Review

Molecular mechanism of endocrine-disruptive effects induced by Bisphenol A: The role of transmembrane G-protein estrogen receptor 1 and integrin αvβ3

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ARTICLE INFO

ABSTRACT

Bisphenol A (BPA) is one of the highest volume industrial products worldwide and has been widely used to make various products as the intermediates of polycarbonate plastics and epoxy resins. Inevitably, general population has been widely exposed to BPA due to extensive use of BPA-containing products. BPA has similar chemical structure with the natural estrogen and has been shown to induce a variety of estrogen-like endocrine effects on organism *in vivo* or *in vitro*. High doses of BPA tend to act as antagonist of estrogen receptors (ERs) by directly regulating the genomic transcription. However, BPA at environmentally relevant low-dose always disrupt the biological function *via* a non-genomic manner mediated by membrane receptors, rather than ERs. Although some studies had investigated the non-genomic effects of low-dose BPA, the exact molecular mechanism still remains unclear. Recently, we found that membrane G protein-coupled estrogen receptor 1 and integrin αvβ3 and its relative signal pathways participate in the induction of male germ cell proliferation and thyroid transcription disruption by the low-dose BPA. A profound understanding for the mechanism of action of the environmentally relevant BPA exposure not only contributes to objectively evaluate and predict the potential influence to human health, but also provides theoretical basis and methodological support for assessing health effects trigged by other estrogen-like environmental endocrine disruptors. Based mainly on our recent findings, this review outlines the research progress of molecular mechanism on endocrine disrupting effects of environmental low-dose BPA, existing problems and some consideration for future studies.

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Introduction

In recent years, people pay more and more attention to the potential hazards of environmental estrogens to human health. Environmental estrogens, also called xenostrogens due to their similar chemical structures with the physiological estrogens, belong to typical environmental endocrine disruptors (Daston et al., 1997). Through simulating or antagonizing physiological and biochemical effects of endogenous estrogens, environmental estrogens disrupt hormone receptors or interfere the generation of endogenous estrogens and subsequently affect the normal functions of endocrine and reproductive systems resulting in the reproductive and developmental disease and malignant tumor (Stancel et al., 1995). Representative environmental estrogens include Bisphenol A (BPA), polychlorinated biphenyls (PCBs), and phthalates (Morgan et al., 2017). Recently, the potential influence of BPA on human health at environmentally relevant low-dose has attracted much concern (Rochester, 2013; ANSES, 2013, 2017, 2018; ECHA, 2015; EFSA, 2015; Heindel et al., 2015) Thus, we have carried out a series of work in this area, here we will summarize our recent findings and further review the current progress of the molecular mechanism for human health effects exposed to environmentally low concentration of BPA, existing problems and some thoughts toward future studies.

1. Wide application and environmental exposure of BPA

BPA is known as 2,2-bis(4-hydroxyphenyl) propane, a phenol derivative composed of a rigid planar aromatic ring and a flexible nonlinear aliphatic side chain. BPA is mainly used as intermediate for the production of polycarbonate plastic, epoxy resin and other polymer materials, which has been widely used in food and beverage packaging materials (such as bottles) and liner, digital media (such as CDs and DVDs), automobile, electronic devices, sports safety equipment and medical instruments (such as dental sealants) (Jiao et al., 2008). In addition, a small amount of BPA is also used in the manufacture of phenolic resins, unsaturated polyester resins, thermal paper additive, flame retardants (such as tetrabromobisphenol A), heat and carbonless paper coating (ANSES, 2013; ECHA, 2015; EFSA, 2015). The extensive use of BPA in different products has increased its global demand by 6%–10% per year, especially in countries with rapid economic growth, such as China (Jiao et al., 2008; Huang et al., 2012; Yu and Jia, 2008).

The BPA polymers connected by ester bonds are easy to hydrolysis under heat, acidic or basic conditions, which leads to the potentially extensive exposure of general population

(ANSES, 2013; EFSA, 2015; Ndaw et al., 2016). According to the investigation of Centers for Disease Control and Prevention in the United States, BPA was detected in 92.6% of urine samples from 2500 in the U.S. general population and the level of BPA were significantly higher in children than in adults (4.5 ng/mL *vs.* 2.5 ng/mL) (Calafat et al., 2008). In addition, the detection rate of urinary BPA was as high as 91%–99% in the general population of North America and many Asian countries (Calafat et al., 2008; Zhang et al., 2011). Notably, recent studies have shown that BPA is also present in the blood, saliva, breast tissue, breast milk, amniotic fluid, neonatal blood, placenta, arterial blood (ANSES, 2013; Vandenberg et al., 2007, 2010). Although there are few studies on human and environmental exposure monitoring to BPA in China, it has been foreseen that the environmental BPA exposure is universal due to extensive use of BPA in China (Huang et al., 2012). Indeed, BPA was detected in some areas, rivers, surface waters and general population in different extent in China (Huang et al., 2012). Many lines of epidemiological evidences have shown that workers occupationally exposed have higher urinary BPA concentrations, likely associated with the thyroid hormone disruption, male infertility, and alterations of female reproductive hormone levels in China (Liu et al., 2015; Miao et al., 2014, 2015; Wang et al., 2012).

2. Potential influence of BPA on human health

A large number of *in vivo* and *in vitro* studies have indicated that BPA has adverse effects on the reproductive function, mammary gland development, cognitive function and metabolism (ANSES, 2018; Beausoleil et al., 2018). BPA has been shown to have a negative effect on the estrous cycle, including irregular and prolonged cycles and changes of estrous cycles dynamic in rats and mice (ANSES, 2018). The data from both rodents *in vivo* and rodent/human cells *in vitro* demonstrated that BPA affects the ovarian estrogen production (Lee et al., 2013; Kwintkiewicz et al., 2010; Mansur et al., 2016). So, it is speculated that BPA is likely to affect the ovarian cycle in humans by disrupting the ovarian follicle activity since the estrogens have the similar functions in rodents and humans (ANSES, 2018). Kurian et al. (2015) have recently shown that BPA can influence the release of hypothalamic neuropeptide kisspetin and pulsatility of gonadotrophin-releasing hormone (GnRH), resulting in the alteration of hypothalamic neuroendocrine function. The kisspetin is also shown to play a role in the HPG axis control in human. Thus, it is believed that BPA have a possibly potential effect on the hypothalamic kisspetin/GnRH system and estrous cyclicity in human (ANSES, 2018). There is emerging evidences that BPA can alter the epithelium-stroma interactions and increase the terminal end

