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Effects of tamoxifen on the sex determination gene and the activation of sex reversal in the developing gonad of mice

Mingxi Yu[a], Jingyun Wang[b], Wei Liu[a,*], Junwen Qin[c], Quan Zhou[d], Yongan Wang[a], Huihui Huang[c], Wenli Chen[e], Chao Ma[f]

[a] Key Laboratory of Industrial Ecology and Environmental Engineering (MOE), School of Environmental Science and Technology, Dalian University of Technology, Dalian, Liaoning 116024, China
[b] School of Life Science and Biotechnology, Dalian University of Technology, Dalian, Liaoning 116024, China
[c] Department of Developmental and Regenerative Biology, Key Laboratory for Regenerative Medicine (MOE), Jinan University, Guangzhou, Guangdong 510632, China
[d] Medical Imaging Center, First Affiliated Hospital of Jinan University, Guangzhou, Guangdong 510630, China
[e] College of Biophotonics, South China Normal University, Guangzhou, Guangdong 510631, China
[f] College of Life Science, South China Normal University, Guangzhou, Guangdong 510631, China

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ABSTRACT

Tamoxifen, as well as most endocrine-disrupting chemicals, affects the reproductive system and sexual development, but little is known about its disruption of the molecular pathways regulating mammalian sex determination. In fetal mice, the expression levels and pattern of key genes involved in controlling sexually dimorphic balance were analyzed both *in vivo* and *in vitro* by using whole-mount *in situ* hybridization and quantitative-PCR. Developmental tamoxifen exposure induced abnormal up-regulation of the testis differentiation marker *Pdfgra* in Leydig cells and of *Sox9* and *Fgf9* in Sertoli cells in XX gonad. Immunohistochemistry analysis confirmed the over-expression of SOX9 protein. Accordingly, the ovary development marker *Foxl2* was depressed at both the mRNA and protein levels. The increase in testosterone and the reduction in 17β-estradiol and progesterone were observed by using the *in vitro* assay with organotypic cultures. Taken together, results indicated that tamoxifen induced the ectopic expression of well-established sex-specific genes during the critical developmental period, thus resulting in abnormal testicular development in the XX gonad of mammals. This study facilitates a better understanding of the molecular mechanisms of antiestrogens and possibly of compounds that interrupt estrogen signaling by other modes of action, and the association with the pathogenesis of human sexual developmental disorders.

1. Introduction

Tamoxifen (TAM) is one of the most representative antiestrogens applied in endocrine therapy for the treatment of breast cancer, gynecomastia, and oligozoospermia (Fisher et al., 1998; Adamopoulos et al., 2003; Khan et al., 2004). The use of TAM as an anti-cancer treatment by out-patients at home has risen. Moreover, TAM has also been commercially used for sex manipulation in aquaculture (Singh, 2013). Consequently, hospital and pharmaceutical effluents are no longer the main expected entry routes of this compound into the environment. TAM has been detected in sewage treatment plant (STP) effluent or surface water in France and the UK (Ashton et al., 2004; Coetsier et al., 2009; Roberts and Thomas, 2006). The application of digested sludge or effluents from municipal STP on agricultural areas may result in the contamination of soil and ground water, further contaminating drinking water and food.

TAM exposure in humans is suspected to be associated with cell apoptosis, gene mutations and a variety of pathophysiologic alterations, such as deep vein thrombosis, pulmonary embolism, and carcinogenicity (Huang et al., 2009; Kawamura et al., 2013; Shah et al., 2012). In animal experiments for non-mammalian vertebrates, a notable effect of TAM is the genetic transformation of female individuals into males by blocking the effects of estradiol (Iela et al., 1975; Lance and Bogart, 1991; Singh, 2013).

* Corresponding author. Tel.: +86 0411 84709160.
E-mail addresses: yumingxi2003@aliyun.com (M. Yu), wangjingyun67@126.com (J. Wang), liu_wei@dlut.edu.cn (W. Liu), junwen@ims.u-tokyo.ac.jp (J. Qin), tzq@jnu.edu.cn (Q. Zhou), chenwl@scnu.edu.cn (W. Chen), macauhello@126.com (C. Ma).

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However, a complete description of the mode of TAM action on mammalian sex determination and differentiation remains lacking.

The health risk presented by TAM is closely associated with its endocrine-disrupting effects. Endocrine-disrupting chemicals (EDCs) have attracted considerable public concern because they pose a serious risk on the environment and human health (Hotchkiss et al., 2008; Schug et al., 2011). Recent studies indicate that EDCs are capable of interacting with steroid and non-steroid receptors, altering the production of endogenous hormones and even inducing intersex (Cao et al., 2013; Ji et al., 2013; Lange et al., 2009; Zhao and Hu, 2012). In non-mammalian vertebrates, the sexual development process is vulnerable to EDC exposure and other environmental factors, whereas the sex determination mechanism for mammals is genetically controlled (Fernandez et al., 2007). However, EDCs might also directly affect genes, which are less well known as one of the mechanisms (Schug et al., 2011). Hence, the potential toxic effects on sexual development in mammals are of significance for a better understanding of the mechanism and health risk caused by TAM and other EDCs.

