins including the Vtg signal and each reporter gene, *enhanced green fluorescent protein* (*EGFP*) or firefly *luciferase* (*LUC*)-fused *EGFP*, in the liver driven by a liver-specific *choriogeninH* (*chg*H) promoter. Each reporter signal was detected from the fertilized eggs spawned by the transgenic females, showing successful transportation of the proteins into the eggs with the Vtg signal. This is the first report demonstrating that the Vtg signal has capability to deliver exogenous proteins into eggs. Because Vtg is a highly conserved protein among most of oviparous organisms, our findings hold promise for establishing bioreactor systems viable in a wide range of organisms.

Keywords Vitellogenin · Fish eggs · Bioreactor · Transgenesis · Firefly luciferase · Medaka

Introduction

To date, various bioreactor systems have been established for the production of recombinant proteins. Bacteria can be extensively used owing to the ease of culturing at any scale, but they cannot effectively produce glycosylated proteins of vertebrates, and also often form misfolded recombinant proteins as the inclusion body (Villaverde and Carrió 2003). Although yeasts and insect cell lines such as Sf9 can perform post-translational modifications (PTMs) to enhance the capability of proteins, it is difficult to completely reproduce the glycosylation pattern of vertebrates; the bioactivities of the produced recombinant proteins may not fully correspond to those of the native forms in vertebrates (Brooks 2004). Mammalian cultured cell lines such as 293T can achieve complex PTMs; however, culture of the cell lines is so expensive that the recombinant protein cannot be produced cost-effectively. As each technique has both advantages and disadvantages, it is necessary to select the appropriate system depending on the properties of the recombinant proteins.

On the other hand, fish also have the following desirable features for use as bioreactor systems; (1) low cost for maintenance, (2) ease of collecting eggs, and (3) availability of the technology for gene modification. Indeed, two studies demonstrated that recombinant proteins could be purified from fish eggs into which the expression plasmid was injected. One is human coagulation factor VII (hVFII) expressed by the cytomegalovirus (CMV) promoter in nile tilapia *Oreochromis niloticus* embryos, and the other is the

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recombinant goldfish luteinizing hormone (LH) expressed by the medaka β-actin promoter in rainbow trout *Oncorhynchus mykiss* embryos (Hwang et al. 2004; Morita et al. 2004). Both recombinant proteins were successfully expressed in fish embryos; however, these studies did not investigate whether the eggs containing each respective recombinant protein can be stably produced from the female transgenic founders. Moreover, Hu et al. (2011) reported that the F_2 fertilized eggs from intercrossed F_1 transgenic zebrafish could produce mature tilapia insulin-like growth factors (IGFs) proteins using the zona pellucida (*zp3*) promoter, which promotes oocyte-specific expression. This report indicated that transgenic fish can stably produce and store exogenous proteins in their eggs. Most teleost fish, however, can generate the precursors of the egg envelope and/or yolk proteins in the liver, and then deliver them into eggs for the formation of matured eggs (Liu et al. 2006; Hara et al. 2016). These results prompted us to examine the possibility of a novel bioreactor system; liver-expressed recombinant proteins may be efficiently transported into eggs, thus allowing for the mass accumulation of the proteins in the eggs. The liver generally plays a role in producing the various proteins in quantity, and thus has the potential to stably and abundantly generate recombinant proteins for delivering into eggs as well.

Vitellogenin (Vtg) is a major precursor of egg yolk protein for embryonic development in oviparous organisms (Romano et al. 2004). In general, teleost fish produce Vtg in the liver with the stimulation of estradiol-17β (E_2), and then it is secreted into the bloodstream (Hara et al. 2016). The secreted Vtg is incorporated into the growing oocytes via specific membrane receptors during vitellogenesis (Hiramatsu et al. 2004; Mizuta et al. 2013; Mushirobira et al. 2015), and then it is proteolytically cleaved by cathepsin D to generate multiple egg yolk proteins such as lipovitellin (Lv) and phosvitin (Pv) (Hiramatsu et al. 2002). Functional analyses of domains of Vtg have depended mainly upon in vitro experiments and biochemical approaches. For example, a previous study using the yeast two-hybrid system revealed that a peptide sequence (HLTKTKDL) in the Lv domain of the Vtg of blue tilapia *Oreochromis aureus* played a critical role in binding to the Vtg receptor (Li et al. 2003). However, the critical sequence of Vtg that enables the transportation of proteins secreted from the liver into the eggs in vivo remains unknown. For easier construct design and expression, it is necessary to identify more precisely the effective region in the full length of Vtg, which is designated as the ‘‘Vtg signal’’ in this study.

Here, we aimed to establish a novel system for transporting foreign proteins into eggs by using the Vtg signal in medaka. First, we generated transgenic medaka lines that expressed enhanced green florescent protein (EGFP) with or without the Vtg signal (N-terminal part of Vtg protein) in their liver using the liver-specific *chg*H promoter (Kurauchi et al. 2005). Second, we demonstrated that EGFP was accumulated in eggs only when used the Vtg signal. We also confirmed that a larger size of exogenous protein (EGFP-fused luciferase: 798 amino acids, 88 kDa) than EGFP (240 aa, 27 kDa) could be transported into eggs with the Vtg signal. Our results showed that the Vtg signal is a valuable tool for a bioreactor.