buds number, lateral branching, ductal and intraductal hyperplasia in female mammary gland after prenatal or/and postnatal exposure, resulting in an increased susceptibility to chemical carcinogens (ANSES, 2013, 2014; Soto et al., 2013). Based on the fact of the similar structure and common hormonal regulation to cell proliferation between humans and rodents, it is assumed that early-life exposures of BPA may likewise induce the abnormal development of mammary gland to increase the susceptibility to breast cancer in humans. BPA is also implicated in the impairment of learning and memory performance and alteration of histological structures in a sex-dependent manner in rodents. In non-human primates experiments, BPA has been shown to have adverse effects on the midbrain dopaminergic system and spine synapses of the hippocampus after prenatal exposure (Elsworth et al., 2013), and a significant cognitive impairment when exposed in adult (Elsworth et al., 2015). In addition, many lines of evidences indicate that BPA is also able to affect the behavior and spine density of rodents (Xu et al., 2015; Inagaki et al., 2012) and the synaptogenesis induced by estradiol in the hippocampus and prefrontal cortex in non-human primates (Leranth et al., 2008). Therefore, it is believed that impairment of BPA to learning and memory in rodents possibly occurs in humans due to their similarities in the regulated effects of sex steroids on cognition. BPA is also found to induce metabolic disturbances including diabetogenic and obesogenic effects following various exposure (ANSES, 2014).There is *in vivo* evidences that BPA is able to affect the insulin synthesis and/or release of β-pancreatic cells, or insulin related signaling in insulin-sensitive organs, increasing the incidence of type-2 diabetes (ANSES, 2018). Many *in vitro* studies also demonstrate that BPA have detrimental effects on adipocyte differentiation and function by modifying the leptin or adiponectin (ANSES, 2018). In addition, prenatal exposure to BPA has altered the glucose homeostasis in dams, leading to the severe glucose intolerance and insulin resistance, hyperinsulinemia and hyperleptinemia. The effect of BPA on metabolism in rodents is believed to be relevant for human due to similar homeostatic regulation of insulin production and sensitivity between rodents and human (ANSES, 2018).

The estrogen-like endocrine disrupting effects of BPA in both *in vivo* and *in vitro* studies have been well established (Wetherill et al., 2007; Richter et al., 2007; ANSES, 2018). However, the internal exposure of BPA is very low in the real environment (Vandenberg et al., 2010). Ikezuki et al. (2002) reported that the level of unconjugated BPA in serum and urine was only between 0.1 and 10 ppb. So, does the low concentration of BPA actually pose a potential hazard to human health? In recent years, more and more studies have found that BPA have low dose effects on experimental animals, that is, the toxic effects such as nerve and reproductive endocrine disrupting effects induced by BPA are obvious at a low concentration, but decreases or even completely disappears at high concentration (Wetherill et al., 2007; Richter et al., 2007). However, there are still debates about the health and safety of low-dose BPA since the biological effects of low-dose BPA are difficult to replicate in human; the extent and action scope of BPA on organisms vary with species, exposure doses and patterns, and present epidemiological evidence are insufficient to show that low-dose BPA exposure is directly associated with certain diseases (Wetherill et al., 2007; Richter et al., 2007; Myers et al., 2009; Rochester, 2013). In view of the importance of BPA in industrial production and its potential harm to human health at low doses of exposure, it is urgent to perform a deeper and more comprehensive evaluation of the potential health hazards and its molecular mechanism under low-dose BPA exposure (Borrell, 2010; Brucker-Davis et al., 2001). On the one hand, a profound understanding for molecular mechanisms not only contributes to the prediction of toxic effects *in vivo*, but also can perform the comparison of biological effects (such as critical signaling pathways) between different species, which will make the assessment and prediction of the potential impact of BPA on human health more objective and the prevention and intervention more targeted. On the other hand, BPA is a typical estrogen-like endocrine disruptors, in-depth study on molecular mechanism for its low dose effects will also provide theoretical basis and technical support for evaluating the health effects of other similar estrogen-like endocrine disruptors. More and more researchers have been paying close attention to the low dose effect of BPA in recent years, published a series of high-quality papers on the low-dose mechanism of BPA and performed a more in-depth discussion about low-dose BPA endocrine disruption mechanism (Bouskine et al., 2009; Prins et al., 2017; Lejonklou et al., 2017; Vom Saal and Hughes, 2005; Cabaton et al., 2011; Sheng et al., 2011; Jenkins et al., 2011; Pfeifer et al., 2015).

3. Non-genomic regulation of environmentally relevant low-dose BPA

In vivo and *in vitro* studies have shown that the majority of estrogen-like compounds at low concentrations have weak binding ability to estrogen receptors (ERs), up to 1000–10,000 times lower for BPA than that of estradiol (Bonefeld-Jørgensen et al., 2001). However, at the same concentrations, these compounds cause biological effects on humans and animals (Wetherill et al., 2007; Brucker-Davis et al., 2001; Vandenberg et al., 2007). Environmental estrogens are generally considered to exert endocrine disrupting effects by competing with estrogens to bind ERs. Therefore, it is speculated that another mechanism may be involved in the endocrine disrupting effects of low-dose estrogen-like compounds when low-dose estrogen-like compounds have weak ability to bind to ERs. In recent years, many studies have shown that estrogens not only regulate gene expression *via* ERs, but also promote cell proliferation and apoptosis through rapid activation of ERs-independent signaling pathways mediated by membrane receptors in a non-genomic way (Watson et al., 2010; Kelly and Levin, 2001; Vasudevan and Pfaff, 2007). Because non-genomic regulation is mainly through the receptor-mediated signaling pathway or network to control important cellular events but not involving gene transcription or protein expression, so it has the advantages of fast biological response, precise regulation and is essential for exerting many important cellular functions. Based on the mechanism of this non-genomic regulation of estrogen, a large number of *in vitro* studies have suggested that low-dose BPA was shown to have a rapid activation effect on islets, endothelium, breast and pituitary through non-genomic regulation (Alonso-Magdalena et al., 2005; Nadal et al., 2000; Noguchi et al., 2002). However, it is still unclear which specific membrane

receptors mediate the low-dose effects of non-genomic regulation. Membrane receptors, as the direct targets and starting point of non-genomic regulation of low-dose BPA, not only play an important role in the study of low-dose effect mechanisms of BPA, but also provide action sites and theoretical basis for the further intervention of this low-dose non-genomic regulation. Therefore, the identification and determination of the membrane receptor and the related mechanisms involved in the non-genomic regulation of low-dose BPA have become the focus of academic attention and the key scientific problems that need to be solved urgently. Recently, we have carried out a series of work in this area and found that the cell membrane G protein-coupled estrogen receptor 1 (GPER1), integrin $\alpha v \beta 3$ and its mediated signaling pathway played a key role in the endocrine disrupting effects of low-dose BPA.

