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Effects of environmental pollutants on signaling pathways in rat pituitary GH3 adenoma cells

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Abstract

An increased rate of acromegaly was reported in industrialized areas, suggesting an involvement of environmental pollutants in the pathogenesis and behavior of GH-secreting pituitary adenomas. Based on these premises, the aim of the study was to evaluate the effects of some widely diffused pollutants (i.e. benzene, BZ; bis(2-ethylhexyl) phthalate, DEHP and polychlorinated biphenyls, PCB) on growth hormone secretion, the somatostatin and estrogenic pathways, viability and proliferation of rat GH-producing pituitary adenoma (GH3) cells. All the pollutants induced a statistically significant increase in GH secretion and interfered with cell signaling. They all modulated the expression of SSTR2 and ZAC1, involved in the somatostatin signaling, and the expression of the transcription factor FOXA1, involved in the estrogen receptor signaling. Moreover, all the pollutants increased the expression of the CYP1A1, suggesting AHR pathway activation. None of the pollutants impacted on cell proliferation or viability. Present data demonstrate that exposure to different pollutants, used at *in vivo* relevant concentrations, plays an important role in the behavior of GH3 pituitary adenoma cells, by increasing GH secretion and modulating several cellular signaling pathways. These observations support a possible influence of different pollutants *in vivo* on the GH-adenoma aggressiveness and biological behavior.

Keywords: pollution; benzene; GH; DHEP; PCB; somatostatin receptor; pituitary adenoma.

Abbreviations: AF2 = activation function 2; AHR = aryl hydrocarbon receptor; AIP = aryl hydrocarbon receptor-interacting protein; A; BZ = benzene; CYP1A1 = cytochrome-P 1A1; DEHP = bis(2-ethylhexyl) phthalate; ERα = estrogen receptor α; FIPA = familial isolated pituitary adenoma; FOXA1 = Forkhead box protein A1; GH = growth hormone; PAC1-R = adenylate cyclase activating polypeptide type 1 receptor; PCB = polychlorinated biphenyls; PPARγ =

peroxisome proliferator-activated receptor γ; PR = progesterone receptor; PRL = prolactin; SD = standard deviation; SRC1 = steroid receptor co-activator 1; SS = somatostatin; SSA = somatostatin analogs; SSTR = somatostatin receptor; ZAC1 = zinc-finger protein.

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1. Introduction

Pituitary adenomas account for 10-25% of intracranial tumors (Dworakowska and Grossman, 2009). They derive from clonal expansion of highly differentiated anterior pituitary cells and are mainly sporadic (Dworakowska and Grossman, 2009; Melmed, 2003). Although the great majority of pituitary tumors are histologically benign, they may have a very aggressive behavior characterized by rapid cell proliferation, infiltration of surrounding nervous and vascular structures, and resistance to medical treatment. GH-secreting adenomas, clinically manifesting with signs and symptoms of acromegaly, are the third most common type of pituitary adenoma after prolactinomas and non-functioning adenomas. Though rare, their prevalence and incidence is reportedly increasing (Beckers, 2010).

Hormone secretion and growth of GH-adenoma cells are under the control of many signaling pathways, including somatostatin (SS) pathway that mainly exerts an inhibitory function. SS and its analogs (SSA) – considered the gold-standard medical treatment for acromegaly - bind to five different seven-domain G-protein-coupled receptors, SSTR1-5, through which SS regulates a complex downstream pathway, that includes the zinc-finger protein (ZAC1), the aryl hydrocarbon receptor (AHR), the aryl hydrocarbon receptor-interacting protein (AIP), and several AIP-interacting proteins (Trivellin and Korbonits, 2011; Gadelha et al., 2013). Furthermore, it has been demonstrated that estrogens directly stimulate GH synthesis and secretion through the estrogen receptor and the classic nuclear hormone receptor pathway (Avtanski et al., 2014).

Although several genetic, such as germ-line mutations of AIP, (Chahal et al., 2010; Guaraldi et al., 2011; Cazabat et al., 2012) and epigenetic factors have been suggested (Melmed, 2006), the exact ethiopathogenesis underlying the development of pituitary adenomas, and the mechanisms responsible for their different behavior are not completely understood. Pollution has significant impact on human health and it is implicated in different types of cancers (Engström et al., 2015; Hashim and Boffetta, 2014). Among several pollutants, benzene (BZ) is widespread and relevant

worldwide (World Health Organization, 2015). It derives from industrial effluents, vehicle emissions and cigarette smoking. Its carcinogenic and non-cancerous systemic toxic effects have been largely demonstrated, and they appear secondary to the cytotoxic effect of free radicals and quinone metabolites produced by its metabolism, as well as to genotoxic effects and altered global DNA methylation (Bahadar et al., 2014; Snyder et al., 1993). Moreover, a large number of chemicals have been identified as endocrine disruptors, compounds that alter the normal functioning of the endocrine system of both humans and wildlife. Bis-(2-ethylhexyl)-phthalate (DEHP) is the most commonly used plasticizer in plastic industries and it has been detected in different environmental compartments (Magdouli et al., 2013). Polychlorinated biphenyls (PCB) are banned from production and use in most countries but they are persistent organic pollutants of concern for environment and health. Adverse effects of PCB and their metabolites include neurotoxicity, immunosuppression, effects on thyroid hormones and retinoic acid transport to target tissues, reproductive effects, porphyria and carcinogenicity (AMAP, 2004; Vorkamp, 2016).