Recent advances in the understanding of the molecular sex determining pathway in mammals (MacLaughlin and Donahoe, 2004), prompted the investigation of the interaction between EDCs and the genes involved in the mammalian sexual development. Normal testicular development in fetus depends on the differentiation and development of Leydig and Sertoli cells, which are necessary for the subsequent development of reproductive tract (Lejeune et al., 1998; Magre and Jost, 1991). Platelet-derived growth factor alpha (*Pdgfrα*) signaling is essential for Leydig cell differentiation and testis cord formation (Brennan et al., 2003). Moreover, as the main Sertoli cell marker, *Sry*-related high-mobility group box 9 (*Sox9*) is proved to be critical for directing the testicular architecture and initiating a series of male-specific molecular pathways, including activating anti-Mullerian hormone (*Amh*) expression (Carmona et al., 2009). Other key testicular determination genes include *Fgf9* and *Jag1*, become male-specific after *Sry* is expressed (Kim et al., 2006; Jeays-Ward et al., 2003). Forkhead transcription factor gene (*Foxl2*) is a key ovarian development gene that is up-regulated in the somatic cells of the developing gonad and shares an antagonistic relationship with *Sox9* (Uhlenhaut and Treier, 2006). The disruption of each gene can affect gonadal differentiation and ultimately lead to developmental disorders.

This study aims to evaluate the effects of TAM on the expression pattern of the key genes controlling sexually dimorphic balance during the critical stages of gonadal development in mice. Considering that the target genes and proteins localized in somatic cells have a wider function in the development of the genitourinary system aside from the sex determination, we evaluated multiple factors including testicular markers *Pdgfrα*, *Sox9*, *Amh*, *Fgf9*, and *Jag1*, as well as female-specific *Foxl2*. Moreover, two complementary experimental systems, *in vivo* and *in vitro*, were conducted. Protein amount and hormone production were also analyzed. The employment of whole-mount *in situ* hybridization (WISH) facilitates the three-dimensional overview of gene expression pattern and offers particular advantages for the mouse embryo. The present study provides multiple lines of evidence, as well as the molecular mechanism, of the activation of the fetal female-to-male pathway in mammals induced by TAM.

2. Materials and methods

2.1. Animals

ICR mice were acclimatized to the laboratory for one week and maintained in a humidity (30–40%) and temperature (24 °C)-controlled room on a 12 h light, 12 h dark cycle. The animals were given access to food and water *ad libitum*. Males were caged with females and the morning of vaginal plug identification was designated as 0.5 d post coitum (dpc).

2.2. In vivo TAM treatments

The first 13 d of mouse gestation is a sensitive period for ovary development, due to the proliferation and apoptosis of germ cells. Previous studies suggested that 1.0 mg/kg·bw TAM is the highest dose tolerated without lethality in embryo stage of mice (Danielian et al., 1998). Therefore, for *in vivo* studies, 1.0 mg/kg body weight (bw) TAM (Sigma, St Louis, MO, USA) or corn oil (served as control group) was injected intraperitoneally into pregnant mice at 10.5 dpc. At 13.5 dpc, the pups were dissected, and the gonads were collected. The mRNA levels of female and male-specific molecular markers in gonads were measured by whole-mound *in situ* hybridization (WISH) and real-time quantitative PCR (Q-PCR). The levels of protein were measured by immunohistochemistry.

2.3. Whole-mount in situ hybridization

WISH was performed to analyze *Pdgfrα* expression. Gonads harvested from *in vivo* TAM exposure were processed following the protocol described by Rosen and Beddington (1993). Briefly, *Pdgfrα* cDNA template for RNA probe synthesis was generated by PCR, cloned into pGEM-T easy vector, and then confirmed by sequencing. The mouse *Pdgfrα* cDNA clone plasmid was linearized with *Sal*I to serve as template for T7 RNA polymerase (Promega, Madison, WI) to give rise to a *Pdgfrα* antisense RNA probe. To obtain the sense RNA probe, the plasmid was digested with *Sph*I and transcribed with Sp6 RNA polymerase (Promega). Sense and antisense *Pdgfrα* probes were labeled with alkaline phosphatase-conjugated digoxigenin. NBT/BCIP was used as a substrate of alkaline phosphatase for color development. The probe was purified on a Quickspin G50 column and stored at −80 °C. Samples were examined using a microscope (CX31, Olympus, Japan), and images were captured by a Canon A640 camera.

2.4. Immunohistochemistry

Protein levels of the key sex-specific factor, FOXL2, AMH, and SOX9 in the gonads after exposure to TAM were evaluated. Immunohistochemistry was performed on six replicates of XX or XY gonad samples from both the control and TAM treatment groups. After dissection, gonads were fixed in 4% paraformaldehyde overnight at 4 °C. After washing with PBS several times, gonads were dehydrated and rehydrated *via* a series of alcohol treatments and then embedded with paraffin under vacuum. Sections of 7 μm were cut on an RM2155 rotary microtome (Leica, Nusslock, Germany) and stained with hematoxylin and eosin. Five random sections of each gonad sample were examined. The antibodies used in immunohistochemistry were: rabbit anti-SOX9 (1:1000, Pharmingen, San Diego, CA, USA), goat anti-AMH (1:500, Pharmingen), and rabbit anti-FOXL2 (1:500, Abcam, Cambridge, MA, USA). The primary antibody was detected by incubation with an appropriate secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) followed by incubation with avidin–biotin peroxidase complex (1:50 in PBS, Vector Laboratories). Stained slides were examined with a microscope (CX31, Olympus), and images were captured by a Canon A640 camera equipped with an ISIS imaging system.