3.1. Effects of low-dose BPA on male germ cell function via GPER1 receptor

Estrogen plays an important role in the maturation and functional regulation of the reproductive system. Therefore, the potential biological effects of BPA on the reproductive system have attracted more and more attention. Testes are an important part of the male reproductive system. A large number of studies have shown that BPA can penetrate the blood testes barrier into the testicular tissue directly damaging spermatogenic cells, support cells and testicular stromal cells, inhibiting testosterone secretion, affecting sperm development and maturation and finally resulting in the decrease of sperm count and activity decrease and increase of sperm malformation. (Peretz et al., 2014; Manfo et al., 2014; Tinwell et al., 2002). In addition, perinatal exposure to low-dose BPA can cause significant damage to testicular development and spermatogenesis in offspring male rats (Salian et al., 2011), suggesting that low doses of BPA can have a lasting effect on the reproductive capacity of the offspring. However, the mechanism underlying the action of low-dose BPA on male germ cells remains unclear.

As mentioned above, the low-dose effect of BPA was thought to be mediated by membrane receptor by the non-genomic way. But what kind of the membrane receptor participating in the non-genomic effects of low-dose BPA is still unclear. O'Dowd et al. (1998) found a third estrogen receptor, GPER1, which is not homologous to the traditional estrogen receptor ER-α and ER-β in the study of rapid non-genomic effects. The GPER1, a seven transmembrane G protein-coupled receptor, consists of 375 amino acid residues and mediates rapid non-genomic effects mainly through activating the second messenger adenosine monophosphate (cAMP) and epidermal growth factor receptor (EGFR) protein kinase pathway (Prossnitz and Maggiolini, 2009). Many studies indicate that GPER1 is involved in the regulation of various life activities and is closely related to energy balance, lipid metabolism, cardiovascular function, neurological memory regulation, especially the occurrence and development of various estrogen-related tumors (Prossnitz and Barton, 2011).

A large number of environmental estrogens exhibit some binding affinity for GPER1, and BPA is one of them whose affinity to GPER1 is similar to ERs (Thomas and Dong, 2006). A recent study showed that environmentally relevant doses of BPA directly activated membrane receptors through non-genomic way and induced the human spermatogonial cell

proliferation by the membrane receptor-mediated protein kinase G (PKG) and protein kinase A (PKA) signaling pathway (Bouskine et al., 2009). In this study, although the specific signal transduction pathway of BPA activation was identified, the key membrane receptors that mediate the signaling pathways were undetermined and the associated mechanisms were still unknown. Based on the above analysis, we hypothesized that GPER1-mediated signaling pathways may be involved in the spermatogonia proliferation induced by low-dose BPA. To test our hypothesis, we used mouse spermatogonial GC-1 cells as an experimental model because these cells display specific features common to type B spermatogonia and early spermatocytes (Bellve et al., 1977). In addition, the GC-1 cells express the GPER1 protein, which was helpful to investigate the role of GPER1 in the non-genomic effects induced by BPA.

Through mutual verification between cell viability and proliferation test, we found that environmentally relevant doses of BPA (10^{-11} to 10^{-7} mol/L) could significantly induce GC-1 cells proliferation in an inverse U-shaped dose–response manner (Fig. 1). In these dose ranges, BPA have no effects on caspase-3 activity, which plays an important role in apoptosis. Thus, we ruled out the possibility that GC-1 cell proliferation induced by BPA is due to its inhibition on apoptosis. Further exploration suggested that BPA promoted GC-1 cell proliferation mainly through activating both PKG and EGFR-ERK (extracellular regulated kinase, ERK) pathway. G protein $G\alpha i/G\alpha q$ is an important part of intracellular membrane G protein-coupled receptors (GPCRs) and involved in the activation of downstream signaling pathways. In our experiments, the $G\alpha i/G\alpha q$ inhibitor PTX significantly prevented GC-1 proliferation by BPA, suggesting that GPCR might be involved in BPA-induce proliferation. In non-genomic regulation mode, PKG and ERK signaling pathways participate in cell proliferation by rapid activation of cell cycle-regulated key transcription factors, cAMP responsive element-binding protein (CREB) and retinoblastoma (Rb) (Mayr and Montminy, 2001). BPA was found to promote phosphorylation of CREB and Rb by activating ERK and PKG at 15 min. At the same time, we also found that E2-BSA plus BPA induced an effect equivalent to that of E2-BSA or of BPA alone, indicating that there is no synergistic or antagonistic effect on GC-1 cells when both substances coexist. E2-BSA is a non-transmembrane E2 complex and exerts its estrogenic effect by acting on the membrane receptor. Therefore, the same proliferation effects observed above suggests that the cell proliferation may be induced by similar membrane receptors and signaling pathways. However, an interesting phenomenon is that the combination of BPA and E2 significantly promoted the proliferation effects of E2, suggesting that ERs may play a role in the GC-1 proliferation by BPA. Further data reveals that ER antagonist ICI can significantly inhibit the proliferation of GC-1 cells by E2 and BPA, but not E2-BSA, indicating that the proliferation of GC-1 cells by BPA may depend on both ER-α (GC-1 cells express ER-α, but not ER-β, and ER-α just has an intracytoplasmic location) and G protein–coupled receptor-epidermal growth factor receptor–extra cellular regulated kinase (GPCR-EFGR-ERK) signaling pathways. However, 10^{-9} mol/L BPA failed to induce ER-α transcriptional activation in GC-1 cells transfected with ER-α reporter gene and ERs-negative SkBr3 breast cancer cells. In addition, BPA could not activate the ER-α or ER-β that contains the yeast
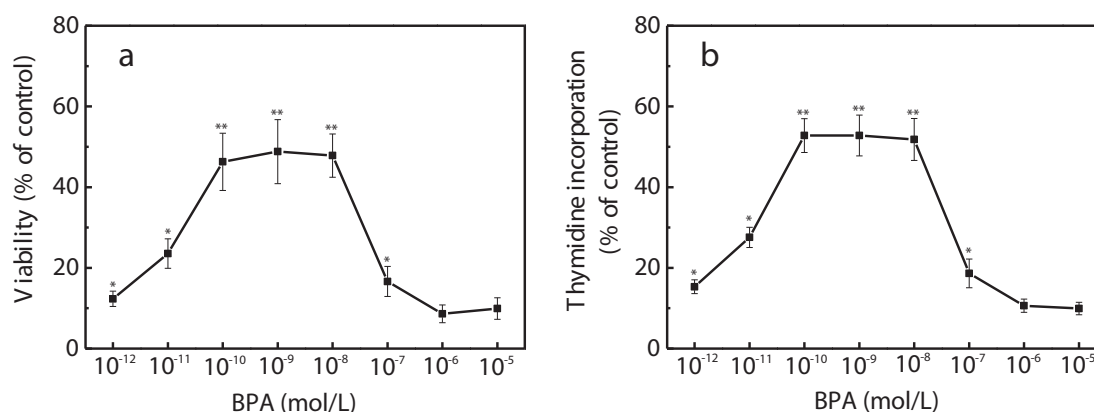
Fig. 1 – Proliferative effects of BPA on GC-1 cells. Cells were exposed to the indicated concentrations of BPA incubated for 12 hr, and cell proliferation was assessed by 3-(4,5-dimethyl-thiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT) assay (a) and [^{3}H]-TdR incorporation analysis (b). The results of three independent experiments performed in triplicate were shown as mean ± SD. *$p < 0.05$, compared with control; **$p < 0.05$, compared with 10^{-12}, 10^{-11}, 10^{-7}, 10^{-6}, or 10^{-5} mol/L BPA (Bisphenol A).