Materials and methods

Fish

The Cab inbred closed colony of medaka *Oryzias latipes* was used in this study. The fish were kept under a 14/10-h light/dark cycle at 26 °C. Fish handling and sampling methods were approved by Kyoto University (No. 26–71). All efforts were made to minimize suffering.

Identification and isolation of the *vtg* signal in medaka

To identify the Vtg signal from the full-length amino acid sequence, the secretory signal of the medaka vitellogenin (NCBI, accession number AY074891) was analyzed using Signal Peptide Predictor software (SignalP, http://www.cbs.dtu.dk/services/SignalP/). In addition, the amino acid sequence of the medaka Vtg was aligned with the Vtg receptor-binding region (HLTKTKDL) of the blue tilapia *Oreochromis aureus* Vtg to investigate the presence of conserved regions using the sequence alignment tool ClustalW (http://clustalw.ddbj.nig.ac.jp/). The resulting 300 amino acid N-terminal region containing the predicted secretory signal and the receptor-binding region was designated as the Vtg signal. The coding sequence (*vtg* signal) of the Vtg signal was isolated by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the liver of a sexually matured female with an RNeasy Plus Mini kit (Qiagen). One microgram of the total RNA was used for synthesizing first strand cDNA with random hexamers and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA was used for PCR amplification of the *vtg* signal with KOD-FX DNA polymerase (Toyobo) using primers Asp718I-vtg-Fw and vtg-NcoI-Rv (Table 1). The PCR condition was as follows: incubation at 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 min, 58 °C for 30 s and 68 °C for 30 s. The PCR products purified with 1% agarose gel electrophoresis and the DNA sequence was confirmed by direct sequencing analysis (data not shown).

Table 1 Oligonucleotide sequences used in this study

Name	Sequence (5′–3′)	Usage
Asp718I-vtg-Fw	AGGTGGTACCATGAGGGGGCTGATTCTTGC	RT-PCR
vtg-NcoI-Rv	ATCGCCATGGTCTCAACTGGCGTTTGGAGGAGC	RT-PCR
NotI-Backbone-Fw	CGTGCGGCCGCGACTCTAGATCATAATC	Construction of plasmid
Backbone-XhoI-Rv	GCACTCGAGTTTCCCCGTATCCCCCCAGG	Construction of plasmid
XhoI-chgH-Fw	CTGCTCGAGACACGTTATTTTGTGATAAC	Construction of plasmid
EGFP-NotI-Rv	GCAGCGGCCGCTTTACTTGTACAGCTCGT	Construction of plasmid
NcoI-LUC-Fw	AGCTCCATGGAAGACGCCAAAAACAT	Construction of plasmid
LUC-Linker- SpeI-Rv	AGCACTAGTAGTTCCACCGCTACCTCCCACGGC GATCTTGCC	Construction of plasmid
EGFP-Fw	TGCCACCTACGGCAAGCTGA	RT-PCR
EGFP-Rv	TGTTGCCGTCCTCCTTGAAG	RT-PCR
ef1a-Fw	CAGGACGTCTACAAAATCGG	RT-PCR
ef1a-Rv	AGCTCGTTGAACTTGCAGGC	RT-PCR

