Pharmacological,toxicological and genetical studies on zebrafish gonad

学 位 論 文 要 約

Summary of Doctoral Thesis

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論文題目：ゼブラフィッシュ生殖巣に関する薬理学的、毒性学的、遺伝学的研究
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論文要約：

Summary：Previously, we examined whether aromatase inhibitor (AI) treatment induces a sex change in adult female zebrafish. A 5-month AI treatment resulted in retraction of the ovaries and testes formation. After changing the diet to an AI-free food, a large number of normal sperm were obtained after eight weeks. Artificial fertilization using sperm from the sex-changed females was successful. These results demonstrated that sex plasticity remains in the mature ovaries of zebrafish. However more than 7 months were necessary and thus paring was unsuccessful.

In this study, we tried to induce sex change by injection of AI to shorten the time during the course of sex change. When AI solution was directly injected into abdomen of zebrafish, retraction of ovary was induced within 2 months. Natural mating of sex changed female with normal female was successful at 3 months. Although fertilization rate was low, juveniles from the mating developed normally. We succeeded to establish the way to induce sex change of adult zebrafish within 3 months. The procedure will support the study to analyze the mechanism how sexual plasticity persists in adult zebrafish following sex differentiation, and identification of undifferentiated stem cells.

Female-to-male sex change is associated with a decrease in estrogen levels (R. K. Bhandari, Komuro, Nakamura, Higa, & Nakamura, 2003). Aromatase inhibitors (AIs) have been developed to reduce estrogen synthesis as pharmaceuticals for the treatment of cancers (Steele, Mellor, Sawyer, Wasvary, & Browne, 1987). Among third-generation AIs, exemestane, an oral steroidal-type aromatase inhibitor, is very effective in the treatment of metastatic breast cancer (Dank, 2002). Exemestane inhibits aromatase with a chemical structure resembling that of the natural substrate androstenedione. Studies have shown that sex change can be induced in many types of fish by aromatase inhibitor (AI) treatment during sex differentiation. In Japanese flounder (*Paralichthys olivaceus*) and tilapia (*Oreochromis niloticus*), brief treatment with an AI during sex differentiation causes a type of sex reversal in which genetic females develop into phenotypically normal males(Kitano, Takamune, Nagahama, & Abe, 2000; Kwon, Haghpanah, Kogson-Hurtado, McAndrew, & Penman, 2000). Furthermore, successful sex change in sex-differentiated fish has been achieved in Medaka, tilapia and zebrafish by long-term treatment with AIs(Paul-Prasanth et al., 2013; Sun et al., 2014; Takatsu et al., 2013).The sex differentiation of zebrafish is known to involve juvenile hermaphroditism. Initially, undifferentiated ovary-like gonads are formed during gonadal development in all juvenile zebrafish, regardless of genotypic sex (Orban, Sreenivasan, & Olsson, 2009). In genotypically male zebrafish, oocytes disappear from the gonad by apoptosis, and spermatocytes develop concomitant with testicular differentiation (Takahashi, 1977; Uchida, Yamashita, Kitano, & Iguchi, 2002). In contrast, oocyte in the female ovaries continue to grow to maturation. In zebrafish, the gonadal masculinization of juvenile genetic females can be induced by the

dietary administration of an AI (fadrozole) (Uchida, Yamashita, Kitano, & Iguchi, 2004).

In a previous study, we examined whether AI (fadrozole) treatment induces a sex change in adult female zebrafish (Takatsu et al., 2013). Our results support the hypothesis that sexual plasticity persists in adult zebrafish following sex differentiation, indicating that undifferentiated stem cells are maintained in adult fish that do not undergo a sex change under natural conditions. Female-to-male sex change in adult fish can be categorized as secondary sex reversal (SSR) (M. H. Li, Sun, & Wang, 2019).It has been proposed that the sex change induced by AI after sex differentiation is a form of SSR rather than primary sex reversal (PSR), in which treatment with an AI is initiated before sex differentiation. In female-to-male SSR, testis formation starts on the ventral side of the ovary. Our previous work in zebrafish showed that newly synthesized testes formed separately on the ventral side of the ovaries after ovarian degeneration (Takatsu et al., 2013). The results suggested that undifferentiated germ stem cells that remained alongside the ovaries developed into testes under these conditions (Akhter et al., 2016).