transcription factor Gal4 DNA binding domain and estrogen binding domain.

As was the case with the effect of BPA plus E2-BSA to GC-1 cell proliferation, we also found that BPA plus the specific GPER1 agonist induced the same effect as each of them alone, indicating that there may be a similar signaling pathway activated by BPA and G1. However, the combination of the specific ER-α agonist propyl pyrazole triol (PPT) and BPA or G1 displayed greater proliferative effects than PPT, BPA and G1

Fig. 2 – Silencing of gper1 or ers-1 inhibits BPA-induced cell proliferation. GC-1 cells were transfected with control scrambled (CS-ODN), GPER1 (gper1/AS-DON), ER-α (ers-1/AS-DON) antisense oligonucleotides, and then treated with BPA (10^{-9} mol/L), E2 (10^{-9} mol/L), and PPT (each at 10^{-7} mol/L). Cell proliferation was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT) assay. The results of three independent experiments performed in triplicate were shown as mean ± SEM. *$p<0.05$, compared with control; *$p<0.05$, compared with E2; #$p<0.05$, compared with BPA; ^$p<0.05$, compared with propyl pyrazole triol (PPT).

alone did. In addition, the silencing of gper1 or ers-1 by antisense oligonucleotides significantly blocked GC-1 cell proliferation induced by BPA, E2, G1 and PPT (Fig. 2). But the silencing of gper1 could not completely inhibit the GC-1cell proliferation induced by E2 and PPT (Fig. 2), suggesting that GPER1 was involved in BPA-induced proliferation and there may be cross-talk between GPER1 and ER-α. The regulation of GPER1 and ERs has not been reported until now and may have important biological significance.
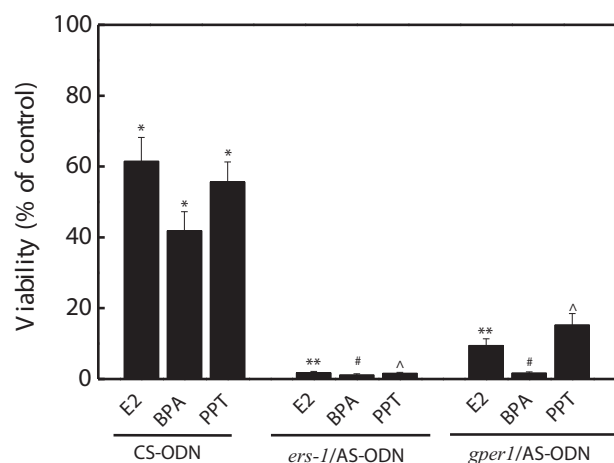
ER transcriptional activation involves hormone dependent and non-dependent induction (Metzger et al., 1995). Transcriptional activation of hormone-independent ERs requires phosphorylation at the Ser118 site in the AF-1 region of ERs (Joel et al., 1995). This site is usually activated by mitogen activated protein kinase (MAPK) to promote ERs-mediated signaling pathway activation and tumor growth (Atanaskova et al., 2002). In our study, 10^{-9} mol/L BPA was found to significantly phosphorylate the Ser118 site of ER through GPER1 and EFGR-ERK pathway (Sheng et al., 2013). What's more, the expression of c-Fos was markedly induced (up to 3–8 times) by BPA *via* the EGFR-ERK signaling pathway activated *via* ER-α and GPER1. Nuclear transcription factor c-Fos regulates the expression of genes associated with cell survival, proliferation, invasion and differentiation (Shaulian and Karin, 2002), and the small changes of its expression can have a significant effect on the phenotype (Coulon et al., 2010; Johnson et al., 1992; Piechaczyk and Blanchard, 1994). Notably, recent studies have shown that c-Fos can be recruited to the activator protein-1 (AP-1, the promoter region of the gper1 gene) site and induce GPER1 and c-Fos expression through a GPER1-EGFR-ERK-c-Fos positive feedback loop (Albanito et al., 2008). Therefore, we presumed that there might be such a positive feedback loop in the induction of cell proliferation by low-dose BPA. To investigate our hypothesis, we performed a series of reporter gene and chromatin immunoprecipitation experiments and found that 10^{-9} mol/L BPA recruited c-Fos to the promoter region (a 5′ region of the 358 base fragment, containing c-Fos binding sites of AP-1) of gper1 to induce gper1 transcription through the same pathway (Fig. 3), and
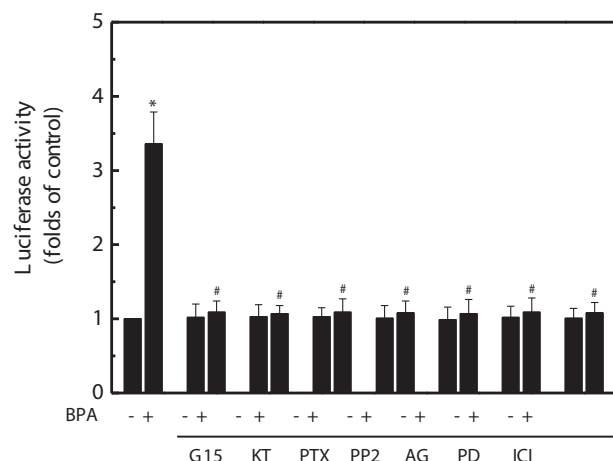
Fig. 3 – Low-concentration BPA transactivates the AP-1 site of the *gper1*–5′-flanking region *via* the GPER1, PKG, ER-α, and EFGR-ERK pathways in the GC-1 cells. Cells were transfected with a reporter plasmid encoding the *gper1*–5′-flanking region, and exposed to 10^{-9} mol/L BPA for 12 h with or without pretreatment with G15, KT5823, PTX, PP2, AG-1478, PD98059 and ICI (inhibitors of GPER1, PKG, Gαi/Gαq, Src, epidermal growth factor receptor (EFGR), mitogen-activated protein kinase (MAPK) and ER-α, respectively). The value for vehicle-treated cells was set as 1-fold induction upon which the activity induced by treatments was calculated. Values shown (mean ± SD) represent the results of three independent experiments performed in triplicate. $^{*}p < 0.05$, compared with control. $^{\#}p < 0.05$, compared with BPA treatment alone.