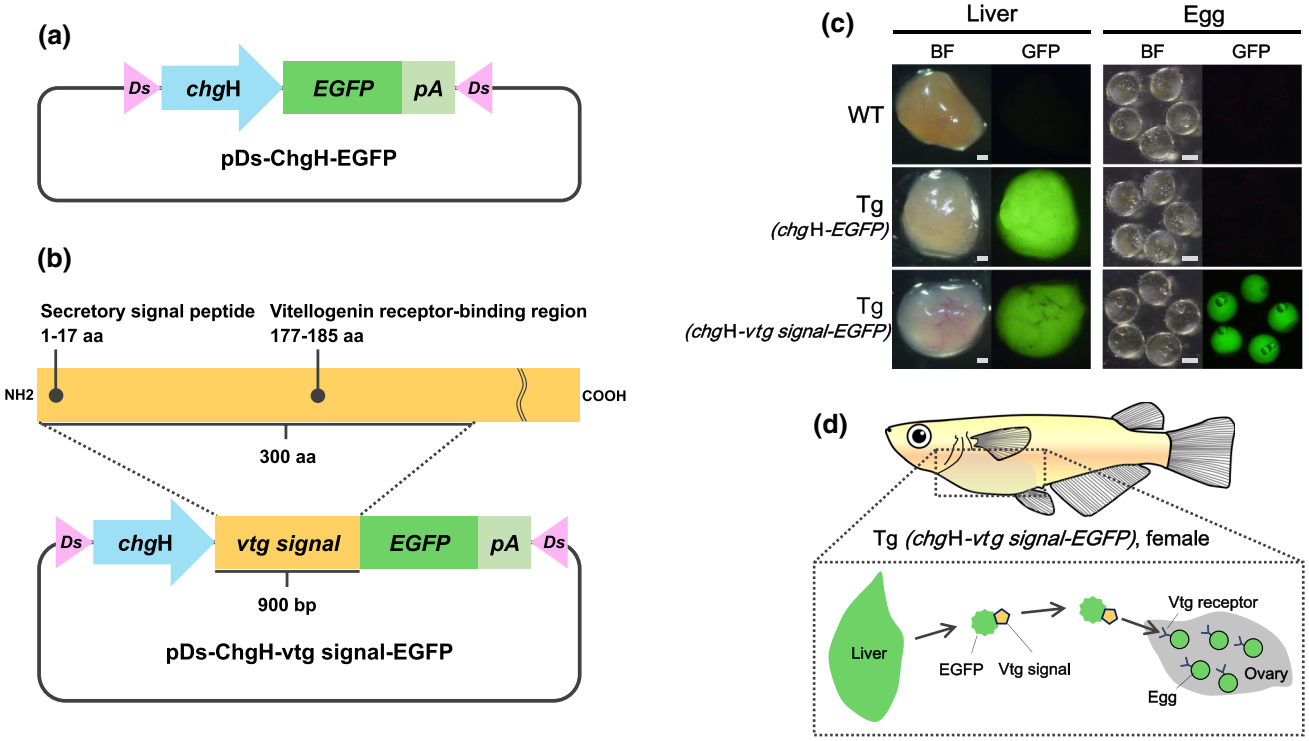

Fig. 1 The Vtg signal-mediated delivery of EGFP secreted in the liver into eggs. **a** DNA construct of the pDs-ChgH-EGFP for establishing Tg (*chg*H-*EGFP*). The *Ds* transposon sequences flanking the plasmid were used to enhance the efficiency of genomic integration. The *chg*H promoter can drive the downstream *EGFP* in liver in response to stimulation by 17-β estrogen. **b** Schematics of the full-length amino acid sequence of the medaka Vtg, and the pDs-ChgH-vtg signal-EGFP for establishing Tg (*chg*H-*vtg signal-EGFP*). The *vtg* signal is 900 bp in length encoding 300 aa containing the secretory signal peptide and the receptor-binding region of the Vtg. **c** Fluorescent images of livers and eggs of sexually mature females in the Cab (WT), Tg (*chg*H-*EGFP*), and Tg (*chg*H-*vtg signal-EGFP*). Each female was mated with a wild-type male to observe the green fluorescence of the fertilized eggs on the day of spawning. There was no GFP signal in the liver or eggs of the Cab (WT). In Tg (*chg*H-*EGFP*), a GFP signal was detected from the liver but not from the eggs, though Tg (*chg*H-*vtg signal-EGFP*) exhibited GFP signals in both the liver and eggs. The transgenic lines in the Figure carried each transgene heterozygously. BF: bright field, GFP: GFP fluorescent image, scale bar: 200 µm. **d** Schematics of delivery of EGFP secreted in the liver into the eggs in Tg (*chg*H-*vtg signal-EGFP*) females. The Vtg signal enabled transportation of EGFP secreted in the liver into the eggs

