

Original article

# Expressions of dual vitellogenin mRNAs in rainbow trout liver tissue exposed to estradiol-17 $\beta$ and/or insulin-like growth factor I *in vitro*

Haruna Amano<sup>1,\*</sup>, Masahiro Nomura<sup>1</sup>, Ryo Ohgushi<sup>1</sup>, Miho Yokoyama<sup>1</sup>, Kazuya Kawashima<sup>1</sup> and Shunsuke Moriyama<sup>1</sup><sup>1</sup> School of Marine Biosciences, Kitasato University, Sagami-hara, Kanagawa, 252-0373, Japan

\* Correspondence: a-haruna@kitasato-u.ac.jp; Tel.: +81-42-778-7545

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## Introduction

Vitellogenin (Vtg) is a main precursor of egg yolk proteins, which is synthesized in the liver of reproductive female by the stimulation of estradiol-17 $\beta$  (E2). Multiple Vtg proteins and/or transcripts have been identified in teleosts [1]. They are typically classified into two or three subtypes [2]. In our previous study, when multiple Vtgs are induced by E2 in the serum of Sakhaline taimen, *Hucho perryi*, the induced levels of VtgC appeared to be much lower than its physiological levels induced in reproductive females; this suggested that other factors (e.g., growth factors, etc.) might be involved in the natural production of VtgC [3].

Physiological roles of circulating insulin-like growth factor I (IGF-I) seems to be linked to those of E2, perhaps reflecting physiological regulation between reproduction and growth. It regulates E2 synthesis in teleosts including salmonids [4, 5]. In mature female tilapia, *Oreochromis mossambicus*, E2 increased Vtg and decreased IGF-I in the plasma [6], suggesting that a negative effect of E2 on the IGF-I production may reflect the slow growth associated with sexual maturation. However, the role of IGF-I for synthesis of multiple Vtgs is unknown.

Our hypothesis is that treatment of IGF-I in presence or absence of E2 may influence on the profiles of E2-mediated Vtg synthesis; in addition, effects of such treatments may be different depending on Vtg subtypes, maturities, and genders of fish. The objective of this study is to investigate possible effects of IGF-I on the E2-mediated Vtg synthesis in salmonids. Dual Vtg (*vtgAs* and *vtgC*) mRNAs in rainbow trout liver tissue exposed to E2 and/or IGF-I *in vitro* were measured.

## Materials and methods

For total RNA isolation, three male fish reared at Nanae Fresh-Water Station, Hokkaido University, were

injected with E2 at a dose of 2 mg/kg body weight. Injections were performed again following a 2 days interval. Livers were taken 7 days after the first injection. Total RNA from the livers of E2-injected males was prepared using ISOGEN (Nippon-Gene). Poly(A) + RNA was isolated from total RNA by using Oligo(dT)-latex beads (Oligotex dT30, Takara). 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA were synthesized by using SMARTer RACE cDNA Amplification Kit (Clontech). On the basis of the *vtgC* sequence of cutthroat trout, *Oncorhynchus clarki*, (Accession No. JX683452), two primer pairs were synthesized (Table 1) and PCRs were carried out. Then, the PCR product were subcloned into the pTAC-2 vector (BioDynamics Laboratory Inc.) and sequenced. On the basis of these partial sequences, two gene specific primers (GSP) were synthesized (Table 1) and 5'-RACE and 3'-RACE were carried out. A contiguous cDNA encoding a full-length rainbow trout *vtgC* was cloned using pCRII-TOPO vector (Invitrogen) and sequenced (Accession No. LC315692).

**Table 1.** Primers used in the sequence and qRT-PCR

Primer	Sequence
VtgC F1	5'-AGTACAGATATGAGGGAATGGTGAA-3'
VtgC R1	5'-ACGAACCAAATTTAGGGGCCAGGA-3'
VtgC F2	5'-ACTCCGGAGAACCATTCTCCCAA-3'
VtgC R2	5'-TCATATGACTGACACTCAGCTCCCCAT-3'
VtgC GSP F5	5'-AAGCCAGAGGGTTATGAAGT-3'
VtgC GSP R5	5'-CAACCCGTCCTTGGCGTACTCGAA-3'
VtgAs SP5	5'-GAAAGGCTGATGGCGAAAAAC-3'
VtgAs ASP5	5'-GAAGGAAGCACCCAGGAATG-3'
VtgC SP5	5'-ACTGATCGCCTGGGACAAC-3'
VtgC ASP5	5'-TCTCAGTCCGCACCACAC-3'

Amounts of mRNAs for *vtgAs* and *vtgC* were determined by qRT-PCR using standard sense RNAs prepared as follows. The sequence information of rainbow trout VtgAs (Accession No. HQ333133) was obtained from GenBank. Fragments of *vtgC* cDNAs were amplified from liver cDNA. Primer sets were described in Table 1. The amplified cDNAs were ligated into the pTAC-2 vector and sequenced. These recombinant