the AP-1site from -210 to -204 bp was required for the transactivation. Furthermore, the induction of GPER1, *p*-ERK1/2, *p*-Ser118-ER-α expression and cell proliferation was significantly inhibited after c-Fos lost its binding capacity to AP-1 by genetic manipulation. It is worth noting that c-Fos dysfunction did not completely inhibit BPA-induced cell proliferation, suggesting that the expression of GPER1 induced by BPA just promotes but not stimulates the GC-1 cell proliferation. The positive feedback loop could increase the amplitude and prolong the activation state of signaling pathways in response to exogenous stimuli (Freeman, 2000). Thus, the "overexpression" of GPER1 induced by environmentally relevant dose of BPA *via* positive feedback loop was likely to play an important role in promoting the transformation and carcinogenesis of malignant germ cells. GPER1 is found to inhibit cell proliferation activated by EGFR-ERK pathway through PKA in normal cells and thus maintains the normal physiological function of cells (Filardo et al., 2002). However, our study suggests that BPA dose not activate PKA through GPER1, which will make BPA exert a long-lasting effect on cell proliferation due to the lack to the inhibition of the EGFR-ERK pathway.

GPER1 is highly expressed in estrogen-associated tumors and is closely related to the occurrence, progression and prognosis of various tumors (Langer et al., 2010; Prossnitz et al., 2008; Filardo et al., 2006; Smith et al., 2009). Recently, environmentally relevant dose of BPA has been found to antagonize the efficacy of

chemotherapeutic drugs against breast cancer (Lapensee et al., 2009). Based on our results, we speculate that BPA-promoted proliferation *via* a positive feedback loop of GPER1 is likely to be the main mechanism of anti-chemotherapeutic efficacy, which are being performed in our lab.

ER-β is involved in the control of male reproductive cell cycle after birth and its down-regulation is closely related to testicular seminoma (Hirvonen-Santti et al., 2003; Pais et al., 2003). Therefore, gonocytes lacking the active ER-β isoform until the prenatal period may be strongly sensitive to the BPA-mediated proliferation effects (Gaskell et al., 2003). As a result, the exposure to BPA during embryonic phase may pose a potential risk to male germ cells. A group of epidemiological data show that the blood BPA levels of pregnant mothers and their fetuses were 0.3–18.9 ng/mL (1.31×10^{-9}–8.28×10^{-8} mol/L) and 0.2–9.2 ng/mL (8.76×10^{-10}–4.29×10^{-8} mol/L), respectively (Schonfelder et al., 2002). These serum concentrations of BPA are similar to those used in our current study, suggesting that exposure to low doses of BPA during pregnancy may have an adverse biological effects on the fetus.

Taken together, our study shows that environmentally relevant doses of BPA activates PKG and EGFR-ERK-c-Fos positive feedback pathways by a cross-talk between membrane receptor GPER1 and nuclear receptor ER-α, inducing and promoting the mouse spermatogonial cell proliferation (Scheme 1) (Sheng and Zhu, 2011; Sheng et al., 2013). The study not only provides new evidence and mechanism for the occurrence and development of germ cell carcinoma induced by BPA at environmentally related doses, but also provides novel theoretical basis regarding the endocrine disrupting effects of other estrogen-like compounds whose structures resembles BPA displayed on the reproductive system.

3.2. Effects of low-dose BPA on thyrotropin-regulated transactivation *via* integrin αvβ3 receptor

Thyroid hormone (TH), including triiodothyronine (T3) and thyroxine (T4), are important for basal metabolism, especially brain growth and development and its mild abnormality can lead to multiple cognitive disorders (Bernal, 2002). Therefore, environmental pollutants that can change TH production or interfere with TH signaling pathways are likely to have a profound effect on body development, and understanding the effects of environmental compounds on TH signaling pathways is critical to human health.