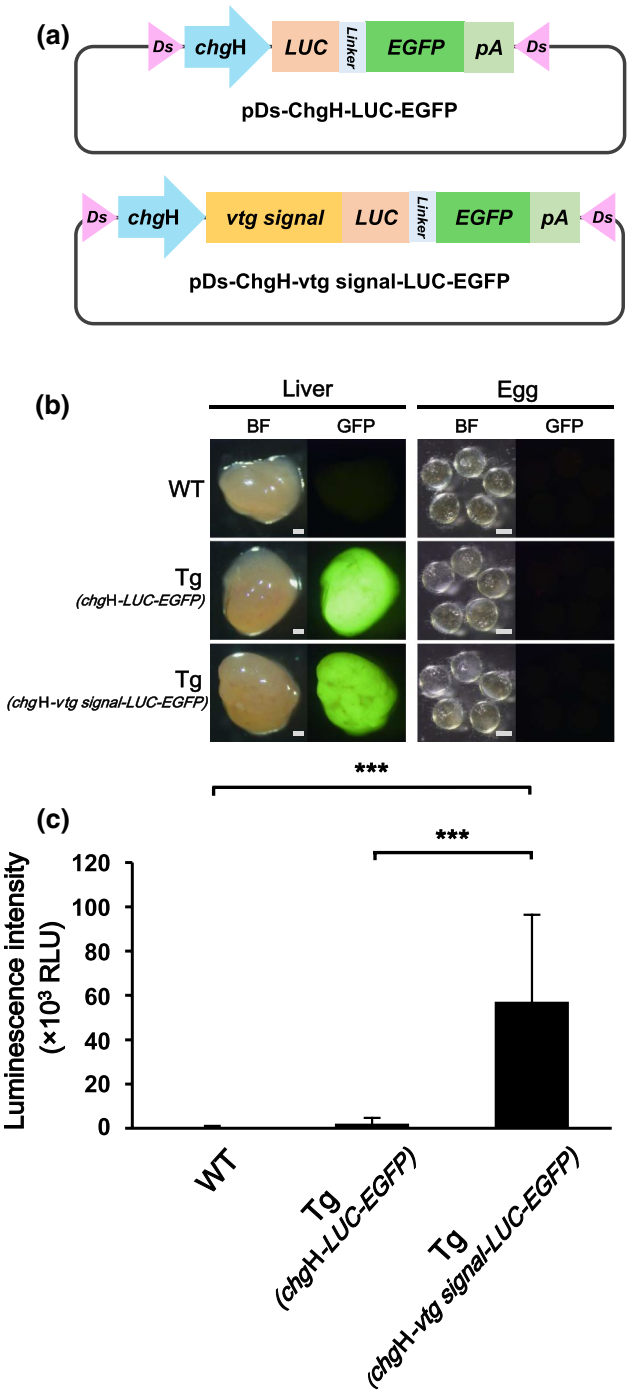

Fig. 2 The transportation of the EGFP-fused LUC expressed in the liver into eggs using the Vtg signal. **a** DNA construction of the pDs-ChgH-LUC-EGFP for establishing Tg (*chg*H-*LUC-EGFP*), and the pDs-ChgH-vtg signal-LUC-EGFP for establishing Tg (*chg*H-*vtg signal-LUC-EGFP*). A linker sequence (GGSGGT) was located between *LUC* and *EGFP* to increase the flexibility of the fusion protein, LUC and EGFP. **b** Fluorescent images of livers and eggs of sexually mature females in the Cab (WT), Tg (*chg*H-*LUC-EGFP*), and Tg (*chg*H-*vtg signal-LUC-EGFP*). The females were crossed with wild-type males to observe the green fluorescence of the fertilized eggs on the day of spawning. No GFP signal was detected in the liver or eggs of the Cab (WT). GFP fluorescence was detected from the livers of Tg (*chg*H-*LUC-EGFP*) and Tg (*chg*H-*vtg signal-LUC-EGFP*), but not from the eggs of either transgenic lines. BF: bright field, GFP: GFP fluorescent image, scale bar: 200 µm. **c** Detection of LUC activity in the eggs derived from females of the Cab (WT), Tg (*chg*H-*LUC-EGFP*), and Tg (*chg*H-*vtg signal-LUC-EGFP*). The luciferase activity in the eggs was normalized by the concentration of total protein extracted from egg samples, and expressed as relative luminescence units (RLU). Data are presented as mean ± SD of triplicate determinations from a single experiment ($n = 9$/group). Stars (***) indicate that the values are significantly different between each group by one-way ANOVA followed by Tukey's multiple comparisons ($P < 0.001$). All transgenic fish in the Figure carried each transgene heterozygously

Plasmid construction

pDs-ChgH-EGFP (Fig. 1a): a backbone fragment containing 5′- and 3′-*Ds* sequences, a poly (A) signal, a pUC replication origin, and an ampicillin resistance gene was amplified from a plasmid pDs-actb2 k-EGFP (Uemura et al. 2015) by PCR using a primer pair (NotI-Backbone-Fw and Backbone-XhoI-Rv) (Table 1) with the condition described above. Also, an insert fragment containing a *chg*H promoter (approximately 2 kbp) and *EGFP* was amplified from a plasmid pChgH-EGFP by PCR using a primer pair (XhoI-chgH-Fw and EGFP-NotI-Rv) (Table 1) (Kurauchi et al. 2005). These two fragments were digested with XhoI/NotI, and then ligated to obtain pDs-ChgH-EGFP. The *chg*H promoter (approximately 2 kbp), which was isolated from the 5′-upstream sequence of the medaka *choriogeninH* gene, induces liver-specific gene expression in response to endogenous and exogenous 17-β estrogen (E2) (Kurauchi et al. 2005).

To generate the plasmid of pDs-ChgH-vtg signal-EGFP, the PCR-amplified *vtg* signal which contains Asp718I and NcoI recognition sites at 5′ and 3′, respectively, was digested and inserted into the Asp718I/NcoI site of the pDs-ChgH-EGFP construct (Fig. 1b).

To generate the pDs-ChgH-LUC-EGFP construct (Fig. 2a), first the fragment of firefly *Photinus pyralis* luciferase (LUC) was amplified from the pGL 4.13 plasmid (Promega) by PCR using a primer pair (NcoI-LUC-Fw and LUC-Linker-SpeI-Rv) (Table 1), and then digested with NcoI/SpeI. The resultant LUC fragment was ligated into the NcoI/SpeI site of the pDs-ChgH-EGFP construct to generate the pDs-ChgH-LUC-EGFP construct. LUC and EGFP were produced as a fusion reporter protein by this construct.

The *vtg* signal fragment (pDs-ChgH-vtg signal-LUC-EGFP) was obtained by digesting the pDs-ChgH-vtg signal-EGFP with Asp718I/NcoI, and then ligating it into the the pDs-ChgH-LUC-EGFP construct at the Asp718I/NcoI site (Fig. 2a).

All constructed plasmids were extracted using the NucleoSpin Plasmid QuickPure kit (Macherey–Nagel) according

to the manufacturer's direction. To eliminate residual RNase activity of the extracted plasmids, the plasmids in 50 µl of 5 mM Tris–HCl buffer (pH 8.5) were incubated with 2 µl of Proteinase K (20 mg/ml) and 5 µl of 10% sodium dodecyl sulfate (SDS) at 55 °C for 30 min, and then purified using NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel) with the buffer NTB.