The first description of the detrimental effects of environmental pollutants on the pituitary dates back to late 1950s, when Iannacone and Cicchella (1958) described the histological changes associated with benzene intoxication in rats. To our knowledge, no other studies were performed in this field up to 2008, when a modest increase in the incidence of pituitary tumors was reported in the population from Seveso (Italy), exposed thirty years earlier to intermediate-high concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a strong toxic and carcinogenic agent, because of an industrial accident. The involvement of AHR signaling pathway was suggested, although not experimentally demonstrated (Pesatori et al., 2008). Soon later, Cannavò et al. (2010) demonstrated a significantly greater rate of acromegaly in a highly industrialized area close to Messina (Italy) with respect to farther areas and the general population, not attributable to genetic/family predisposition. The authors therefore hypothesized the involvement of environmental pollutants in the pathogenesis of somatotropinomas. Recently, Tapella et al. (2016) demonstrated *in vitro* that the

pollutants BZ and DEHP are able to interfere with normal rat pituitary cell proliferation and to promote gene expression changes at AIP and AHR levels, providing a link between epidemiological and genomic findings in pituitary tumors. However, to date, there are no other studies about the influence of these environmental chemical pollutants on the behavior of pituitary tumors in terms of aggressiveness and potentially drug therapy resistance. Based on these premises, aim of the study was to evaluate the *in vitro* effects of BZ, DEHP and PCB on GH production, gene and protein expression of the main constituents of the somatostatin and estrogen signaling pathways, and on cell growth and viability, using a GH-producing pituitary adenoma cell line (GH3).

2. Materials and Methods

2.1. *Chemicals*

Benzene (BZ), bis(2-ethylhexyl) phthalate (DEHP), and polychlorinated biphenyls (PCB) were purchased from Sigma-Aldrich Co. (St. Louis, MO). BZ was dissolved in DMEM-F12 medium added with dextran-charcoal stripped sera (12.8 μM), PCB was purchased in solution (10 μg/ml in isooctane); DEHP was dissolved in ethanol (2.5 μM). Further dilutions were made in culture medium. PCB concentration is given in grams since PCB is a mixture of compounds # 28, 52, 101, 138, 153, 180 and 209, and it was not possible to calculate the molarity of the solution. Experimental doses were within the ranges observed in blood of exposed people (Hines at al., 2009; Yan et al., 2009; Hays et al., 2012; Chovancova et al., 2012; Hsu et al., 2014).

2.2. *Cell cultures*

Rat pituitary adenoma GH3 cells, obtained from the American Type Culture Collection (ATCC), were maintained in DMEM-F12 culture medium (Lonza, Switzerland) supplemented with 2.5% fetal calf serum and 15% horse serum (Gibco, UK), at 37° C in a humidified atmosphere of 95% O_2 and 5% CO_2. Antibiotics (100 UI/ml penicillin and 100 μg/ml streptomycin) were added to growth media. Cells were routinely screened for mycoplasma contamination, and kept in serum-free medium for the 24 hours preceding the treatment with pollutants. Cells were cultured into polystyrene plasticware from Greiner Bio-One (Kremsmünster, Austria). As reported elsewhere, no significant release of bisphenol A from polystyrene at 37° C occurred (Biswanger et al., 2006).

2.3. *Gene expression profile*

Real Time PCR was used to evaluate the expression of growth hormone (GH), prolactin (PRL), somatostatin receptor type 1 to 5 (SSTR1-5), ZAC1, AIP, AHR, cytochrome-P 1A1 (CYP1A1), estrogen receptor α (ERα), progesterone receptor (PR) and Forkhead box protein A1 (FOXA1)

genes. To assess gene expression profile, GH3 cells were seeded in cell culture flasks (25 cm^2) at a density of 1 x 10^6 cell/flask, and then treated with 130 pM BZ, 250 pM DEHP, and 100 ng/l PCB for 24, 48 and 72 hours.

Total RNA was extracted using TRIzol Reagent (Invitrogen Ltd, Paisley, UK), and 1 µg of the total RNA reverse-transcribed with iScript cDNA Synthesis Kit (BioRad Laboratories, Inc.), following manufacturer's protocol. Specific primers were designed using Beacon Designer 5.0 software; primers sequences are shown in **Supplementary Table 1**.

Real-time PCR was performed using a BioRad MiIQ Detection System (BioRad Laboratories, Inc.) with SYBR green fluorophore. Reactions were performed in 15 µl volume, which included 7.5 µl IQ SYBR Green Supermix (BioRad Laboratories, Inc.), 0.3 µl each primer at 10 µM concentration, 1.90 µl RNAse-free distilled water, and 20 ng/5 µl of the previously reverse-transcribed cDNA template. For each primer set, the reaction was optimized using seven serial 5X dilutions of template cDNA obtained from cells in basal conditions (100, 20, 4, 0.8 and 0.16 ng). A melting curve analysis was performed following each run to ensure a single amplified product for each reaction. All reactions were carried out at least three times for each sample. Every gene expression level was normalized on the expression of three house-keeping genes (β-Actin, L13A and β-2-microglobulin), and expressed as relative expression fold *vs* untreated controls. Before RNA extraction viable cells were counted by Trypan Blue exclusion assay in order to assess absence of cytotoxicity induced by pollutants exposure.

