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Research paper

Medaka *igf1* identifies somatic cells and meiotic germ cells of both sexes

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ABSTRACT

Insulin-like growth factors (IGFs) play significant roles in regulating cell proliferation, differentiation and reproduction in diverse organisms. Here, we report the identification of medaka *igf1* and the expression pattern of *igf1* RNA in adult medaka gonads. Using RT-PCR, *igf1* RNA was easily detected in several somatic organs and adult gonads of both sexes. Using chromogenic *in situ* hybridization, *igf1* RNA in the ovary was found in stage I-IV oocytes and in the somatic cells throughout oogenesis. In the testis, *igf1* RNA was present in meiotic products and the somatic cells surrounding male germ cells throughout spermatogenesis. We performed fluorescent *in situ* hybridization and immunostaining for the Vasa protein as a germ cell marker on gonadal cryosections. We showed that *igf1* RNA in the ovary was most abundant in theca cells and meiotic oocytes at stage I-IV, which was detectable in granulosa cells and infrequently occurred in the mature oocytes at stage V but was absent in oogonia. The *igf1* RNA in the testis was most prominent in Leydig cells and Sertoli cells and meiotic spermatids and sperm as well as detectable in spermaotgonia and spermatocytes. We conclude that differential *igf1* RNA expression identifies medaka somatic cells and meiotic germ cells of both sexes.

1. Introduction

The insulin-like growth factor (*igf*) signaling pathway is highly conserved in diverse organisms and plays significant roles in regulating growth and reproduction (Reinecke, 2010; Stubbs et al., 2013). *Igfs* signaling includes ligands, receptors and high-affinity binding proteins. Signaling occurs when *igfs* are combined with their cognate receptors, tyrosine kinases, which then triggers a series of downstream cascades, such as the mitogen-activated protein kinase and phosphoinositide-3-kinase-Akt pathways (Guntur and Rosen, 2013; Zhao and Zheng, 2017). *Igfs* are single chain polypeptides and are highly structurally similar to proinsulin. Mature *igfs* peptides contain four distinct domains (A, B, C and D), which separate them from proinsulin, which only has A, B and C domains. For all known insulin-like peptides, IGFs are synthesized as preprohormones with an N-terminal signal peptide that is targeted for secretion and a C–terminal E domain. The signal peptide and E domain undergo proteolytic processing to produce the mature *igfs* peptides (Wood et al., 2005; Filus and Zdrojewicz, 2015).

Igf1 has been well studied in fish in addition to mammalians. *Igf1* is primarily produced in the liver, which is the primary source of circulating IGFs. The expression of *igf1* was also detected in numerous extrahepatic sites, such as the gonads, in which their functions occur *via* paracrine/autocrine mechanisms (Reinecke, 2010). *Igf1* mRNA or peptides were reported in the ovary and testis of multifarious fish species, including rainbow trout, tilapia and sea bass (Le Gac et al., 1996; Reinecke et al., 1997; Berishvili et al., 2006; Vinas and Piferrer, 2008); red seabream (Kagawa et al., 1995), gilthead seabream (Perrot et al., 2000), tilapia (Schmid et al., 1999; Berishvili et al., 2006) and zebrafish (Li et al., 2015a; Xie et al., 2016). In addition, studies found that *igf1* also has some important physiological functions in catfish (nee Pathak et al., 2015), tilapia (Baroiller et al., 2014) and zebrafish (Morais et al., 2013; Li et al., 2015a; Xie et al., 2016). However, the expression and function of *igf1* varies among different fish species and the precise role of the *igfs* system in the fish gonadal development remains unclear. Much more study on other fish is needed.

Medaka (*Oryzias latipes*) is a model organism to study vertebrate development (Wittbrodt et al., 2002) and embryonic stem cells (Hong et al., 1998; Yi et al., 2009; Hong, 2010). In addition, medaka is also a good model for studying reproductive biology and biotechnologies, it is the first non-mammalian vertebrate for which the male sex-determining

Abbreviations: aa, amino acid residues; *igf*, insulin-like growth factor; ISH, *in situ* hybridization; FISH, fluorescence ISH; nt, nucleotide; PGCs, primordial germ cells; *cyp19a*, P450 aromatase; *egfr*, epidermal growth factor receptor; cAMP, cyclic adenosine monophosphate; *igf1r*, the *igf* type 1 receptor; GH, growth hormone; 11-KT, 11-ketotestosterone

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gene has been identified (Matsuda et al., 2002; Nishimura et al., 2015). Medaka has germline stem cells (Hong et al., 2004; Nakamura et al., 2010; Li et al., 2015b) and many transgenic fish, in which transgenes are specifically expressed in the gonads (Li et al., 2009; Li et al., 2012; Hong et al., 2016). We have investigated a lot of genes whose functions are distinct and varied in regulating germ cells development. For example, knock down of *piwi* or *vasa* in medaka gives rise to defects in primordial germ cells (PGCs) migration (Li et al., 2009; Li et al., 2012); While depletion of *dazl* or *dnd* abolishes PGCs specification (Hong et al., 2016; Li et al., 2016a). Germ cells transplantation for surrogate production by germline replacement is also established in medaka (Li et al., 2016b). Thus, medaka is an interesting model to study the involvement of *igfs* in reproduction.

In this study, *igf1* was cloned and identified from medaka. The structure of the *igf1* was determined, and its expression pattern during embryogenesis and in adult tissues was analyzed by RT-PCR. In addition, *igf1* RNA *in situ* hybridization and immunohistochemistry on gonadal sections was performed by using the germ cell marker, Vasa antibody (αVas). We show that *igf1* RNA is expressed in both somatic cells and meiotic germ cells of medaka gonads.