In this study, we tried to shorten the time period required for sex change by the injection of an AI (exemestane). We succeeded in shortening the total time from 7 to 3 months. Furthermore, we successfully paired sex-changed females with normal females. The method established in this study might provide a good model for purposes such as the analysis of changes in sex behavior or the identification of remaining undifferentiated germ stem cells in adult zebrafish.

Endocrine disrupting chemicals (EDCs) that may interfere with the body's

endocrine system and produce adverse developmental, reproductive, neurological and immuno effect in both human and wildlife. Bisphenol A (BPA) is one of the major EDCs that have been used to manufacture of polycarbonate plastics and epoxy resin worldwide. The effects of bisphenol A (BPA) treatment through dietary administration in male zebrafish on reproductive factors, fertility and hatching rate, were examined. Next-generation effects and further transgenerational effects were also examined.

A long term *in vivo* investigation was done by feeding BPA mixed food to zebrafish, *TG (β-actin:EGFP);roy*. Fish were exposed to six different concentrations of BPA, 10, 0.1, 0.01 mg/g, 1 mg/g, 1ng/g and 1pg/g of food. Reproductive performances were investigated by pairing the experimental male with normal female and showed lower percentage of hatching rate in embryos from males of all the experimental groups after three months exposure. Only low percentages but abnormal hatched embryos were found in juveniles from all the group of BPA treated fish. After long term feeding with BPA increased the infertility in treated fish and finally all fish were found as sterile. Next-generation effects and further transgenerational effects were evident in the concentrations from 1 mg/g or higher. Adverse transgenerational effects on the fecundity of F2 and F3 generation fish that grown by normal food were also observed. These results demonstrated that BPA from food intake cause adverse effects on male reproductive functions and the effects of BPA were transferred to subsequent generations through sperm.

Bisphenol A (BPA) is frequently used in numerous commercial applications and has been produced over eight billion pounds per year worldwide (Ji, Hong, Kho, & Choi, 2013). According to recent estimate, 100 ton of BPA per year may end up in the environment (Santangeli et al., 2016). It is widely used in the manufacture of

polycarbonate plastic and epoxy resins but is also as an additive to other plastics (Tiwari & Vanage, 2013), (Burridge, 2003). Due to its extensive use in daily human life, the accumulation of BPA-containing waste in the environment has been a serious concern and a potential risk to the public and wildlife health (Ramji K Bhandari, Vom Saal, & Tillitt, 2015), (Flint, Markle, Thompson, & Wallace, 2012), (Colborn, Vom Saal, & Soto, 1993). However, the presence of BPA in the environment leads to a chronic exposure of living organisms. It has been considered a highly suspect human endocrine disruptor likely affecting both male and female reproduction system (D. Li et al., 2010). It was first recognized as synthetic estrogen in the year 1930s (Dodds & Lawson, 1938). (Kidd et al., 2007). Provided evidence that estrogens dramatically reduced fish abundance (Kidd et al., 2007). Numerous physiological effects also observed after BPA exposure such as, impairment in the fertility of female, inhibition of seminiferous tubule development (Furuya, Adachi, Kuwahara, Ogawa, & Tsukamoto, 2006),and decrease of daily sperm production and testicular weight (Sakaue et al., 2001). Several experiments demonstrated its toxicants in both *in vivo* and *in vitro* (Welshons et al., 2003). Paris *et al.* demonstrated that it works like other receptor protein 15 and binding with nuclear estrogen receptors (ERs), can alters the expression of gene (Kundakovic & Champagne, 2011), (Wetherill et al., 2007). Its ability to the bind with membrane receptors make it harmful even a lowest concentration. Additionally, lifetime exposure to estrogenic compound may have greater impacts on reproduction than acute exposure (Nash et al., 2004), (Fenske, Maack, Schäfers, & Segner, 2005). It is necessary how lifetime exposure affects population dynamics or whether animal can recover reproductive function following transfer to clean environments.

The aim of the study was to understand the lifetime BPA exposure toxicity and its

underlying mechanisms of action. In the present study we conducted a long term feeding experiment using Zebrafish *TG* (b-actin:EGFP*);roy;* were feed BPA mixed diets from the age of nine weeks (after sex differentiation) to forty months and make in vivo observation. Furthermore, histological examination also performed at the end of the mating experiment investigates gonad structure, induced through BPA exposure. The zebrafish has significant advantage over the species. Zebrafish has gained merits as a model species over the past two decades and is now becoming a popular model for use in toxicology (Maack & Segner, 2004) and pharmaceutical screenings (Kawahara et al., 2011), (Y. Li, Huang, Huang, Du, & Huang, 2012),(Rihel & Schier, 2012),(Tang, Xie, & Feng, 2015) because of its similar cellular and physiological characteristics with higher vertebrates.