2.4. *Western blot*

GH3 cells were seeded in cell culture flasks (25 cm^2) at a density of 1 x 10^6, and treated with 130 pM BZ, 250 pM DEHP, and 100 ng/l PCB. At different times after treatment, cells were scraped from the flask in the presence of 1 ml lysis buffer (containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 10 mg/ml PMSF, 30 l/ml aprotinin and 100 mM sodium orthovanadate). Cell lysates were incubated in

ice for 30–60 min. At completion, tubes were centrifuged at 4 °C for 20 min at 15,000 × g. Clear supernatants were stored at −80 °C until use. Proteins were separated on 10% SDS-PAGE for ZAC1 and SSTR2 or 8% SDS-PAGE for GH, transferred to PVDF and probed with the following antibodies: polyclonal anti-ZAC1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-SSTR2 (1:500 Santa Cruz Biotechnology, Santa Cruz, CA, USA), and monoclonal anti-GH (2 μg/ml, R&D Systems, Minneapolis, MN, USA).

The membranes were then stripped and re-probed with a rabbit polyclonal anti-GAPDH antibody (1:10000, Sigma, Saint Louis, MO, USA) to check protein loading. Proteins were detected with Pierce Super Signal chemiluminescent substrate. Bands were photographed and analyzed using Kodak 1D Image Analysis software.

2.5. *Growth hormone secretion*

GH3 cells were seeded in cell culture flasks (25 cm^2) at a density of 1 x 10^6, and treated with 130 pM BZ, 250 pM DEHP, and 100 ng/l PCB. After a 72h exposure, cell medium was centrifuged and collected and secreted GH was measured in the medium using the Rat Growth Hormone ELISA Assay (KRC5311, Invitrogen Ltd, Paisley, UK), following manufacturer's instructions.

2.6. *Cell viability assay*

Cell viability was assessed using Cell Proliferation Reagent WST-1, a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells - (Roche Applied Science, Penzberg, Germany). Cells were seeded in 96-well plates at a density of 1 x 10^4 cell/well. After 72 hours cells were treated with: 1 pM - 1 nM BZ, 2.5 pM - 25 nM DEHP, and 1 ng/l - 10 μg/l PCB; untreated cells were used as control. At the different time points, (i.e. 0, 24, 48 and 72 hours) 10 μl of WST-1 was added to each well and after 1 hour incubation, absorbance at 450 nm was measured using a plate reader (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA). Four replicate wells were used to determine each data point.

2.7. *Cell proliferation assay*

GH3 cells were seeded in 96-well plates at a density of 5 x 10^3 cell/well. Thereafter, cells were treated with the different pollutants, as in cell viability assay. At given time points, cell proliferation was assessed using a colorimetric immunoassay based on the measurement of the pyrimidine analogue BrdU incorporation during DNA synthesis (Roche Applied Science, Penzberg, Germany), following manufacturer's instruction. Briefly, cells were labelled using 10 mM BrdU/well and incubated at 37°C for 2 hours. Thereafter, by adding FixDenat solution, the cells were fixed and DNA was denatured. Cells were then incubated with anti-BrdU-POD antibody for 90 minutes, washed and, finally, the substrate solution was added. After 15 minutes, absorbance was measured at 450 nm using a plate reader (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA). Eight replicate wells were used to determine each data point.

2.8. *Statistical analysis*

Data are expressed as mean ± SD, calculated on at least three independent experiments (N=3). Comparison between groups was performed with analysis of variance (two-way ANOVA), and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at $p<0.05$.

3. Results

3.1. *Effects of pollutants on cell signaling pathways*

GH3 cells were treated with each of the pollutants for 24, 48 and 72 hours. No time-dependent changes in both gene (**Supplementary Figure 1**) and protein (**Supplementary Figure 2**) levels were observed in untreated control cells.

3.1.1. GH and PRL Expression and Production (The Secretion Pathway)

After 72 hours of exposure, BZ caused a statistically significant increase compared to control cells in the expression of *GH* gene (1.5 fold, $p<0.05$; **Figure 1A**), as well as of GH protein recovered from cell lysates (1.7 fold, $p<0.01$: **Figure 1B and 1C**) and in the level of GH secreted in the medium of GH3 cells (basal vs BZ, $p<0.01$; **Table 1**). DHEP and PCB did not influence *GH* gene expression (**Figure 1D and 1G**) while both caused an increase compared to control cells of GH protein recovered from cell lysates (for DEHP: 1.6 fold, $p<0.001$; **Figure 1E, and 1F;** for PCB: 2 fold, $p<0.001$; **Figure 1H and 1I**) and of GH level in the medium of GH3 cells (basal vs DEHP, $p<0.05$; basal vs PCB, $p<0.01$; **Table 1**). No modification of *PRL* gene expression was observed in GH3 cells exposed to the three pollutants at any treatment time (data not shown).

3.1.2. SSTRs, ZAC1, AIP and AhR expression and production (The Somatostatin Pathway)

The pollutants effects on genes involved in the somatostatin signaling pathway were then evaluated. In **Figure 2** we report the effects on *SSTR2*. BZ caused a statistically significant increase compared to control cells in *SSTR2* gene expression (1.7 fold, $p<0.01$: **Figure 2A**) after 24 hour treatment, and then the expression progressively returned to basal levels. On the other hand, SSTR2 protein (**Figure 2B and 2C**) was reduced at every time point (0.7 fold at 24 and 48 hours, $p<0.00$; 0.6 fold at 72 hours, $p<0.001$). DEHP also caused a statistically significant increase compared to control cells in SSTR2 gene expression but after 48 hour treatment (1.3 fold, **Figure 2D**), and SSTR2 protein (**Figure 2E and 2F**) was reduced after 72 hours (0.6 fold, $p<0.01$). Finally, PCB induced a

statistically significant increase of SSTR2 gene expression compared to control cells after 48 hour treatment (1.4 fold, $p<0.01$; **Figure 2G**) and, again, SSTR2 protein (**Figure 2H and 2I**) was reduced after 72 hours (0.6 fold, $p<0.001$).

