Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES www.jesc.ac.cn

Reference gene validation for quantification of gene expression during final oocyte maturation induced by diethylstilbestrol and di-(2-ethylhexyl)-phthalate in common carp

Yanyan Shi[1], *Jie Lu*[2], *Yilei Wang*[1], *Shuhong Wang*[1,*]

1. *Fisheries College, Jimei University, Xiamen 361021, China*
2. *School of Physics and Mechanical & Electrical Engineering, Xiamen University, Xiamen 361005, China*

ARTICLE INFO

Article history:
Received 19 August 2015
Revised 28 September 2015
Accepted 16 December 2015
Available online 19 March 2016

Keywords:
Cyprinus carpio
Reference gene
Final oocyte maturation
Diethylstilbestrol
Di-(2-ethylhexyl)-phthalate

ABSTRACT

Final oocyte maturation is the key step to successful spawning and fertilization. Quantitative real-time PCR (qPCR) is the technique of election to quantify the abundance of functional genes in such study. Reference gene is essential for correct interpretation of qPCR data. However, an ideal universal reference gene that is stable under all experimental circumstances has not been described. Researchers should validate their reference genes while performing qPCR analysis. The expression of 6 candidate reference genes: *18s rRNA*, *28s rRNA*, *Cathepsin Z*, *Elongation factor 1-α*, *Glyceraldehyde-3-phosphate dehydrogenase* and *β-actin* were investigated during final oocyte maturation induced by different compounds (DES and DEHP) in common carp (*Cyprinus carpio*). Four softwares (Bestkeeper, geNorm, NormFinder and RefFinder) were used to screen the most stable gene in order to evaluate their expression stability. The results revealed that *EF1α* was highly stable expressed when final oocyte maturation was induced by DES, while *gapdh* was the most stable gene when final oocyte maturation was induced by DEHP. Stable expressed reference gene selection is critical for all qPCR analysis to get accurate target gene mRNA expression information.

Introduction

Teleost oocytes, growing within the ovarian follicles, are arrested at the first meiotic prophase after they complete their growth. When environmental conditions such as light, temperature are suitable, the fully grown oocytes become ready for the next process (Devlin and Nagahama, 2002). This process, called final oocyte maturation (FOM), occurs prior to ovulation and is a prerequisite for successful fertilization. The duration of FOM usually lasts very short, generally no more than 24 hr, but it consists of several events including breakdown of the germinal vesicle (GVBD), chromosome condensation, assembly of the meiotic spindle, and formation of the first polar body (Nagahama, 2008). Since 1980s, hormones regulating teleosts FOM have been investigated by a number of researchers, as well as the molecular mechanism of FOM (Goetz, 1983; Kime et al., 1992; Hyttel et al., 1997; Sirard et al., 2007). Several studies also showed that chemicals could impact reproduction through FOM. Tokumoto (2004) investigated the effects of many endocrine-disrupting chemicals (EDCs) by GVBD ratio. It turns out some EDCs may have an influence on fish oocyte maturation like hormone does. Wang et al. (2008) suggests that hypoxia can inhibit FOM. These results indicated that FOM could be a meaningful ecological biomarker to access the effects of environmental stressor on reproduction.

* *Corresponding author*. E-mail: shwang@jmu.edu.cn (Shuhong Wang).

http://dx.doi.org/10.1016/j.jes.2015.12.030

The pituitary glycoprotein hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) act as high-order controls of the gonads in vertebrates. Gonadotropin control of the growth and function of the gonads is mediated by the gonadotropin receptors, FSH receptor and LH receptor (LHR). Genes encoding these receptors are expressed primarily in the accessory cells (follicular and interstitial cells) of the gonads and directly affect gonadal steroidogenesis (Kwok et al., 2005). In the present study, we used LHR which was cloned before (not published) as the target gene of 6 candidate reference genes for describing FOM.

The determination of variation in transcript abundance is an important element in the uncovering of the underlying processes during FOM and quantitative real time PCR (q-PCR) is a technique of choice. To date, qPCR has become an important method for the detection and quantification of nucleic acids in biological samples due to the advantages of its extreme sensitivity, large dynamic range and outstanding accuracy (Cao et al., 2012). However, the accuracy of the results obtained by this method strongly depends on accurate transcript normalization using stably expressed genes, known as reference genes (Gutierrez et al., 2008). Traditionally, most qPCR studies use housekeeping genes including *18s ribosomal RNA* (*18s*) or *actin* as reference genes. Unfortunately, a lot of studies have shown that these widely used housekeeping genes are not always stably expressed in some circumstances (Stürzenbaum and Kille, 2001; Small et al., 2008; Albershardt et al., 2012; Park and Kwak, 2012). Using unstable reference gene to analyze qPCR results could lead to serious deviation. More and more studies suggest that no single reference gene has been discovered in a universal and invariant level (Huggett et al., 2005; Gutierrez et al., 2008; Guénin et al., 2009). Therefore, reference genes must be carefully validated for each experimental situation (Schmittgen and Zakrajsek, 2000).