DNAs were cut with EcoRI. RNA synthesis was performed using MAXIscript SP6 Kit (Ambion). The synthesized cRNAs were quantified and serially diluted in yeast tRNA solution (50 ng/ $\mu$ l). qRT-PCR for dual *vtgs* were developed using KAPA SYBR FAST One Step qRT-PCR Kit (Kapa Biosystems). The reaction was started with reverse transcription at 42°C for 5 min, followed by amplification: 95°C for 10 s (denaturation), and 63°C for 30 s (annealing and extension). In the assays, several doses of standard *vtgAs* and *vtgC* sense RNAs (*vtgAs*:  $1.93 \times 10^3 \sim 1.93 \times 10^7$  copies, *vtgC*:  $1.96 \times 10^3 \sim 1.96 \times 10^7$  copies) and unknown total RNA samples (25 ng) were subjected in duplicate. The amount of mRNAs was calculated as copies per ng total RNA.

For liver tissue culture, mature male and immature female trout were bought from a fish farm. Following dissection of livers from one mature male or one immature female, they were cut into small pieces (3×3 mm) in ice-cold culture medium (MEM, Gibco) containing Kanamycin (60  $\mu$ g/ml). Four liver pieces obtained from each male and female were placed into a well in 24-well culture plate containing 0.5 ml of medium containing Kanamycin (30  $\mu$ g/ml) and pre-incubated for 24 h at 10°C with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Following a medium change, the incubation with E2 ( $10^{-9}$  and  $10^{-8}$  M) and/or IGF-I ( $10^{-8}$  M) was carried out for 24 h under the same conditions mentioned above. Control wells contained no hormones. The total RNA was extracted from liver piece using the RNeasy Mini Kit (Qiagen) with QIAcube (Qiagen). The RNA samples were diluted to 12.5 ng/ $\mu$ l and subjected in qRT-PCR.

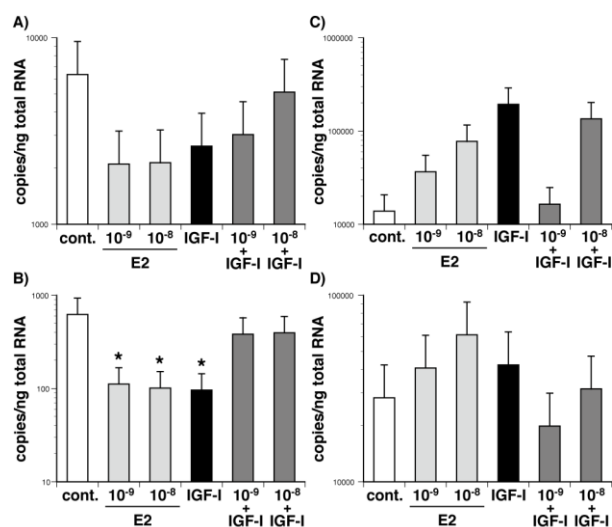
Levels of dual *vtg* mRNAs in each hormone treatment were analyzed for their statistical difference against the control using student *t*-test performed by Microsoft Excel. Differences were considered to be significant at  $P < 0.05$ .

## Results and discussion

A contiguous cDNA encoding full-length rainbow trout *vtgC* was obtained in this study. It (4,128 bp; Accession No. LC315692) contained an open reading frame of 3,847 bp encoding 1,281 amino acid residues including 15 residues of the predicted signal peptide. A BLASTP search confirmed that the rainbow trout *vtgC* clone was an ortholog of teleost *vtgC*, exhibiting the highest identity (99%) with cutthroat trout *vtgC*. Meanwhile, the deduced amino acid sequence of rainbow trout *vtgC* showed extremely low identities with the complete *vtgs* (28% with *vtgAs* of both trouts).

The results of qRT-PCR were shown in Fig. 1. The levels of both *vtg* subtypes in cultured immature female liver tended to be lower in groups of E2 alone or IGF-I alone comparing to the levels in control group, as well as to those in E2 and IGF-I combination group (Fig. 1A,

1B). Especially *vtgC* in single hormone groups were significantly lower than it of control. Meanwhile in cultured mature male liver, both *vtg* subtypes tended to be higher in each single hormone group comparing to the control and combination groups with low E2 dose of  $10^{-9}$  M (Fig. 1C, 1D). These data suggested that transcriptional patterns of trout dual *vtgs* seemed to be synchronous regardless of single or combination of hormone stimulation, while the responses were rather changed depending on gender and/or maturity.



**Fig. 1.** Expression levels of dual *vtgs* genes in cultured liver tissues. (A) *vtgAs* expression in immature female; (B) *vtgC* expression in immature female; (C) *vtgAs* expression in mature male; (D) *vtgC* expression in mature male. \*Significantly different from the control at  $P < 0.05$ .

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