Ac RNA preparation for microinjection

The *Ds* sequences in the above plasmids contain the cis-required terminal repeats that can be transactivated by an *Ac* transposase (Lazarow et al. 2013). Introduction of plasmids with the *Ds* elements and the *Ac* RNA in medaka embryos enhances the efficiency of germline transmission in transgenic medaka (Boon Ng and Gong 2011). The *Ac* RNA for microinjection was synthesized and purified as described previously (Inoue et al. 2016). Briefly, the linearized pAcII plasmid with BamHI was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel) for in vitro transcription. The capped *Ac* RNA was transcribed from the purified DNA template using the Message mMachine SP6 Kit (Life Technologies, Gaithersburg, MD). The synthesized RNA was purified using RNeasy Mini Kit (Qiagen) for microinjection.

Generation of transgenic medaka line

In the present study, 4 different transgenic lines that harbor pDs-ChgH-EGFP, pDs-ChgH-vtg signal-EGFP, pDs-ChgH-LUC-EGFP and pDs-ChgH-vtg signal-LUC-EGFP are designated as Tg (*chg*H-*EGFP*), Tg (*chg*H-*vtg signal-EGFP*), Tg (*chg*H-*LUC-EGFP*), and Tg (*chg*H-*vtg signal-LUC-EGFP*), respectively. To establish the 4 transgenic lines, injection mixtures containing 10 ng/µl of each plasmid and 100 ng/µl of *Ac* RNA were injected into fertilized eggs at the one-cell stage as previously described (Inoue et al. 2016). The hatched yolk-sac larvae were screened by observing EGFP expression in the liver after overnight exposure to 100 ng/µl of E2. The screened larvae were reared to adulthood as founders (F_0s), and then mated with wild-type fish to obtain the F_1 transgenic fish. In this study, $F_{1\text{-}8}$ heterozygous females were used for verifying the accumulation of foreign proteins in the fertilized eggs.

Observation of GFP fluorescence

The fluorescence of the liver and fertilized eggs was observed using a fluorescence stereomicroscope MZFLIII (Leica Microsystems) with a GFP2 filter set. Microscopic images were captured using a digital color-cooled charge-coupled camera and the VB-7010 image control system (Keyence).

Reporter assay for the detection of LUC activity

To investigate Vtg signal-mediated accumulation of LUC in eggs, bioluminescence was detected with a Luciferase Assay System Kit (Promega) and GloMax 96 microplate luminometer (Promega) as described by Saito et al. (2009). Egg samples for this analysis were collected from three groups: wild-type, Tg (*chgH-LUC-EGFP*), and Tg (*chgH-vtg signal-LUC-EGFP*). In each group, 9 females were mated with wild-type counterparts to obtain 5 fertilized eggs per 1 female. The eggs of each transgenic line were prepared as follows: (1) 3 founders (F_0s) of Tg (*chgH-LUC-EGFP*) or Tg (*chgH-vtg signal-LUC-EGFP*) were mated with wild-type counterparts to establish 3 independent heterozygous lines (F_1s) of each transgene. (2) 3 heterozygotes (F_1s) were mated with wild-type counterparts to generate 3 heterozygous females (F_2s) in each line. (3) The resulting 9 females (F_2s) were mated with wild-type males to collect 5 fertilized eggs in each pair. The collected 5 fertilized eggs were pooled in a 1.5-ml microtube containing 300 µl of the Reporter lysis buffer (Promega), and then homogenized with a pestle. After freezing (− 80 °C) and thawing (room temperature), 80 µl of each homogenate was dispensed into 3 wells on a 96-well microplate for triplicate assays. Then, 20 µl of the luciferase assay substrate of the above kit (Promega) was added to each well to measure the bioluminescence with the GloMax 96 microplate luminometer (Promega). The measured bioluminescence intensity was normalized by the concentration of total protein extracted from each homogenate, and expressed as relative luminescence units (RLU). The protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Scientific) according to the manufacturer's protocol.

Evaluation of transcripts of foreign genes in ovary

RT-PCR was performed as described in the above "Identification and isolation of the *vtg* signal in medaka" section to evaluate the transcripts of the elongation factor-1α (*ef-1α*) gene and *EGFP* gene in the ovary. Briefly, the expression of *ef-1α* was analyzed by PCR using KOD-FX DNA polymerase (Toyobo) with primers ef1α-Fw and ef1α-Rv (Table 1) to evaluate the possibility of contamination of genomic DNA (gDNA). This primer set gives different lengths of PCR products from gDNA (519 bp) and cDNA (374 bp). The expression analysis of *EGFP* was performed by PCR with KOD-FX DNA polymerase (Toyobo) and primers EGFP-Fw and EGFP-Rv (Table 1) to evaluate the presence of transcripts of each foreign gene, GFP or GFP-LUC, in the ovary. The ovary of a transgenic female (*42Sp50-EGFP*) expressing EGFP in the oocyte was used as a positive control to confirm the detection of *EGFP* in the RT-PCR analysis (Kinoshita et al. 2009). The PCR conditions were as follows: one cycle

at 94 °C for 2 min followed by 35 PCR cycles of 98 °C for 10 min, 58 °C for 30 s and 68 °C for 30 s. The resulting PCR products were analyzed by 1% agarose gel electrophoresis.