The classical TH genome regulation involves the complex formation of TH and the nuclear thyroid hormone receptor (TR), which make nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid receptors (SMRT) shedding from the transcriptional complex and then steroid receptor coactivator-1 (SRC-1) recruitment to TR DNA binding domain and consequent gene transcription initiation (Yen et al., 2006). *In vitro* studies have shown that BPA has a relatively low affinity to TR (Wetherill et al., 2007), but it can antagonize the gene transcriptional regulation by TH-mediated *via* TR while BPA does not show the TH activities (Kitamura et al., 2005; Iwamuro et al., 2006; Moriyama et al., 2002). *In vivo* studies have also confirmed that developmental exposure to BPA suppress the negative feedback regulation of thyroid hormone in mouse by selective antagonism of the TR-β
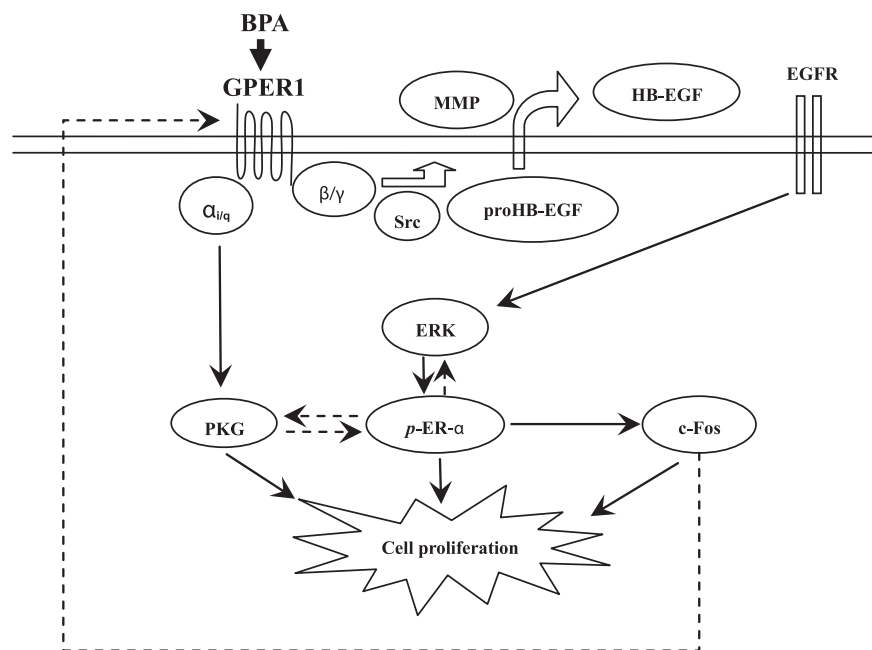
Scheme 1 – Based on the present data and available literature, it was assumed that BPA acted *via* G protein-coupled estrogen receptor 1 (GPER1) to activate the cGMP-dependent protein kinase (PKG) and induce the release of surface-bound membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF) (Filardo et al., 2000), which in turn activated epidermal growth factor receptor–extra cellular regulated kinase/estrogen receptor (EFGR-ERK/ER-α/c-Fos) pathways, finally leading to the stimulation of a mitogenic signaling network (Bunone et al., 1996; Lannigan, 2003).

receptor at the pituitary level, which elevates T4 levels and thyroid hormone regulatory gene protein kinase c substrate/neurogranin expression (Zoeller et al., 2005).

The low affinity of BPA to TR means that a high level of BPA is required to antagonize TH-mediated transcriptional regulation. However, Zoeller et al. (2005) found that perinatal exposure to low dose of BPA in rats had increased their offspring's T4 levels on postnatal day 15 and up-regulated the TH responsive gene in the brain through TH pathways, which led to abnormal brain development such as cognitive disorders and hyperactivity in the offspring. Iwamuro et al. (2006) demonstrated that low dose of BPA (0.1 μmol/L) could significantly antagonize the gene expression of TR in the tail of African Xenopus tadpole, especially when there also existed TH, the antagonistic effect of BPA was even greater. Additionally, low-dose BPA (20 μg/(kg·day)) exposure during pregnancy in rats altered their offspring's cerebral cortex and stimulated the expression of TR-α and its related genes (Nakamura et al., 2006). Since TR is not a direct target (Xu et al., 2007), perinatal exposure to low-dose BPA is considered to affect thyroid hormone function and brain development through a non-genomic regulation. However, the specific mechanism of the TH effect induced by low-dose BPA remains unclear.
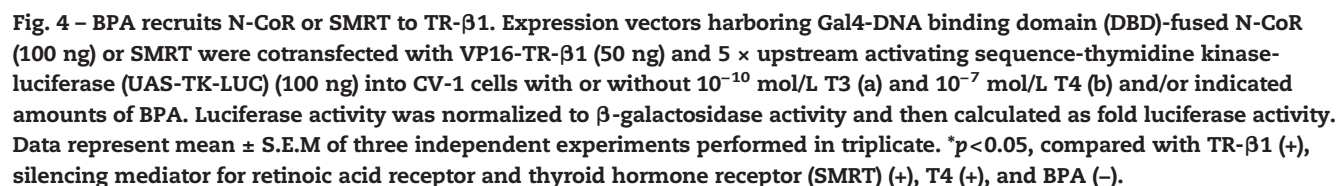
Transcriptional regulation of TH in different cells are not always by the TR pathway, sometimes through non-genomic way which involves the rapid activation of the signaling transduction pathway mediated by membrane receptors, as mentioned earlier (Davis et al., 2008). Davis et al. (2008) found that the non-genomic regulation of TH was at least partially dependent on integrin αvβ3 and the cellular signaling pathway

mediated by it. Integrin αvβ3 is a heterodimeric transmembrane glycoprotein composed of αv and β3 (1:1) subunit, being an important member of the family of adhesion molecules. Both αv and β3 subunit consists of three parts: the extracellular region, the transmembrane region and the cytoplasmic region. The extracellular domain binds to specific ligands, and the cytoplasmic domain regulates communication between cells and cells, or cells and extracellular matrix (such as cell extension, migration, and extracellular matrix assembly) as well as bi-directional transmission of intracellular and extracellular signals (such as focal adhesion kinase-regulated MAPK signaling) by interacting with actin-binding proteins, intracellular signaling proteins, and non-enzymatic proteins (Arnaout et al., 2005). T3 and T4 are agonists of αvβ3 (Bergh et al., 2005), promoting the isolation of N-CoR or SMRT from TR and the transactivation of the related gene through activating αvβ3-mediated MAPK/c-Src pathway since the activated MAPK/c-Src phosphorylate serine 142 of TR-β DNA binding domain.

A large number of studies have shown that low-dose BPA exerts its endocrine disrupting effects in a non-genomic manner mainly by directly controlling the membrane receptors-mediated intracellular signaling pathways (Wetherill et al., 2007). Based on these studies, we hypothesized that the interference effects of environmentally related low-dose BPA on TH is likely to be associated with its direct action on integrin αvβ3-mediated signaling pathway. Therefore, we used the African cercopithecus aethiops monkey kidney cell line CV-1 as the experimental model to investigate our hypothesis. Since CV-1 cells facilitate genetic manipulation and lack TR but expresses αvβ3, it is an ideal experimental model to explore the role of αvβ3 and its signaling pathway in BPA-induced TH/TR transcriptional antagonism.