Any chemicals that can cause adverse health effects on the progeny of the exposed individuals, periods after exposure, are the source of serious concern (Baker, Peterson, & Heideman, 2014). Though the abnormal health outcomes are, because prior generation exposure rather than direct exposure, these are said to a transgenerational phenotype (Ramji K Bhandari et al., 2015).

In a series of report, transgenerational inheritance of the effects of endocrine disruptors has been demonstrated. The experimentally induced abnormality in juveniles was first described in mouse (Anway, Cupp, Uzumcu, & Skinner, 2005), similar infertility and epigenetic inheritance was observed in laboratory rates induce by a variety of environment toxicants ((Nilsson & Skinner, 2015). In fish a line of evidence of transgenerational effect were reported in many species, including parental exposure of EE2 during reproductive season reduced eggs, embryos and F1 juvenile production in fathead minnows (Schwindt, Winkelman, Keteles, Murphy, & Vajda,

2014), embryonic exposure to BPA or EE2 to medaka caused transgenerational abnormalities and health outcomes at later life stage (Ramji K Bhandari et al., 2015) and more recently deregulation of epigenetic patterns by BPA treatment was observed (Santangeli et al., 2016), although the mechanisms mediating these effects remains an active area of research.

As stated above, a substantial's number of laboratory animal's studies suggested that the plasticizer BPA might act as a reproductive toxicant for humans (Richter et al., 2007). Thus, we should investigate real effect of BPA on organisms. Previously found in our lab that juveniles from BPA treated female phenotypically seemed like males, as oocytes in ovaries did not develop well and filled with pre-vitellogenic oocytes.

In this study we confirmed the preliminary results of next generation's effect and further addressed transgenerational effect of BPA from dietary exposure on zebrafish. It is necessary to understand how chemical exposure affects population dynamics, or weather animals can recover reproductive function after being transferred to clean environments.

My present results showed that transgenerational effect of BPA continue further generation up to F3. The results indicated that some factors or changes in genome inherit through germ cells to next generations.

This study aims to determine the involvement of progestin membrane receptor (mPR) in the non-genomic reaction pathway, which is a novel pathway of steroid hormone. mPR localized on the cell membrane during egg maturation is a molecule that causes a non-genomic response of the egg maturation induction signal.

Our laboratory cloned four subtypes α, β, γ-1, γ-2 of goldfish mPR, among which mPRα, mPRβ functions as a receptor for MIH was suggested by knockdown experiment by antisense morpholino. It was also revealed that the mPR subtype groups are expressed in all tissues including the brain and kidney. The mPR gene is conserved in vertebrates and is drawing attention as a molecule mediating a different pathway from the action of commonly known steroid hormones. That is, it was suggested that nongenomic reaction pathway through a membrane receptor different from the intracellular nuclear receptor-mediated genomic reaction pathway which has been considered as the sole action pathway of steroid hormone exists. However, in addition to the induction of egg maturation as described above, the physiological function of mPR was proved by only antisense, although there was experiment in cultured cells. In recent years, proof of functional inhibition by gene disruption has become required as a definite proof. So, I attempted to prove the function of mPR molecule by reverse genetically by generating mPR gene mutated zebrafish. By the knockdown experiment with antisense morpholino showed that zebrafish mPRα also functions as a receptor for MIH.

In this study, the establishment of a gene knock-out zebrafish strain of paqr5b among seven PAQR (progestin and adipo Q receptors) family members (mPRαs; paqr7a and paqr7b, β; paqr8,ε; paqr9, γ; paqr5a and paqr5b) was advanced by CRISPR/Cas9 method. Zebrafish is a small fish and used as a model organism in a lot of studies. CRISPR/Cas9 method of DNA double strand digesting which induce repair mistake that resulted in missing of several nucleotides. Phenotypic analysis is even possible in F2 homozygous individuals by mating F2 homozygous. I established homozygous mutant fishes of paqr5b. Interestingly paqr5b mutant showed serious phenotype.