Recently, reference gene selection softwares like Bestkeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and RefFinder (Xie et al., 2011) appeared subsequently. These softwares can help researchers find suitable reference genes in different experiments. These four softwares also can give the right number of reference genes needed for accurate quantification (Brinkhof et al., 2006). geNorm calculates and compares gene expression stability (M value) and pairwise variation ($Vn/n + 1$) of all candidate gene. Similar to geNorm, NormFinder attempts to identify the optimal reference genes out of a set of reference genes. Bestkeeper assesses the stabilities of candidate reference genes based on the inspection of calculated variation. RefFinder is an online comprehensive software, which is integrated by geNorm, NormFinder, Bestkeeper and delta threshold cycles (*Ct*) method. It gives comprehensive assessment for the results of the four kinds of analysis methods, avoiding the one-sideness of single analysis method, but it only could be a good reference result unless the amplification efficiency of each primer pair was incorporated.

Endocrine-disrupting chemicals (EDCs) are chemicals that can potentially interfere with endocrine systems which regulate growth, development, reproduction and other physiological processes of animals (Park and Kwak, 2012). Diethylstilbestrol (DES), a nonsteroidal xenoestrogen, could trigger oocyte maturation in fish as an EDC. The morphology (the time course of GVBD) and an intracellular molecular event (the de novo synthesis of cyclin B) induced by DES have been confirmed indistinguishable from those induced by a natural maturation-inducing hormone, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) (Tokumoto, 2004). Phthalates are ubiquitous environmental contaminants because of their use in plastics and other common consumer products. Di-(2-ethylhexyl)-phthalate (DEHP) is the most abundant phthalate and it impairs fertility by acting as an endocrine disruptor, and researchers also found out that it can affect oocyte maturation (Ambruosi et al., 2011).

The present study aimed to identify suitable reference genes for robust analysis of molecular changes during FOM induced by DES or DEHP. Six commonly used reference genes including *18s*, *28s ribosomal RNA* (*28s*), *Cathepsin Z* (*CTSZ*), *Elongation factor 1-α* (*EF1α*), *Glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and *β-actin* were selected and their expression stabilities during FOM under different compoundexposures were analyzed by four statistical algorithms, geNorm, NormFinder, Bestkeeper and RefFinder, respectively. The stability analysis results will provide useful guidelines for the optimal reference gene selection and make it possible to obtain more reliable results of mRNA expression levels of target genes during FOM in common carp (*Cyprinus carpio*).

1. Materials and methods

1.1. Sample preparation

Six gravid adult female common carp (body weight, 500–800 g) was collected from a local aquatic market in Xiamen from December to next February. Fish were killed by decapitation. Ovaries mostly composed of post-vitellogenic follicles were removed and dissected manually with fine forceps. Full grown oocytes with follicles of 0.5–0.7 mm diameter were collected and then incubated in Cortland's solution (Stoeckel and Neves, 1992; Sen et al., 2002) (mmol/L: NaCl 160, KCl 2.55, $CaCl_2$ 1.56, $MgSO_4$ 0.93, $NaHCO_3$ 17.85, NaH_2PO_4 2.97, glucose 5.55) containing streptomycin (100 μg/mL) and penicillin (100 IU/mL) adjusted to pH 7.4. These follicles were divided into different groups based upon different compounds exposed: 0.1, 1, and 5 μmol/L Di-(2-ethylhexyl)-phthalate (DEHP, TCI, Tokyo), 0.1, 1 and 2 μmol/L Diethylstilbestrol (DES, Sigma, USA) (Carnevali et al., 2010; Tokumoto, 2004). Follicles incubated in different compounds were collected at 0, 3, 6, 9 and 12 hr after incubation. The rate of GVBD to total oocytes was determined after fixing them in clearing solution of acetic acid–ethanol–formalin (1:6:3, V/V/V). For each collection, pools of ~20 oocytes per individual were ground in liquid nitrogen and stored at −80°C.

1.2. RNA extraction and cDNA synthesis

Total RNA was extracted from oocytes using trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. Potentially contaminating DNA was eliminated by treatment with DNase I digestion. The quality of RNA was assessed on 1.0% agarose gel electrophoresis and the concentration was determined by A260/280. RNA samples used for studies fulfilled the defined criterions. From each sample, 3 μg of total RNA was reverse transcribed using Reverse