All the pollutants produced a statistically significant increase in the expression of *ZAC1* gene after 48 hours (for BZ: 4.4 fold, $p<0.001$, **Figure 3A**; for DEHP 10.8 fold, $p<0.001$, **Figure 3D**; for PCB 2.4 fold, $p<0.001$, **Figure 3G**). As far as ZAC1 protein is concerned, BZ caused a statistically significant increase compared to untreated controls after 48 hours (1.5 fold, $p<0.001$, **Figure 3B and 3C**), whereas it caused a statistically significant reduction at 72 hours (0.5 fold, $p<0.001$, **Figure 3B and 3C**), such as both DEHP (0.6 fold, $p<0.001$, **Figure 3E and 3F**) and PCB (0.7 fold, $p<0.001$, **Figure 3H and 3I**).

No modifications occurred in *AIP* gene expression (**Figure 4A, 4D and 4G**). An early and transient increase of *AHR* expression was observed only in BZ treated cells at 24 hours (1.4 fold, $p<0.05$, **Figure 4B**), but not after DEHP and PCB treatment. Nevertheless, all three pollutants caused a significant increase of CYP1A1 gene. BZ induced CYP1A1 earlier that DEHP and PCB and the induction was sustained throughout the entire period evaluated (at 24 hours: 1.4 fold, $p<0.001$; at 48 and 72 hours: 1.5 fold, $p<0.001$; **Figure 4C**). DEHP and PCB induced CYP1A1 only after a 72 hour exposure, but at a much greater extent (for DEHP 2.4 fold, $p<0.01$, **Figure 4F;** for PCB 2.6 fold, $p<0.05$, **Figure 4I**).

3.1.3. ERα, PR and FOXA1 (The Estrogenic Pathway)

A statistically significant increase in *ERα* expression compared to untreated control cells occurred after 48 and 72 hours of treatment with DEHP (at both times: 1.48 fold, $p<0.05$; **Figure 5D**) and after 48 hours of treatment with PCB (1.4 fold, $p<0.05$, **Figure 5G**), while *ERα* expression levels never reached any significant increase during BZ treatment (**Figure 5A**). Only BZ treatment induced a statistically significant increase of *PR* after 72 hours (1.4 fold, $p<0.05$; **Figure 5B**), while

neither DEHP (**Figure 5E**) and PCB (**Figure 5H**) affected it. All three pollutants caused a statistically significant increase of the expression of transcription factor *FOXA1* but with a different temporal pattern: *FOXA1* expression increase was observed at 24 hours in cells treated with BZ (4 fold, $p<0.001$, **Figure 5C**), at 24 (2.3 fold, $p<0.01$) and 48 (2.1 fold, $p<0.05$) hours in cells treated with DEHP (**Figure 5F**), and at 48 hours in cells treated with PCB (2.3 fold, $p<0.01$, **Figure 5I).**

3.2. *GH3 Cell Viability and Proliferation*

No statistically significant modification of cell viability or proliferation was detected after treatment with the three pollutants at the different concentrations (**Supplementary Figure 3**). Moreover, viable were not modified by the pollutants under study even when evaluated as internal control in gene expression experiments (**Supplementary Table 2**).

4. Discussion

Air, water and soil pollution has been demonstrated to induce important and pleiotropic pathological conditions, i.e. cancer, respiratory, immune and neurological diseases, birth defects, infertility and other endocrine-metabolic dysfunctions (Briggs, 2003). Several original studies and reviews have been published on the detrimental effects of various pollutants and '*endocrine disruptors*', the latter defined as exogenous substances, of natural or chemical origin, that alter the function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub) population (International Programme on Chemical Safety, 2002). Some hundreds of substances are currently recognized as endocrine disruptors, although only some have been investigated in depth as regards their pathogenic mechanisms (Wielogorska et al., 2015; Maqbool et al., 2016).

Studies traditionally focused on the estrogen signaling pathway (Kiyama et al., 2015) while, more recently, the effects on other pathways, controlling differentiation, growth and secretion of the endocrine system cells, have gained attention. In particular, a potential relationship between the development and behavior of somatotropinomas and pollution has been suggested (Pesatori et al., 2008; Cannavò et al., 2010). Based on these evidences, among the hundreds of suggested environmental pollutants we chose BZ, DEHP and PCB, for which the risk of contamination in daily life is considered high (World Health Organization, 2015; Magdouli et al., 2013; Vorkamp et al., 2016).