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism (MDF). *P* values less than 0.05 were considered statistically significant.

Results

There was no accumulation of EGFP without the *vtg* signal in eggs spawned by Tg (*chg*H-*EGFP*).

To evaluate whether EGFP produced in liver can be delivered into eggs, we established a Tg (*chg*H-*EGFP*) line, expressing EGFP in the liver under the regulation of the *chg*H promoter (Fig. 1a and Online Resource, Fig. S1). The sexually mature transgenic fish were mated with wild-type counterparts to generate the heterozygous females. The resultant females exhibited intense green fluorescence in the liver, whereas no GFP signal was detected from the fertilized eggs obtained by mating with wild-type males (Fig. 1c), showing no accumulation of EGFP in the eggs. Thus, to achieve the transport of EGFP from the liver into the eggs, we focused on Vtg that is synthesized in the liver and delivered into the eggs.

Identification and cloning of the *vtg* signal in medaka

We tried to identify a region of Vtg sufficient for uptake of maternal factors into medaka. The in silico analysis using SignalP (http://www.cbs.dtu.dk/services/SignalP/) nominated the possible secretion signal peptides in the first 17 aa (^{1}MRGLILALSLALVAANQ^{17}) of Vtg. In addition, the alignment analysis of Vtg amino acid sequence of various fish species revealed that a similar amino acid sequence (identical in 7 of the 8 amino acids) to the receptor-binding region (HLTKTKDL) identified in tilapia Vtg (Li et al. 2003) was located between amino acids 178 and 185 (^{178}HLSKTKDL^{185}) of the medaka Vtg. From these results, we hypothesized that the 300 aa N-terminal portion of Vtg ("Vtg signal") was sufficient to deliver the foreign proteins produced in the liver into eggs. The 900 bp of *vtg* signal encoding the Vtg signal (300 aa) was isolated from cDNA of the female liver by RT-PCR, and then cloned into reporter gene plasmids (Figs. 1b, 2a) to generate transgenic medaka.

Transportation of EGFP expressed in liver into eggs by the Vtg signal

To investigate whether the Vtg signal could be effective for the transportation of secreted EGFP from the liver into eggs, we observed the green fluorescence in the liver and fertilized eggs of Tg (*chg*H-*vtg signal-EGFP*) female fish. The liver sample, which was dissected from a sexually mature female of the transgenic line, showed the intense green fluorescence of introduced *EGFP* driven by the *chg*H promoter similar to the liver of Tg (*chg*H-*EGFP*) (Fig. 1c). In contrast, there was a large difference in EGFP fluorescence in fertilized eggs between the transgenic lines with and without the *vtg* signal. Fertilized eggs of Tg (*chg*H-*vtg signal-EGFP*) fish exhibited intense green fluorescence, whereas those of Tg (*chg*H-*EGFP*) had no green fluorescence (Fig. 1c). These findings show that the Vtg signal can promote the transportation of EGFP expressed in the liver into eggs.

Transportation of LUC-EGFP expressed in liver into eggs by the Vtg signal

To test whether a larger molecule than EGFP (240 aa) can be delivered into eggs by the Vtg signal, we compared females of two transgenic lines, Tg (*chg*H-*LUC-EGFP*) and Tg (*chg*H-*vtg signal-LUC-EGFP*) that stably expressed the fused protein (798 aa) of LUC and EGFP in the liver. The transgenic line without the *vtg* signal, Tg (*chg*H-*LUC-EGFP*), was used as a negative control to assess the effect of the *vtg* signal. The dissected livers of both heterozygous transgenic females appeared bright green under the fluorescence microscope (Fig. 2b). In contrast, there was no EGFP fluorescence in fertilized eggs from either transgenic lines of the heterozygous females, showing that the transportation efficiency of Tg (*chg*H-*vtg signal-LUC-EGFP*) expressing a fusion protein LUC and EGFP (798 aa) significantly decreased in comparison to that of Tg (*chgH-vtg signal-EGFP*) producing a single protein EGFP (240 aa) (Figs. 1c, 2b). Additionally, we measured the LUC activity in the fertilized eggs using the luciferase reporter assay system because luminescence of luciferase and luciferin is more sensitive than fluorescence of EGFP. As a result, the eggs of Tg (*chg*H-*vtg signal-LUC-EGFP*) showed a significantly higher value of luminescence than those of wild-type and Tg (*chg*H-*LUC-EGFP*) lacking the Vtg signal (Fig. 2c). Our data show that the Vtg signal enables the delivery of not only EGFP (240 aa) but also the fusion protein of EGFP and LUC (798 aa) into eggs.

Evaluation of transcripts of the transgene, *EGFP*, in the ovary by RT-PCR

To evaluate the quality of RNA extracted from the ovary, RT-PCR with specific primers of *ef-1α* was performed.

As shown in Fig. 3a, smaller PCR products (374 bp), which were derived from e*f-1α* RNA, were observed in WT and transgenic lines, whereas larger PCR products (519 bp), which are amplified from genomic DNA, were not detected. These results showed that the contamination of gDNA was eliminated in our RNA extract.