Using gene reporter assays, we found that BPA at concentration ranging from 10^{-9} to 10^{-7} mol/L significantly inhibited the physiological concentration of T3-mediated TR-β1 transcription. Since BPA at this concentration range had no toxic effect on CV-1 cells, we eliminated the possibility that the transcriptional repression of BPA was due to decreased cell viability. BPA did not suppress retinoic acid X receptor-mediated or glucocorticoid receptor-mediated transcription, indicating that BPA was specific for TR transcriptional inhibition. Furthermore, we also observed that in the absence of T3, BPA of 10^{-9} to 10^{-7} mol/L did not affect the basal transcription, TR-β1-mediated basic transcription, and N-CoR-mediated TR-β1 transcription inhibition, suggesting that the inhibition of tr transcription by BPA was not due to its direct binding to TR. Indeed, the affinity of BPA at present concentration to TR was far lower than that of T3 (Moriyama et al., 2002). These results indicated that the inhibitory effect of low-dose BPA on TR transcription may not attribute to its competition with TH for binding to TR. Using mammalian two-hybrid and glutathione S-transferase pull-down experiments, we demonstrated that BPA did not directly influence the binding of T3-mediated TR-β1 to SRC-1 when co-transfected with transcriptional factor SRC-1, excluding the possibility that transcription inhibition of BPA due to directly affecting the interaction between TR-β1 and SRC-1.

When co-exposed to physiological concentrations of T3/T4, BPA of 10^{-7} mol/L can promote N-CoR/SMRT binding to TR-β1 (Fig. 4). Moriyama et al. (2002) found that high doses of BPA also could recruit N-CoR to TR by directly acting on TR, causing the suppression of the TH transcriptional regulation. Interestingly, their data showed that the inhibitory constant of BPA for T3 binding to TR was about 10^{-4} mol/L, but BPA of 10^{-6} mol/L had significantly suppressed transcription. Furthermore, BPA at 10^{-9}–10^{-5} mol/L could not markedly bind to TR, but strongly recruit N-CoR to TR. The above results suggested that low doses of BPA might recruit N-CoR to TR through other means. Then, are the integrin αvβ3 and its regulated c-Src signaling pathway involved in the recruitment of N-CoR by low-dose BPA? We found that overexpression of αvβ3 integrin and c-Src in the

presence of T3/T4 significantly inhibited the recruitment of N-CoR/SMRT by BPA and the corresponding transcriptional inhibitory effect of TR, suggesting that β3 integrin and signal protein c-Src were involved in the recruitment of N-CoR/SMRT and the related TR-β1 transcription inhibition induced by BPA. It has been shown that T3 binds to both sites of αvβ3 and activates both MAPK and/or c-Src/PI3K pathways, while T4 combines only one site and only activates MAPK (Bhargava et al., 2009). However, we found that c-Src also participate in the T4-regulated recruitment of N-CoR or SMRT to TR-β1 by BPA, indicating an intracellular cross-talk between PI3K and MAPK via αvβ3. These findings may have other important biological significance.

Studies have shown that TH induces serine phosphorylation of TR-β1 by activating the αvβ3/c-Src/MAPK signaling pathway, resulting in the dissociation of N-CoR or SMRT from TR-β1 and activation of transcription (Bhargava et al., 2009). Thus, we further examined whether BPA antagonized TR-β1 transcriptional activation by interfering with TH-activated αvβ3/c-Src/MAPK/p-ser-TR-β1 pathway. In the TR-absence 293T cells, co-immunoprecipitation results showed that the physiological concentration of T3 or T4 could significantly inhibit the co-precipitation of TR-β1 and N-CoR or SMRT. However, in the presence of BPA, MAPK inhibitor PD98059, c-Src inhibitor PP2 and αvβ3 integrin antagonist Tetrac, the TR-β1/N-CoR or SMRT co-precipitation inhibitory effect of T3/T4 on the TR-β1/N-CoR or SMRT co-precipitation was obviously antagonistic, suggesting that integrin αvβ3 and its regulated signaling proteins c-Src and MAPK were involved in the suppression of TH/TR transcription activation mediated by BPA. Furthermore, BPA, PD98059, PP2 or Tetrac could also markedly inhibit the formation of co-precipitation of TR-β1 and tyrosine-phosphorylated MAPK or serine-phosphorylated TR-β1 in the presence of T3/T4, indicating that BPA inhibited TH-mediated MAPK and TR-β1 activation. In addition, BPA, PP2, and Tetrac were found to inhibit the co-immunoprecipitation of αvβ3 and c-Src mediated by TH, but PD98059 was not, suggesting that BPA

Fig. 4 – BPA recruits N-CoR or SMRT to TR-β1. Expression vectors harboring Gal4-DNA binding domain (DBD)-fused N-CoR (100 ng) or SMRT were cotransfected with VP16-TR-β1 (50 ng) and 5 × upstream activating sequence-thymidine kinase-luciferase (UAS-TK-LUC) (100 ng) into CV-1 cells with or without 10^{-10} mol/L T3 (a) and 10^{-7} mol/L T4 (b) and/or indicated amounts of BPA. Luciferase activity was normalized to β-galactosidase activity and then calculated as fold luciferase activity. Data represent mean ± S.E.M of three independent experiments performed in triplicate. $^*p < 0.05$, compared with TR-β1 (+), silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) (+), T4 (+), and BPA (–).

antagonizes the serine phosphorylation of TR-β1 by MAPK/αvβ3/c-Src pathway. These findings not only demonstrated that BPA inhibited TR-β1-mediated transcriptional activation by interfering with TH-regulated αvβ3/c-Src/MAPK/TR-β1 pathway, but also further confirmed that TH promoted the dissociation of N-CoR/SMRT from TR-β1 through αvβ3/c-Src/MAPK/TR-β1 pathway, facilitating further transcriptional activation. Dietary exposure to low-dose BPA in pregnant rats has been shown to elevate levels of serum T4 in their offspring and alter expression of brain protein kinase C substrate/granule protein (Zoeller et al., 2005). In this study, the "flat" dose–response relationship between BPA concentrations and serum T4 levels was thought to be due to the limited amount of TR and co-inhibitory factors. However, based on our current results, it was speculated that the limited quantity of αvβ3 and TR-β1 may partly contribute to the "mild dose effect" of BPA on serum T4 levels.

In summary, low-dose BPA recruited N-CoR/SMRT to TR-β1 by interfering with the physiological concentration T3/T4-mediated αvβ3-c-Src-MAPK signaling pathway, resulting in the suppression of TR-regulated gene transcription in a non-genomic manner (Sheng et al., 2013). Although the effects of BPA on the thyroid axis at least in mammals are not considered at the moment as a recognized effect at the European level, the present study provided a new mechanism for the interference TH effect induced by low-dose BPA.