Our study was primarily aimed at identifying the effects of these pollutants on GH3 cell function, analyzing gene and protein expression. Notably, we observed that all the pollutants we studied increased GH production and secretion. Our finding is of primary importance since the increased GH production subsequent to BZ, DEHP and PCB exposure was demonstrated for the first time in GH-producing adenoma cells; therefore, we suggest that pollution could modify the functional

behavior of pituitary adenomas, and confer additional aggressiveness. As far as the mechanisms of increased GH secretion is concerned, only BZ increased GH expression, while no modification of GH mRNA was observed after DEHP and PCB treatments. GH mRNA was reported to be increased in GH3 cells treated with estradiol (Avtanski et al., 2014) and also with some endocrine disruptors (tert-octyl-phenol (OP), p-nonyl-phenol (NP) or bisphenol A (BPA), that induced GH secretion in the medium too (Dang et al., 2009). Nevertheless, previous studies reported that neither testosterone nor estradiol affected GH mRNA levels *in vivo* and *in vitro* (González-Parra et al., 1996). As epigenetic changes including altered expression of micro RNAs (miRNAs) have been observed after xenobiotic exposure (Marrone et al., 2014), DEHP and PCB may affect GH production by acting at a post-transcriptional level. Furthermore, a recent study by Ling and coworkers (Ling et al., 2016) suggests as a new mechanism of phthalates toxicities, their direct effect on regulation of mRNA translation and protein synthesis.

We did not observe any significant modification of *AIP* expression after BZ, DEHP or PCB treatment. In GH3 cells, it was observed that AIP reduced forskolin-induced, but not baseline, GH secretion, while endogenous *AIP* knockdown caused a slight decrease in GH secretion under basal conditions (Formosa et al., 2013). Even though an interaction between AIP expression and GH production was postulated we cannot confirm a relationship between *AIP* expression and GH secretion after pollutant exposure.

AHR expression levels were not modified by the pollutants we tested, suggesting that its synthesis was not affected. However, all the three compounds determined a significant increase of CYP1A1 expression. CYP1A1, classically activated by AHR, is involved in the extrahepatic metabolism of several xenobiotic, and it is regarded, when overexpressed, to have a role in cancer induction. Benzene is known to be an AHR signaling activator (McHale et al., 2011; Lindsey and Papoutsakis, 2012); on the contrary, multiple mechanisms may underlie the induction of CYP1A1 by DEHP and PCB. In fact, in our study we used a mixture of non-dioxin-like PCB. Their activity on AHR

pathway is by far not as well understood as the one of dioxin like PCB that activates AHR. The non-dioxin-like PCB 180, one of the components of the mixture used in our study, is able to exert its anti-apoptotic activity through the activation of AHR (Raggi et al., 2016). Moreover, a mixture of different compounds can have diverse effects on cellular systems with respect to single component, as the result of synergistic or antagonistic mechanisms, as described by Ferrante et al. (2011) and Raggi et al. (2016). As far as DEHP is concerned, it is a weak activator of AHR, but it's also a ligand of peroxisome proliferator-activated receptors alpha and gamma (PPARα and PPARγ), and phthalates activation of PPAR pathways determine an increase of CYP1A1 expression (Sereè et al., 2004). The issue is further complicated by the fact that the expression of CYP1 genes is also strictly linked to estrogen signaling pathway. E2-dependent induction of CYP1A1 and CYP1B1 has been observed (Frasor et al., 2004); some studies reported that induction of CYP1 may increase the oxidative metabolism of estradiol (Spink et al. 1990, 1992); chromatin immunoprecipitation (ChIP) assays revealed insights on ERα-AHR cross talk and demonstrated the co-recruitment of both transcription factors to the same gene promoters (Beischlag and Perdew, 2005; Matthews et al., 2005). Furthermore, tissue-specific action of PCBs, DEHP and xenobiotics in general also depends on cell- and tumor types (Ghosh et al., 2010; Raggi et al., 2016), and at present, data on pituitary models are unfortunately very limited. Taking into account how much complex the AHR-mediated pathways are (Safe et al., 2013) and limited available data about pituitary cell behavior, further studies will be needed to definitely understand which pathways are involved in pituitary biology and carcinogenesis.

To understand the potential interferences of pollutants on GH-producing adenoma function, we focused our attention on SS pathway. Our data demonstrated an increase of *SSTR2* mRNA after 24 or 48 hours treatment with BZ, DEHP and PCB, followed by the decrease of mRNA levels back to baseline after 72 hours. On the other hand, all the three pollutants caused a reduction of the amount

of receptor detectable at Western blotting after 72 hours, suggesting thus that they may reduce the ability of SSTR2 to mediate SSA efficacy in GH-producing adenomas. As well established, the integrity of SS pathway is essential for a successful treatment with SSA (Melmed, 2003; Melmed 2006). Indeed, *SSTR2* expression at both mRNA and protein level is positively correlated with response to SSA (Ferone et al., 2008; Plockinger et al., 2008). *SSTR2* expression was reported to be lower in non-functioning pituitary adenoma as compared to somatotropinomas and normal pituitary tissue (Visser-Wisselaar et al., 1997). Resistance to SSA in acromegaly was initially suggested to be caused by reduced/absent *SSTR2* expression (Reubi et al., 1989). Estrogens have been shown to up-regulate the expression of *SSTR2* and *SSTR3* in rat pituitary (Visser-Wisselaar et al., 1997), and in breast cancer (Kumar et al., 2005) while, to our knowledge, no data have been reported on the effects of pollutants on SSTRs.

Finally, it is noteworthy the recent report by Cannavò and coworkers (2016) that patients from highly polluted areas (and even more in those carrying *AHR* and/or *AIP* variants) acromegaly is biochemically more severe and especially more resistant to SSA treatment and this last observation is totally consistent with our data.