To confirm that EGFP or LUC-EGFP that had accumulated in the eggs of Tg (*chg*H-*vtg signal-EGFP*) or Tg (*chg*H-*vtg signal-LUC-EGFP*), respectively, was not derived from the transcript of each transgene in the ovary, we evaluated the presence of the transcript of *EGFP* in the ovary, which is an indicator of both GFP and LUC-EGFP RNA. As shown in Fig. 3b, the PCR product derived from *EGFP* (298 bp) was clearly detected in the ovary of Tg (*42Sp50-EGFP*) which expresses EGFP in the oocyte and was used as an EGFP positive sample (Kinoshita et al. 2009). On the other hand, the expression of *EGFP* was not detected in the ovaries of wild-type (WT), Tg (*chg*H-*vtg signal-EGFP*) or Tg (*chg*H-*vtg signal-LUC-EGFP*) (Fig. 3b). These results show that the Vtg signal-fused EGFP or LUC-EGFP was delivered from outside the ovary into the eggs and was not produced by ectopic expression of each transgene in the ovary.

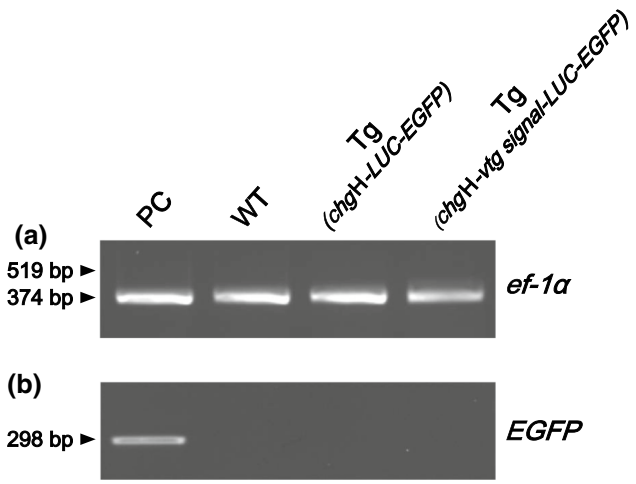

Fig. 3 Expression analysis of *EGFP* in the ovary using RT-PCR. **a** Electrophoresis image of the PCR amplicons of *ef-1α* gene. In all samples, the smaller size of PCR products (374 bp) derived from the exon sequence were observed, whereas the longer size of PCR products (519 bp) derived from the exon and intron sequence were not detected. **b** Electrophoresis image of the PCR amplicons of *EGFP* gene. The PCR amplicons (298 bp) containing the *EGFP* gene were detected only from the ovary of a transgenic female which expresses EGFP in the oocytes and was used as a positive control (PC), but not from that of WT (Cab), Tg (*chg*H-*vtg signal-EGFP*) and Tg (*chg*H-*vtg signal-LUC-EGFP*)

Discussion

In this study, we successfully established a novel bioreactor system in medaka using the N-terminal 300 aa of Vtg, which is a major yolk precursor protein. Based on the results of in silico analysis showing that medaka Vtg contains the secretory signal peptide (^{1}MRGLILALSLALVAANQ^{17}) and the receptor-binding region (^{178}HLSKTKDL^{185}), we predicted that the 300-aa N-terminal portion of Vtg ("Vtg signal") was sufficient to transport the foreign proteins produced in the liver into eggs. To investigate whether the prediction was true or not, we generated transgenic medaka expressing the foreign proteins (Vtg signal-EGFP and Vtg signal-EGFP-LUC) in the liver. The fertilized eggs spawned by both transgenic lines showed the fluorescence of EGFP and the luminescence of LUC, respectively, indicating that the Vtg signal-fused proteins were incorporated into the eggs through the Vtg receptor on the surface of the oocytes (Figs. 1c, d, 2c). We therefore concluded that the identified Vtg signal could be effective for the in vivo delivery of secreted foreign proteins from the liver into the eggs.

Several studies have shown that fish eggs could be used for producing recombinant proteins with transgenesis. For example, Hwang et al. (2004) and Morita et al. (2004) succeeded in obtaining the recombinant protein (human coagulation factor VII or goldfish luteinizing hormone) from the first-generation (G_0) eggs of nile tilapia *Oreochromis niloticus* or rainbow trout *Oncorhynchus mykiss* into which the expression vector containing the CMV promoter or the medaka β-actin promoter was injected, respectively. This method is helpful for obtaining the target proteins quickly, but it is necessary to microinject into a large number of eggs sufficient to reach the desired amount of protein. In contrast, when producing the target proteins stably and continuously over a long period with less work, it is efficient to obtain the protein from the eggs spawned by established transgenic females of subsequent generations (F_1, F_2, and F_3), as shown in the present study. In this respect, small model teleost fish, such as zebrafish and medaka, are promising models for bioreactors because of their frequent spawning and ease of generating and maintaining a large number of transgenic lines. Nevertheless, there have been only a few reports verifying the use of bioreactors for producing exogenous proteins in the eggs of these fish. A previous study using zebrafish reported that transgenic females could produce proteins of tilapia insulin-like growth factors (IGFs) in the eggs under the oocyte-specific zona pellucida (*zp3*) promoter (Hu et al. 2011). Unlike the previous study, we demonstrated for the first time that the proteins expressed in the liver could be delivered into the eggs by using the Vtg signal in medaka. In general,

liver synthesizes large quantities of diverse proteins; thus, liver is suitable for the mass production of recombinant proteins. Additionally, our transgenic medaka appeared normal and had no obvious health or growth problems (up to the F_8 generation); thus, we will be able to routinely obtain the targeted protein from eggs without sacrificing the matured females unless the protein causes toxicity or infertility in the females.

