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A rapid screening test for endocrine disrupting chemicals using primary cell culture of the marine medaka

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ABSTRACT

While endocrine disrupting chemicals (EDCs) pose a significant threat to wildlife worldwide, their diverse chemical structures present a major challenge to their detection, particularly since they are present at very low concentrations in the environment. We here report the development of an *in vitro* system for rapid screening of EDCs, using primary cell cultures (pituitary, ovarian follicular and testicular cells) of the marine medaka (*Oryzias melastigma*). Pituitary, testis and ovary cell cultures were developed and challenged by environmentally relevant concentrations of three well known EDCs (*viz.* estradiol, 2,2′,4,4′-tetrabromodiphenyl ether, and 4-*n*-nonylphenol) as well as hypoxia (which has been shown to be a potent endocrine disruptor). In general, the mRNA expression levels of gonadotropins, their receptors and steroidogenic enzymes exhibited dose response relationships to the four endocrine disruptors in different tissues. The sensitivity and responses were also comparable to *in vivo* responses of whole fish and *in vitro* responses of the H295R human adrenocortical cell line. Our results suggest that the use of marine medaka primary cultured cells can serve as a cost effective tool for rapid screening of EDCs in the marine environment, and at the same time, sheds light on the underlying mechanisms of EDCs by deciphering their specific target sites along the hypothalamus-pituitary-gonad axis of vertebrates.

1. Introduction

Due to their ubiquitous occurrence and threats to wildlife and human health, endocrine disrupting chemicals (EDCs) have become global environmental concerns in the past decades (Colborn et al., 1993; Diamanti-Kandarakis et al., 2009). However, their highly diversified chemical structures, properties and mechanisms of action present a major challenge to their chemical detection/quantification, especially since many EDCs often occur at very low concentrations in the environment (Bezbaruah and Kalita, 2010; Comerton et al., 2009). Previously, attempts have been made to measure the biological effects of EDCs using whole fish exposure systems (Harris et al., 2001; Scholz and Mayer, 2008). Despite the valuable information provided by such studies, most *in vivo* testing systems require long exposure times, a large number of test animals and a large amount of test samples, making them both labor- and resource-intensive, thus limiting the number of affordable testing (Schirmer, 2006). Furthermore, the *in vivo* feedback mechanisms and interactions between various endocrine organs make it difficult to decipher the target organ and specific effect of EDCs. In view of the above difficulties, numerous reports have called for the development of new alternative testing, and the use of live fish cells (in lieu of whole fish) to identify EDCs in water has been suggested (Lee et al., 2008; Part et al., 2010; Schirmer, 2006).

Although 283 cell lines have been established from finfish thus far (Lakra et al., 2011), the vast majority of these are embryonic and fin cell lines, as embryos and fins have high generative ability. Surprisingly, very few of the piscine cell lines have originated from gonadal and pituitary tissues, despite the fact that most EDCs act on the hypothalamic–pituitary–gonad (HPG) reproductive axis (Rempel and Schlenk, 2008). Indeed, a report on chemical testing of the European REACH legislation showed that reproductive-toxicity testing represents about 90% of projected animal use and 70% of projected costs (Hartung and Rovida, 2009). *In vitro* testing using the H295R derived from the human adrenocortical carcinoma cell has now been widely used to study the effect of EDC on reproduction because in this cell line all the genes controlling the steroidogenic pathway are expressed. However, the fact that this cell line is of human adrenal origin makes it not very relevant

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to the observed effects on the HPG axis of fish or on marine lives (OECD, 2010).

Clearly, there is a compelling need to develop an *in vitro* system for quick and cost-effective screening of EDCs, and to elucidate target sites and toxicological mechanisms on specific reproductive tissues. Despite the obvious importance of determining the effects of EDCs on fish reproduction (Le Gac et al., 2001; Lizardo-Daudt et al., 2008), no *in vitro* test has been established using pituitary or gonads of fish to date. Recently, the marine medaka (*Oryzias melastigma*) has been proposed as a universal model for marine ecotoxicological studies (Kong et al., 2008). Here we report the development of the first marine fish cell culture system, targeted for cost-effective and routine screening of EDCs in the environment, which simultaneously enables us to decipher the effects of EDCs along different target sites along the HPG axis.

Three primary cell cultures, *viz.* the pituitary cells, the ovarian follicular cells and the testicular cells, were developed. Being non-transformed cells, primary cell culture offers a platform to study tissue-specific gene expression. Gonadotropins (luteinizing hormone, LH and follicle-stimulating hormone, FSH) from the pituitary play a crucial role in orchestrating gametogenesis and steroidogenesis in the gonad (Nagahama, 1994). Steroidogenesis is the process by which cholesterol metabolism is catalyzed by a series of steroidogenic enzymes to produce important steroid hormones including sex steroids progesterone (P4), testosterone (T) and estradiol (E2), as well as maturation-inducing hormone (MIH) 17α,20β-dihydroxy-4-pregnen-3-one in teleosts (Nagahama, 1994). The synthesis and activity of steroidogenic enzymes have previously been shown to be highly sensitive to different types of EDCs and both have been used as endpoints to detect EDCs in various studies (Sanderson, 2006). Studying gene expression in both pituitary and gonads would therefore provide detailed information on the target site and toxic mechanisms of the EDC in question, including the impacts on biosynthesis of key reproductive hormones in teleosts.