Interestingly, in our study, ZAC1 expression was increased by BZ, DEHP, and PCB. Nevertheless, after a 72 hour treatment with either BZ, DEHP and PCB, both SSTR2 and ZAC1 gene expression levels returned to basal, and protein levels were reduced and this again is in agreement with the suggested increased biochemical aggressiveness and SSA treatment resistance induced by pollutant exposure. ZAC1 is a transcription factor and co-regulator with a crucial role in the regulation of cell proliferation (inducing apoptosis and G1 cell-cycle arrest), hormone synthesis and response to SSA treatment in pituitary adenomas (Theodoropoulou et al., 2006). ZAC1 target genes include adenylate cyclase activating polypeptide type 1 receptor (PAC1-R), peroxisome proliferator-activated receptor γ (PPARγ), and the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p57^{Kip2}$ via

coactivation of p53 and p73 (Theodoropoulou et al., 2010; Theodoropoulou et al., 2009). Its expression is induced by SSTR2 through a G-protein-mediated signaling pathway, and activated by p53 (Theodoropoulou et al., 2010). Experimental evidences suggest that SSA, through GSK3b phosphorylation, activate p53 and increase *ZAC1* expression. If p53 is knocked down, *ZAC1* levels decrease and the effect of octreotide on *ZAC1* transcription is abolished (Theodoropoulou et al., 2006).

It has to be recalled that although ZAC1 mainly acts as a powerful co-activator of several nuclear receptors (i.e. androgen, estrogen, glucocorticoid, and thyroid hormone), it is also a repressor of some other genes in different cell lines (Huang and Stallcup, 2000). We are thus looking at a factor interplaying with several different pathways, and it is likely that BZ, DEHP and PCB exert their effect on many other transcription factors, i.e. FOXA1.

Indeed, all the pollutants led to a remarkable increase of *FOXA1*, also known as HNF3α (hepatocyte nuclear factor 3α), a member of the FOXA transcription factor family, crucial and context-specific regulators of development during embryogenesis and early life. FOXA1 also mediates the action of steroid hormones at nuclear receptor level. In fact, it is involved in estrogen and androgen receptor signaling in normal conditions, and in breast and prostate cancer (Bernardo and Keri, 2012; Augello et al., 2011); it is expressed in rat pituitary, and is involved in tissue-specific expression of the human *GH* gene (Norquay et al., 2006). The increase in *FOXA1* expression, we observed, could contribute to enhanced GH secretion by these cells, but also suggests (together with the effect on ZAC1) that pollutants may have a direct effect on transcription regulators, thus modulating cell functioning at different levels. In addition to the remarkable effect on FOXA1 exerted by all the three chemicals, BZ induced PR expression, while DEHP and PCB enhanced ERα gene expression, suggesting a possible involvement of the estrogenic pathway. Moreover, it has to be reminded that activated AHR associates with unliganded ER, recruiting the co-activator p300 to estrogen-responsive gene promoters, finally activating transcription and estrogenic effects (Ohtake et al.,

2003). It was reported that aryl hydrocarbon receptor nuclear translocator 1 (ARNT1), an obligatory transcriptional partner of AHR, presents a D-box-regulated AF2 domain, which interacts with SRC1 exon 21, that in turn is an estrogen-related binding site for the ERα AF2 domain. These interactions increase estrogen related transcriptional responses (Endler et al., 2012). Both these observations were linked to the estrogenic effect of dioxin, but many other pollutants use the same pathway. Therefore, our findings in GH-secreting pituitary GH3 cells could be related to the interference of BZ, DEHP and PCB with different transcription regulators, linking together different signaling pathways, resulting, finally, in profound alterations of the endocrine balance (hormone production and its control) at a cellular level.

Finally, none of the tested pollutants affected cell growth or viability. Recently, Tapella et al. (2016) reported that 96 hour *in vitro* treatment with either BZ (250 pM- 1.25 nM) or DEHP (250 pM - 1.25nM) was able to increase cell viability and proliferation of primary cultures obtained from normal rat pituitary tissue. This different behavior may be explained by different exposure times or the different models used; pollutants could interfere with the growth of normal cells; whereas, the absence of effects on cancerous cells, may be due to an already dysregulated cell cycle.

5. Conclusions

Our study demonstrated for the first time that benzene, DEHP and PCB, widely diffused environmental pollutants, can modify the behavior of GH-producing rat pituitary cells by increasing GH secretion, and modulating the expression of *SSTR2*, as well as of transcription factors (e.g. *ZAC1* and *FOXA1*) involved in SSA and estrogen hormone signaling.

These observations support a possible influence of different pollutants *in vivo* on the modulation of the GH-adenoma aggressiveness and biological behavior. Future studies are looked-for to elucidate the true impact of environmental pollution on human pituitary adenoma aggressiveness and response to SSA treatment.