4. Key problems need to be further explored

GPER1 is a new and important estrogen receptor on membrane surface, highly conserved between human and mouse, widely expressed in different tissues and cells, and mediates a variety of biological effects in the nervous, reproductive and endocrine systems (Prossnitz and Maggiolini, 2009). Integrin αvβ3, an important member of the cell adhesion molecule family, is expressed in a variety of tissue cells and highly conserved among different species (Arnaout et al., 2005). T3/T4 participates in a variety of physiological and pathological processes *in vivo* through mediating non-genomic regulation (Davis et al., 2008). Based on these facts, we hypothesized that the non-genomic effects of low-dose BPA mediated by membrane receptor GPER1 or/and αvβ3 may play a vital role in inducing endocrine disrupting effects *in vivo*. Notably, low-dose BPA is likely to disrupt human's normal physiological function by interfering with the non-genomic regulation mediated by these two receptors since the structure and function of both the GPER1 and αvβ3 are highly conservative between experimental animals and human beings. In our studies, we have observed an interesting phenomenon, that is, the GPER1-mediated ERK signaling pathway allows the phosphorylation of the serine118 of ER-α to be sustained, likewise, the αvβ3 integrin-mediated ERK signaling pathway activates ER-α in the same manner. Thus, we hypothesize that GPER1 and αvβ3 may co-mediate the low-dose effect of BPA through coordination of different signaling pathways in the presence of T3/T4 *in vivo*. Therefore, the first key problem we need to further explore is that which signaling pathways will be regulated and what kinds of toxic effects will occur *via* GPER1 or/and αvβ3 after environmentally relevant low-dose BPA treatment in the germ and nerve cells highly expressing GPER1 and αvβ3.

In recent years, the impact of environmental endocrine disruptors on epigenetics has been widely concerned (Zhang and Ho, 2011). Epigenetics is a genetic modification that does not involve DNA sequence changes in regulating gene expression and is the result of the interaction of environmental factors with the genetic material. Epigenetics participates in the development and progression of many diseases, especially tumors (Crews and McLachlan, 2006). As an important way of epigenetic modification, DNA methylation is one of the epigenetic events and also the main mode for epigenetic effect caused by environmental endocrine disruptors (Jones and Takai, 2001). DNA methylation can alter the structure of chromatin, the conformation and stability of DNA, and the interaction between DNA and protein, finally regulating gene expression and normal cellular development. It has been shown that environmental estrogen-like endocrine disruptors have positive feedback-regulation effect on growth factors *via* the phosphorylation of AF-1 mediated by ERK signaling pathway, which may be related with DNA methylation modification (Miyagawa et al., 2011). Our studies have shown that environmentally related low-dose BPA controls AF-1 activation and epidermal growth factor through GPER1-ERK positive feedback loop (Sheng et al., 2013). In addition, recent studies have also shown that low doses of BPA can affect the key genes and enzymes participating in the regulation of cell genomic DNA methylation (Tang et al., 2012; Yaoi et al., 2008; Dolinoy et al., 2007). In view of this, we assume that membrane receptor GPER1 or/and αvβ3-mediated non-genomic regulation may have some relationship with DNA methylation modification in the low dose effect of BPA. Based on the above analysis, the second question to be explored is how the environmentally relevant low-dose of BPA regulates DNA methylation through GPER1 or/and αvβ3-mediated signaling pathways. At present, the epigenetic regulation of the germ line is considered to be one of the main ways in individual epigenetic effects of environmental endocrine disruptors (Rajender et al., 2011). The reprogrammed genetic information is most likely to be transmitted to the offspring through the germ cell line resulting in various health hazards to the offspring since the environmental endocrine disruptors reprogram the genetic-development information of germ cell lines (such as spermatozoa, oocytes, *etc.*) by affecting DNA methylation (Walker and Gore, 2011; Guerrero-Bosagna and Skinner, 2012). Therefore, understanding the molecular mechanism of the effects of low-dose BPA on DNA methylation will have important reference values for evaluating the genetic influence of BPA on the offspring of developmental stage.

In vivo, environmental endocrine disruptors display their endocrine disrupting effects mainly through interfering with the physiological functions of endogenous hormones in an antagonism or coordination manner. However, *in vitro*, when it coexists with the physiological concentrations of hormones, it may induce a biological effect different from its single exposure, especially in the case of low dose exposure. In our previous study, BPA alone did not have any effect on cells (since this cell did not express GPER1), but BPA interfered with the T3/T4-induced activation of αvβ3 when the T3 and T4 coexisted (Sheng et al., 2013). In addition, the physiological concentration of estrogen plus BPA could change the phosphorylation state of ERK1/2, a key signal molecule in mouse pituitary cell, but this

effect was not produced by BPA alone (Jeng and Watson, 2011). In developing cerebellar neurons, BPA and estrogen co-injection could dose-dependently inhibit estrogen-induced ERK1/2 rapid activation (Zsarnovszky et al., 2005). Taking together our current results, we believe that the inhibition of estrogen ERK1/2 activation by low-dose BPA may be due to the combined effects of BPA and T3/T4 and estrogen co-exposure: estrogen activates ERK1/2 through membrane surface receptors; because of the saturation activation mechanism, BPA will no longer activate ERK1/2, even if low-dose BPA activates the same signaling pathway through GPER1, but it can interfere with T3/T4-induced activation of ERK1/2 by αvβ3. Thus, the final net result is the inhibition of ERK1/2 signaling pathway. Therefore, we speculate that the endocrine disrupting effect of low-dose BPA observed *in vivo* is likely to be a result of the combined effect of BPA and the physiological concentration of hormones. Studies have shown that estrogen, thyroid hormones and androgen play a key role in the regulation of nervous and reproductive development, which is also mainly affected by BPA (Wetherill et al., 2007). Based on the above analysis, the third question need to explore is: with the physiological concentration of estrogen, thyroxine and androgen coexisted, which signal transduction pathways and correspondingly what cell functions or methylation modification will be affected by the environmentally relevant low-dose BPA through GPER1 or/and αvβ3. Understanding this will contribute to a more real and accurate understanding of the endocrine disrupting effects of low-dose BPA, so as to provide a more reasonable explanation and reliable inference for the whole animal experiment results and effects on human health.

Acknowledgments

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