論文の内容の要旨

論文題目　**Investigation of Biomarkers for the Assessment of Human Health Effects of Bisphenol A Exposure by using Human Cells derived from Congenital Male Genital Abnormalities and Breast Cancer**

（先天性生殖器異常男児由来細胞と乳癌細胞を用いたビスフェノール A 曝露のヒト健康影響評価のためのバイオマーカーの探索）

氏　　名　　秦　咸陽

1. Background

Bisphenol A (BPA) is one of the world's highest production-volume chemicals and used extensively in the plastics produced for food and beverage containers. Recently, exposure to BPA at concentrations detected in humans has been related with human reproductive health and endocrine-related cancers. However, most of this is still controversial due to the unclear underlying mechanism. The aim of this study is to investigate the *in vitro* molecular basis of the effect of low-dose BPA exposure on human health. In this PhD thesis, there are two major targets. The first one is to investigate the potential effects of BPA exposure on the development of congenital male genital abnormalities (CMGAs). In this part, the individual variation of the genetic response to low-dose BPA is specially focused. The second one is to investigate the potential effects of BPA exposure on the development and prognosis of breast cancer. In this part, the *in vitro* molecular mechanism and long-lasting effects of BPA exposure in human cell lines is specially focused.

2. Effect of BPA exposure on the development of CMGAs

During the early stages of development (embryonic, fetal and infant), humans are highly vulnerable to environmental hazards. It has been proposed that *in utero* exposure to EEDs could adversely affect fetal growth and induce several types of CMGAs, such as cryptorchidism (CO) and hypospadias (HS), which are the most

common two types of CMGAs in boys with a global prevalence of approximately 2-9% and 0.2-1%, respectively.

2.1 Association of variants in genes involved in EEDs metabolism and risk of CO and HS

The effect of EEDs would depend on several factors, including the dosage of EED exposure, the developmental stage in which EED exposure occurred, and inter-individual variability in genetic susceptibility to the effects of EED exposure. We hypothesized that SNPs of genes involved in EEDs metabolism might influence the risk of male genital malformations. In this study, we explored for association between 384 SNPs in 15 genes (AHR, AHRR, ARNT, ARNT2, NR1I2, RXRA, RXRB, RXRG, CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP3A4, CYP17A1and CYP19A1) and risk of CO and HS in 334 Japanese males (141 controls, 95 CO and 98 HS). Five SNPs from ARNT2 (rs2278705 and rs5000770), CYP1A2 (rs2069521), CYP17A1 (rs4919686) and NR1I2 (rs2472680) were significantly associated at both allelic and genotypic levels with risk of at least one genital malformation phenotype. These findings indicate that genetic polymorphisms in genes involved in EED metabolism are associated with risk of CO and HS.

2.2 Identification of novel BPA targets in human foreskin fibroblast cells (hFFCs) derived from HS patients

To better understand the molecular basis of the effect of BPA on human reproductive health, a genome-wide screen was performed using hFFCs derived from child HS patients to identify novel targets of low-dose BPA exposure. Gene expression profiles of hFFCs were measured after exposure to 10 nM BPA, 0.01 nM 17b-estradiol (E2) or 1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 24 h. Differentially expressed genes were identified using an unpaired Student's *t* test with *P* value cut off at 0.05 and fold change of more than 1.2. These genes were selected for network generation and pathway analysis using Ingenuity Pathways Analysis, Pathway Express and KegArray. Seventy-one genes (42 downregulated and 29 upregulated) were identified as significantly differentially expressed in response to BPA, among which 43 genes were found to be affected exclusively by BPA compared with E2 and TCDD. Of particular interest, real-time PCR analysis revealed that the expression of matrix metallopeptidase 11 (MMP11), a well-known effector of development and normal physiology, was found to be inhibited by BPA (0.47-fold and 0.37-fold at 10 nM and 100 nM, respectively). Study of hFFCs derived from HS and CO patients (n = 23 and 11, respectively) indicated that MMP11 expression was significantly lower in the HS group than in the CO group (0.25-fold, *P* = 0.0027). These findings suggest an involvement of BPA in the etiology of HS might be associated with the downregulation of MMP11.