Conflict of interest

All authors of this manuscript do not have any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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LEGENDS TO FIGURES

Figure 1. Effect of BZ, DEHP and PCB on GH. mRNA expression of *GH* after treatments with 130 pM BZ (A), 250 pM DEHP (D) and 100 ng/l PCB (G) for 24, 48, 72 hours. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. control (N=3).Western blot for GH (B, E and H), anti-GAPDH was used to confirm equal loading; the image shows a typical experiment. Semiquantitative analysis (C, F and I) of western blot results of three independent experiments (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 2. Effect of BZ, DEHP and PCB on SSTR2. mRNA expression of *SSTR2* after treatments with 130 pM BZ (A), 250 pM DEHP (D) and 100 ng/l PCB (G) for 24, 48, 72 hours. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. control (N=3).Western blot for SSTR2 (B, E and H), anti-GAPDH was used to confirm equal loading; the image shows a typical experiment. Semiquantitative analysis (C, F and I) of western blot results of three independent experiments (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3. Effect of BZ, DEHP and PCB on ZAC1. mRNA expression of *ZAC1* after treatments with 130 pM BZ (A), 250 pM DEHP (D) and 100 ng/l PCB (G) for 24, 48, 72 hours. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. control (N=3).Western blot for ZAC1 (B, E and H), anti-GAPDH was used to confirm equal loading; the image shows a typical experiment. Semiquantitative analysis (C, F and I) of western blot results of three independent experiments (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

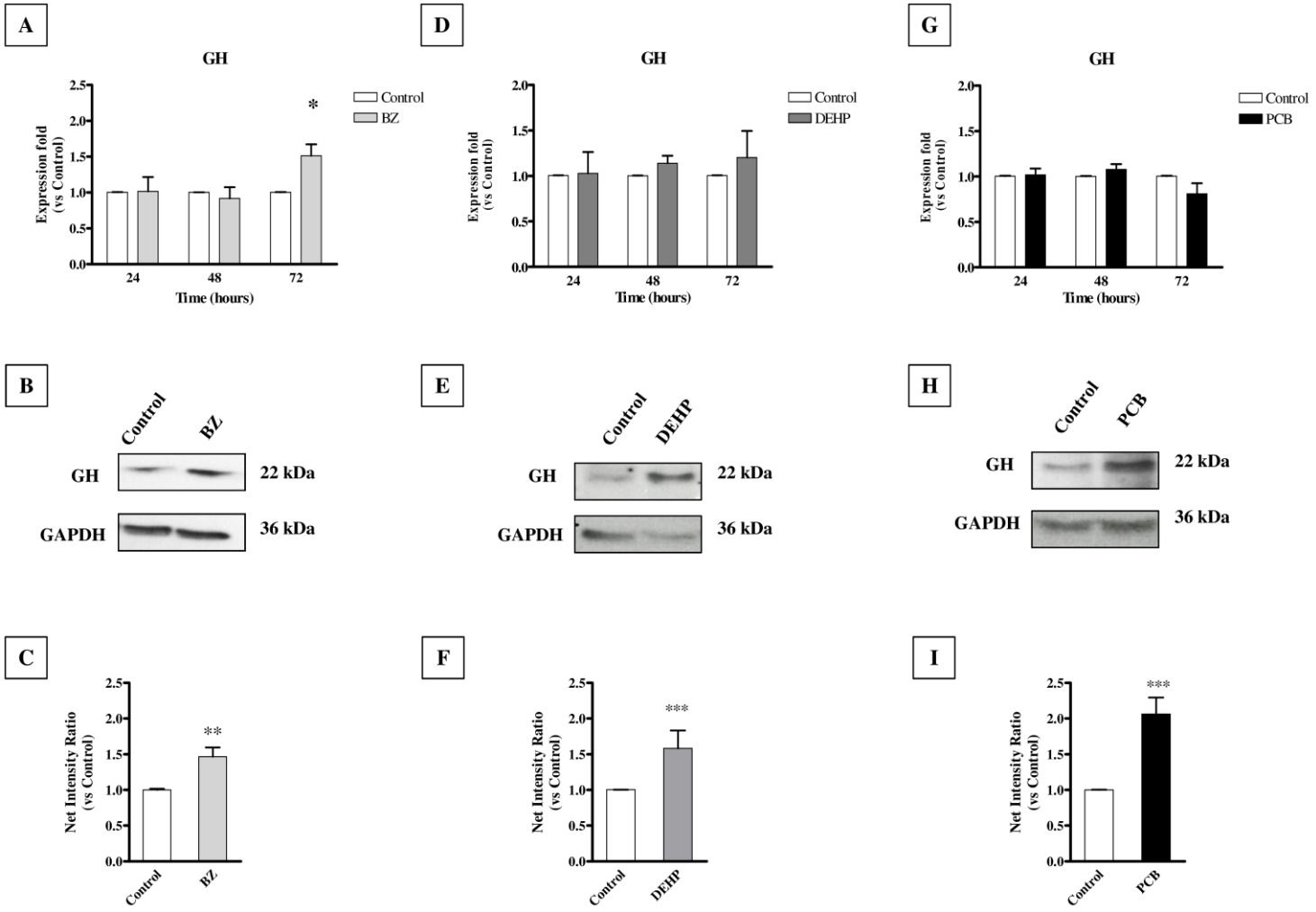
Figure 4. Effect of BZ, DEHP and PCB on *AIP, AHR* and *CYP1A1* gene expression. mRNA expression of *AIP*, *AHR* and CYP1A1 after treatment with 130 pM BZ, 250 pM DEHP and 100 ng/l PCB for 24, 48, and 72 hours. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. control (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 5. Effect of BZ, DEHP and PCB on *ERα, PR, FOXA1* gene expression. mRNA expression of *ERα*, *PR* and *FOXA1* after treatment with 130 pM BZ, 250 pM DEHP, and 100 ng/l PCB for 24, 48, and 72 hours. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. control (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Supplementary Figure 1. Basal gene expression at 24, 48, 72 hours. mRNA gene expression in cells maintained in basal conditions at 24, 48, and 72 hours. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. expression at 24 h (N=3).