The delivery system into eggs with the Vtg signal can potentially be applied for the transportation of other valuable substances into eggs. One example is the genome editing tool such as clustered regularly interspaced short palindromic repeats (CRISPR) and its associated endonuclease (Cas) system. Chaverra-Rodriguez et al. (2018) developed a new technology for gene knockout of progenies (F_1) by transporting the Cas9-guide RNA (gRNA) ribonucleoprotein (RNP), injected into the hemolymph of females (G_0), into the eggs of the mosquito *Aedes aegypti*. The delivery was achieved by injecting a fused protein Cas9 and *Drosophila melanogaster* yolk protein 1 (DmYP1) possessing a similar function to Vtg; the DmYP1 is produced in fat bodies, secreted into the hemolymph, and then transported into the eggs (Bownes et al. 2002). To simplify gene modification in a wide range of organisms, it is essential to develop a delivery system of genome editing tools such as the CRISPR/Cas9 system without conventional embryonic microinjection. Another example of delivery of substances with the Vtg signal is an antimicrobial peptide against a broad spectrum of bacteria. Recently, due to human population growth, health trends, and economic development, the demand for aquaculture products is increasing. Supplying high quality fish seeds is a key factor for meeting this demand; however, bacterial pathogens are still significant threats for sustainable intensive aquaculture (Toranzo et al. 2005). Cecropins, which are antimicrobial peptides isolated from the hemolymph of silk moth *Hyalophora cecropia* pupae, have been proven to increase resistance to infection by bacterial pathogens in channel catfish *Ictalurus punctatus* (Dunham et al. 2002; Sarmasik et al. 2002). The Vtg signal-mediated delivery of antimicrobial peptides such as cecropins into eggs is potentially effective for producing specific pathogen-free fish seeds. Furthermore, in the medical and pharmaceutical field, because of the progress in research on peptides for human health, it is possible to synthesize novel artificial peptides with antitumor activities, and discover the potential effects of existing antimicrobial peptides such as defensins (Papo et al. 2004; Suarez-Carmona et al. 2015). We thus anticipate that these functional peptides can be efficiently generated by the strategies described in this study in the future. To expand the application possibilities of the delivery system with the Vtg signal, we are planning to investigate the versatility of the system for these valuable substances and other fish species including commercially cultured fish.

The present study also found an issue relating to the delivery of substances. As shown in Figs. 1c and 2b in this study, we observed GFP fluorescence in the eggs of Tg (*chgH-vtg signal-EGFP*), whereas we could not detect any GFP signal in those of Tg (*chg*H-*vtg signal-LUC-EGFP*). These results show that the transportation efficiency of Tg (*chg*H-*vtg signal-LUC-EGFP*) expressing a fusion protein LUC and EGFP (240 aa) significantly decreased in comparison to that of Tg (*chgH-vtg signal-EGFP*) producing a single protein EGFP (798 aa). This lower efficiency may be attributable to the weakened dimerization caused by fusing LUC to the Vtg signal and EGFP. The circulating Vtg generally exists in the blood as a dimer; thus, Vtg conformation is considered to play an important role in Vtg incorporation into eggs (Reading et al. 2009). In contrast, in Tg (*chg*H-*vtg signal-LUC-EGFP*), the transduced protein fused LUC may lead to a weakened dimerization of the fusion protein, because LUC is a monomer protein unlike the weakly dimeric EGFP (Shaner et al. 2005). The difference in delivery efficiency between these two transgenic lines indicates the need for further study to identify what protein features, including the structure, electrical charge, polar characteristics, and molecular weight, are critical for being taken up into eggs.

In summary, we demonstrated that the Vtg signal was beneficial for accumulating exogenous proteins (EGFP and LUC-fused EGFP) into the eggs in medaka. This finding would offer a platform for other fish species for establishment of a novel bioreactor generating recombinant proteins, production of healthy seed without pathogens, and comprehension of the uptake mechanism of yolk proteins into eggs. Vtg is a highly conserved protein among almost all oviparous organisms including fish, amphibians, reptiles, birds, and insects; thus, the Vtg signal has great potential for applications as described above in various organisms (Sappington and Raikhel 1998; Romano et al. 2004). Further research into the Vtg-mediated transportation system into eggs would lead to technological innovations in the future.

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Author contributions YM designed and carried out the experiment. TH contributed to the detection of the firefly luciferase. MK conceived the study and guided the overall project. YM and MK wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

Human and animal rights All experiments were performed in accordance with the Regulation on Animal Experimentation at Kyoto University (approval number: 26-71).

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