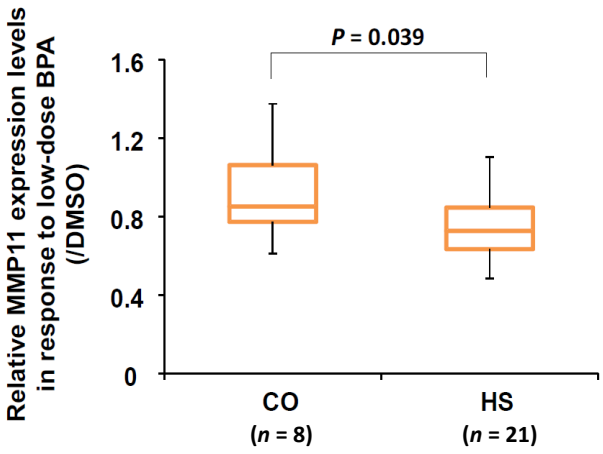

Figure 1. Difference in the genetic response to low-dose BPA in hFFCs derived from child HS and CO patients.

2.3 Individual variation of the genetic response to BPA in hFFCs derived from CO and HS patients

We hypothesized that polymorphic differences among individuals might cause variations in the effect that EEDs have on CMGAs. Then, individual variation in the genetic response to low-dose BPA was investigated in hFFCs derived from child CO and HS patients (n = 8 and 21, respectively). BPA exposure (10 nM) was found to inhibit MMP11 expression in the HS group but not in the CO group (0.74-fold, *P* = 0.0034 and 0.94-fold, *P* =

0.70, respectively) (Figure 1). Significantly lower levels of MMP11 expression were observed in the HS group compared with the CO group in response to 10 nM BPA (0.79-fold, P = 0.039). The effect of SNP rs5000770 (G>A), located within the ARNT2 locus, on individual sensitivity to low-dose BPA was investigated in the HS group. A significant difference in neurotensin receptor 1 (NTSR1) expression in response to 10 nM BPA was observed between AA and AG/GG groups (n = 6 and 15, respectively. P = 0.031) (Figure 2). These findings advance our understanding of the specificity of low-dose BPA effects on human reproductive health. Our results suggest that genetic variability among individuals affects susceptibility to the effects of EEDs exposure as a potential cause of HS.

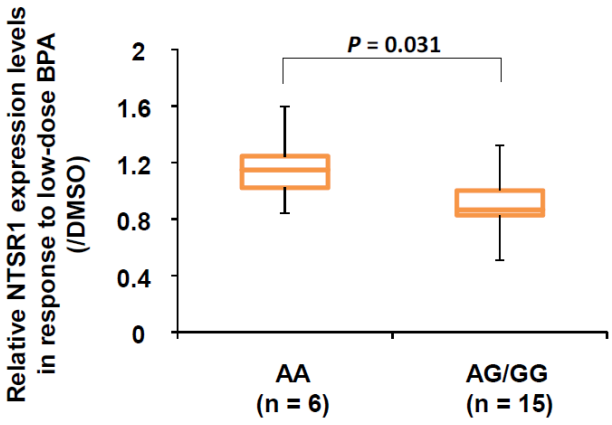

Figure 2. Effect of SNP rs5000770 genotype on the genetic response to low-dose BPA.

3. Effect of BPA exposure on the development and prognosis of breast cancer

Breast cancer is the most common invasive cancer in women (22.9% of all cancers and 13.7% of cancer deaths in women), the trend of which has been attributed to multiple factors including increased exposure to EEDs. Epidemiology studies have highlighted the correlation between BPA exposure and human cancers. Animal study has demonstrated that low doses of BPA *in utero* alter the developing mammary gland and that these subtle changes increase the risk of cancer in the adult. Furthermore, it is reported that BPA can affect the gene signaling related with the tumor aggressiveness and prognosis and increase resistance to anti-cancer treatment in cells derived from breast cancer patients.

3.1 Long-lasting effect of BPA exposure on the proliferation of normal human mammary epithelial cells (HMEC)

The carcinogenic activity of BPA is responsible for stimulating growth in estrogen-dependent breast cancer tissues, cell lines and rodent studies. However, it is not fully understood how this compound promotes mammary carcinogenesis. In this part, I examined the effect of BPA on cellular proliferation and senescence in HMEC. Exposure to BPA for 1 week at the early stage at passage 8 increased the proliferation and sphere size of HMEC at the later stage up to passage 16, suggesting that BPA has the capability to modulate cell growth in breast epithelial cells (Figure 3). Interestingly, the number of human heterochromatin protein-1γ positive cells, which is a marker of senescence, was also increased among BPA-treated cells. Consistent with these findings, the protein levels of both p16 and cyclin E, which are known to induce cellular senescence and promote proliferation, respectively, were increased in BPA-exposed HMEC.