2. Materials and methods

2.1. Fish

All experiments were conducted in strict accordance with the guidance of the Committee for Laboratory Animal Research at Shanghai Ocean University. Medaka was maintained at 26 °C in 14 h light/10 h dark cycle and embryo developmental stages as previously described by (Iwamatsu, 2004).

2.2. Molecular cloning of medaka igf1

By searching the database (http://www.ensembl.org/index.html), we obtained a cDNA encoding *igf1,* and the *igf1* cDNA contained the complete open reading frame (ORF). To further confirm the cDNA sequence, the ORF was cloned and sequenced. The sequences and primers were highlighted in Fig. 1A. A phylogenetic tree was analyzed based on IGF1 protein sequences by using DNAMAN version 8.0 (Lynnon Biosoft, US) and the MEGA 6 program with the neighbor-joining (NJ) (Tamura et al., 2013). Protein alignment was performed with Vector NTI Advance® 11.5 (Thermo Fisher, US).

2.3. RT-PCR analysis

Total RNA was isolated by using TRIzol (Invitrogen, Carlsbad, CA), and the cDNAs were synthesized by using M-MLV reverse transcriptase (Takara, Shiga, Japan) with an oligo$(dT)_{18}$ primer. The primers were used for *igf1* (ATGTCTAACGCTCATTTCTCTCAA and CTACATTCGGTA ACTTCTGCCG), and *β*-actin (TTCAACAGCCCTGCCATGTA and CCTCC AATCCAGACAGTAT) were used for calibration. PCR was performed in a 25 μl reaction system for 35 cycles, with each cycle consisting of denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s and extension at 72 °C for 1 min. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and documented on a bio-imaging system (Bio−Rad, Hercules, CA, US).

2.4. RNA in situ hybridization and immuno-staining

RNA *in situ* hybridization on sections (SISH) and fluorescent *in situ* hybridization (FISH) were performed as described by (Xu et al., 2009; Li et al., 2011). Briefly, the 558 nt ORF for *igf1* was inserted into the pGEM-T vector and sequenced. The plasmid was linearized for the synthesis of antisense and sense probes from T7 or SP6 promoter by using the digoxigenin (DIG) or FITC RNA Labelling Kit (Roche, Basel, Switzerland). The probes were treated with RNase-free DNase and purified by LiCl precipitation at least 2 h at − 20 °C. RNA was stained with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) on sections (SISH) and FISH was performed by using the (TSA™) Plus Fluorescence Systems according to the product manual (Life Technologies, Carlsbad, CA). Nucleus was stained with 4′-6-diamidino-2-phenylindole (DAPI) and slides were mounted with the Gold anti-fade reagent (Invitrogen, Carlsbad, CA). Immunohistochemistry was performed using anti-Vasa antibody (αVas) as described by (Xu et al., 2007; Yuan et al., 2014; Li et al., 2015b).

2.5. Microscopy

Microscopy was performed as described by (Li et al., 2012). Briefly, micrographs were taken under an upright-microscope with a Nikon Ds-Ri2 camera (Nikon, Tokyo, Japan).

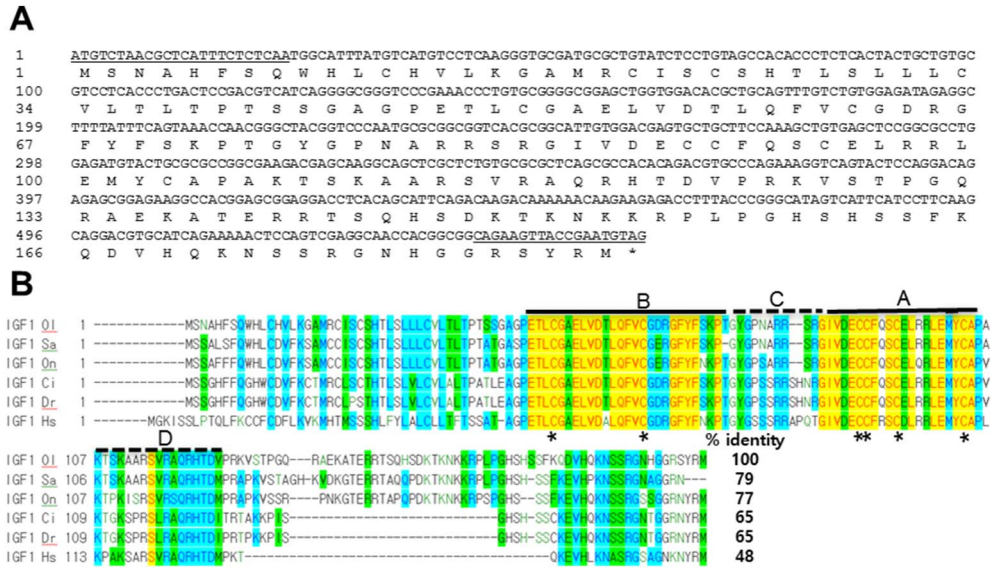

Fig. 1. Sequences of medaka *igf1*. (A) Nucleotide and amino acid sequences of medaka *igf1*.The sequences used for PCR primers are indicated by lines. (B) Alignment of IGF1. Domains A-D of the mature IGF1 are highlighted. Asterisks depict the six conserved cysteine residues within domains A and B. Sequence identitiy values are given at the end of the alignment. Species abbreviations are as follows: Ol, *Oryzias latipes* (medaka); Sa, *Sparus aurata* (seabream); On, *Oreochromis niloticus* (tilapia); Ci, *Ctenopharyngodon idellus* (grass carp); Dr., *Danio rerio* (zebrafish); Hs, *Homo sapiens* (human).