Three common EDCs, (2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 4-nonylphenol (4-NP) and estradiol (E2)), as well as hypoxia (which has been shown to be a potent endocrine disruptor by Wu et al. (2003)) were used as model EDCs for evaluating the response sensitivity of the three types of primary cell cultures developed in the current study. It has been shown in other cell lines or whole fish exposure studies that these EDCs could influence reproductive success by altering the production of gonadotropins and sex steroids (Harris et al., 2001; Shang and Wu, 2004; Shang et al., 2006; Tompsett et al., 2009; Wu et al., 2003). The sensitivity of the current fish cell cultures to EDCs and their reliability were compared with those reported in previous *in vivo* or cell-based systems.

2. Materials and methods

2.1. Animals and chemicals

All animal research procedures were approved by the Committee of the Use of Live Animals in Teaching and Research of the University of Hong Kong. The stock of marine medaka (*O. melastigma*) used in our experiment was obtained from Interocean Industries (Taiwan) and has been reared in our laboratory for over 10 generations. The fish were maintained in flow-through aquaria (26 ± 1 °C, 30‰, 14L:10D) and fed with hormone-free Aquatox Feed (Zeigler Bros. Inc., Gardners, PA) and live artemia. All the chemicals used in cell culture were obtained from Gibco (Carlsbad, CA) unless otherwise specified. BDE-47 was obtained from Chem Service Inc. (West Chester, PA), 4-*n*-NP and E2 were from Sigma–Aldrich (St. Louis, MO). Stock solutions of 10 mM (BDE-47) and 20 mM (E2 and 4-NP) were first prepared in dimethyl sulfoxide (DMSO) and diluted to the working concentrations immediately before use.

2.2. Pituitary cell culture

The pituitary cell culture of marine medaka was prepared based on the protocol modified from the preparation of zebrafish pituitary culture (Lin and Ge, 2009). The pituitaries were isolated from 60 sexually mature marine medaka (3–6 months old) of mixed sexes and placed in ice-cold Leibovitz's L-15 medium. The pituitaries were washed with phosphate buffer saline (PBS) three times in a 1.5 ml centrifuge tube. Tissue dissociation was performed with 0.3% trypsin, 0.2% collagenase type 1 and 0.005% DNase (w/v) (Worthington, Lakewood, NJ) at 28 °C on the Multi-Therm Shaker (Benchmark Scientific, South Plainfield, NJ) at 800 rpm for 15 min. The pituitaries were gently triturated with a pipette to break up cell aggregates. The dissociated cell suspension was transferred to a new tube and fetal bovine serum (FBS) was added to it to stop the enzymatic reaction. Tissue dissociation and trituration were repeated on the remaining undigested pituitary fragments. The cell suspension was filtered through a 40 μM cell strainer (BD Biosciences, San Jose, CA) and washed two times with Dulbecco's Modified Eagle Medium (DMEM). More than 95% of the cell suspension was viable as assessed by trypan blue staining. About 1×10^5 cells were plated in each well of 96-well plates overnight in phenol red-free DMEM supplemented by 10% FBS, 1× Glutamax, 0.15% Fungizone antimycotic, 100 U/ml penicillin and 100 μg/ml streptomycin at 28 °C in 5% CO_2. The attached cells were then exposed to various EDCs in serum-free medium to prevent decreased bioavailability of exposed chemical due to binding to serum albumin (Hestermann et al., 2000).

2.3. Testicular cell culture

Testicular cell culture was developed based on that established for freshwater medaka (*Oryzias latipes*) (Song and Gutzeit, 2003) with modifications. The testes were isolated from 20 sexually mature male medaka and placed into L-15 medium. The connective tissues were removed as much as possible to minimize the growth of fibroblast. The testes were washed with PBS for five times in 1.5 ml centrifuge tube. Tissue dissociation was performed with 0.25% collagenase type 1 and 0.005% DNase (w/v) at room temperature for 10 min. The testes were gently triturated with a pipette to break up cell aggregates. The dissociated cell suspension was transferred to a new tube and FBS was added to stop the enzymatic reaction. The remaining undigested fragments were further dissociated by 0.01% DNase for 5 min with gentle trituration. The cell suspension was filtered through a 40 μM cell strainer and washed three times with DMEM. More than 90% of the cell suspension was viable as assessed by trypan blue staining. About 1×10^6 cells were plated in each well of a 24-well plate overnight in phenol red-free DMEM supplemented by 10% FBS, 1× Glutamax, 0.15% Fungizone antimycotic, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 28 °C in 5% CO_2. Both the suspended and attached cells were then exposed to various EDCs and hypoxia in serum-free medium.

2.4. Ovarian follicular cell culture

The ovarian follicular cell culture of medaka was prepared according to that established for zebrafish (Pang and Ge, 2002) with some modifications. The ovaries were isolated from 30 sexually mature female medaka, dispersed into individual follicles in L-15 medium and the connective tissues were removed as much as possible to reduce fibroblast growth. The follicles were washed with Medium 199 five times and then cultured in incubation medium (phenol red-free Medium 199 supplemented with 10% FBS, 1× Insulin–Transferrin–Selenium-A, 1× Glutamax, 0.15% Fungizone antimycotic, 100 U/ml penicillin and 100 μg/ml