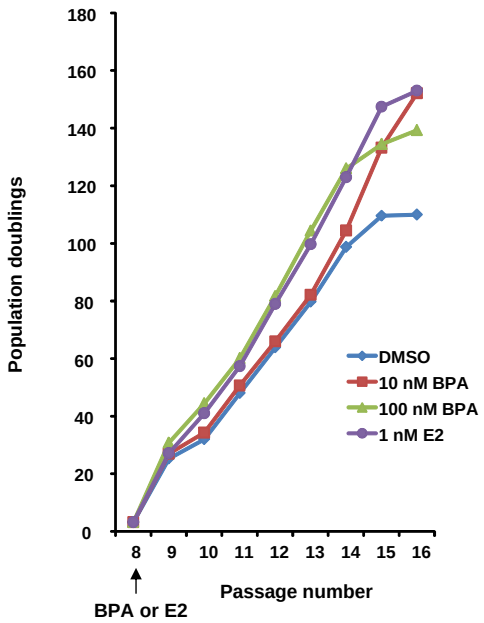

Figure 3. Effects of BPA exposure on the proliferation of HMEC

Furthermore, DNA methylation levels of genes related to development of most or all tumor types, such as BRCA1, CCNA1, CDKN2A (p16), THBS1, TNFRSF10C and TNFRSF10D, were increased in BPA-exposed HMEC. These findings in the HMEC model suggest that the genetic and epigenetic alterations by BPA might damage HMEC function and result in complex activities related to cell proliferation and senescence, playing a role in mammary carcinogenesis.

3.2 Inhibitory effect of BPA exposure on ARNT2 expression in human breast cancer cells MCF7

ARNT2 is believed to play important roles in a variety of physiological processes, including estrogen signaling pathways, which may be involved in the pathogenesis and therapeutic responses of endocrine-related cancers. Recent human studies found that the mRNA expression level of ARNT2 was positively associated with the prognosis of breast cancer. In this part, I investigated whether ARNT2 expression is regulated by BPA in human cancer cell lines. BPA was found to be estrogenic toward BG1Luc4E2 cells by an E-CALUX bioassay. ARNT2 expression was downregulated by BPA in a dose-dependent manner in ESR1-positive MCF7 and BG1Luc4E2 cells, but not in estrogen receptor-negative LNCaP cells. The reduction in ARNT2 expression in cells treated with the xenoestrogens was fully recovered by the addition of a specific ESR1 antagonist, MPP (Figure 4). Furthermore, I used small interfering RNA techniques to knockdown ARNT2 expression in MCF7 human breast cancer cells, and found that an almost 40% downregulation of ARNT2 mRNA expression increased the expression of sensitive to apoptosis gene (3.36-fold), and decreased the expression of von Hippel-Lindau (0.27-fold) and MMP1 (0.35-fold). The metabolite analysis revealed the contents of glucose, glycine, betaine, phosphocholine, pyruvate and lactate involved in the HIF-1-dependent glycolytic pathway were significantly lower in cells treated with siARNT2. These findings show for the first time that ARNT2 expression is modulated by BPA by an ESR1-dependent mechanism in MCF7 breast cancer cells and ARNT2 might play an important role in the modulation of HIF-1-regulated signaling and metabolism.

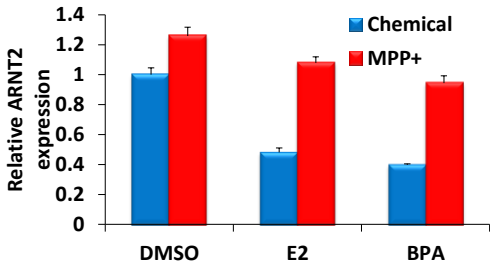

Figure 4. ARNT2 mRNA expression decrease mediated by BPA is increased by MPP in MCF7

4. Conclusion

In this study, I investigated the underlying molecular basis of the effect of BPA exposure on the development of CMGAs and breast cancer in the *in vitro* system. I found that children with different phenotype/genotype might have different genetic response to BPA exposure at concentration detected in humans suggesting that individual variability exist in genetic susceptibility to the low-dose effects of BPA exposure. It can partly explain the controversy in the epidemiological studies regarding the correlation between BPA exposure and human health, which usually ignore the effect of individual variability on the assessment of adverse health effects from environmental hazards. I also found a long-lasting effect of low-dose BPA exposure on the proliferation of HMEC over multiple cellular passages and an inhibitory effect of BPA exposure on the mRNA and protein expression of ARNT2 in human breast cancer cell line MCF7. These findings provide new evidence in human cells that prenatal and/or chronic exposure to BPA in people's daily life might affect human health via genetic and epigenetic dysregulation of cell cycle regulatory genes/tumor suppressor genes, without any significant symptoms, until the accumulated effects possibly lead to tumorigenesis in later life.