Supplementary Figure 2. Basal protein levels at 24, 48, 72 hours. SSTR2 and ZAC1protein levels in cells maintained in basal conditions at 24, 48, and 72 hours. Results are expressed as Net Intensity Ratio vs GAPDH expression (N=3).

Supplementary Figure 3. **Effect of pollutants on cell viability (left panels).** Growth curve of GH3 cells after treatment with BZ (1.3 pM - 13 nM), DEHP (2.5 pM -2 5 nM) and PCB (1 ng/l - 10 μg/l) for 24, 48 and 72 hours, expressed as viability ratio between the considered time point and baseline. **Effect of pollutants on cell proliferation (right panels)**. GH3 cell proliferation after treatment with BZ (1.3 pM - 13 nM); DEHP (2.5 pM - 25 nM) and PCB (1 ng/l - 10 μg/l) for 24, 48 and 72 hours, expressed as 450 nm absorbance.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PCB

Figure 1

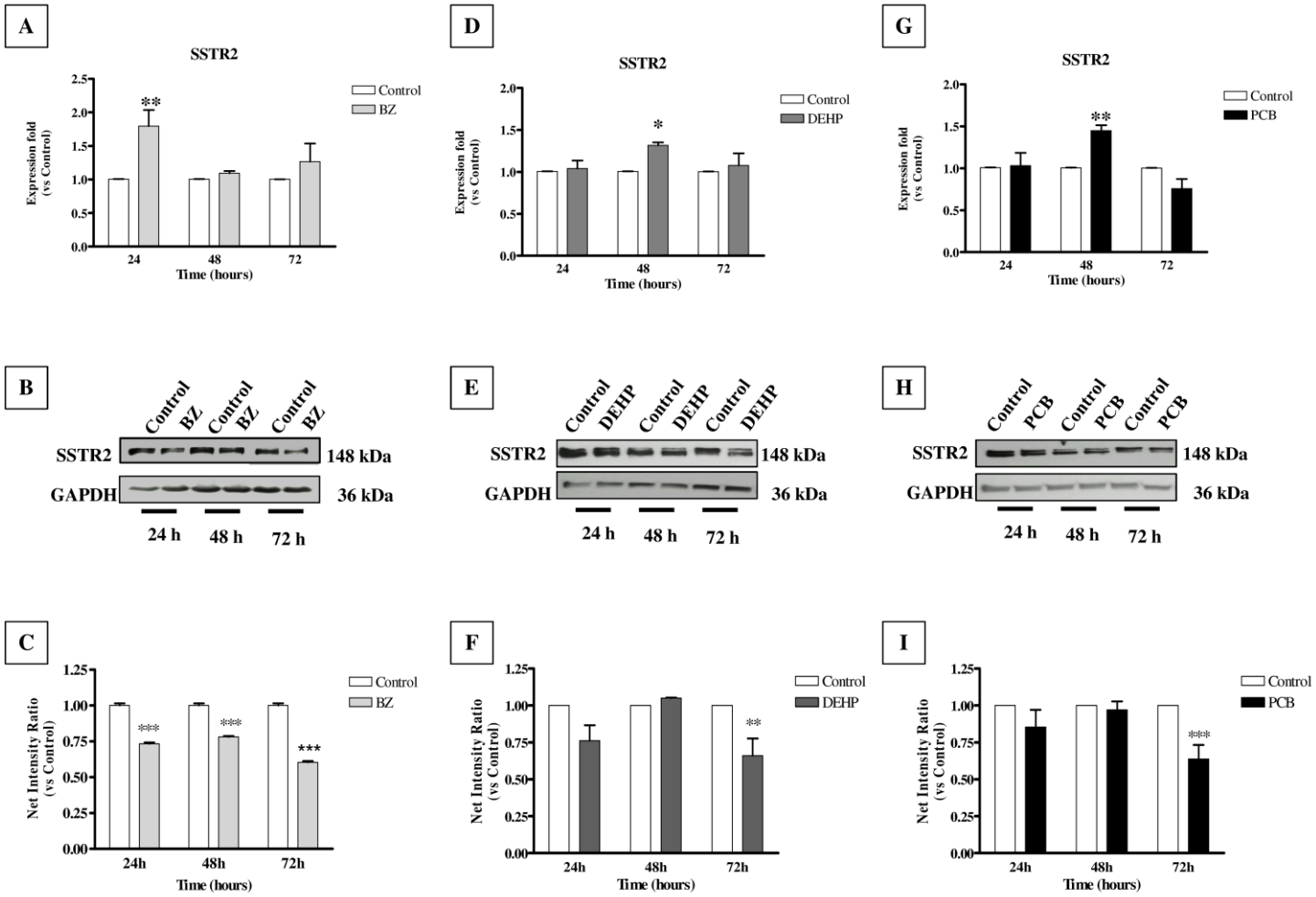

A
SSTR2
Expression fold (vs Control)
Control
BZ
Time (hours)
D
SSTR2
Control
DEHP
G
SSTR2
Control
PCB
B
Control
BZ
SSTR2
148 kDa
GAPDH
36 kDa
24 h
48 h
72 h
E
Control
DEHP
H
Control
PCB
C
Net Intensity Ratio (vs Control)
24h
48h
72h
F
I

Figure 2

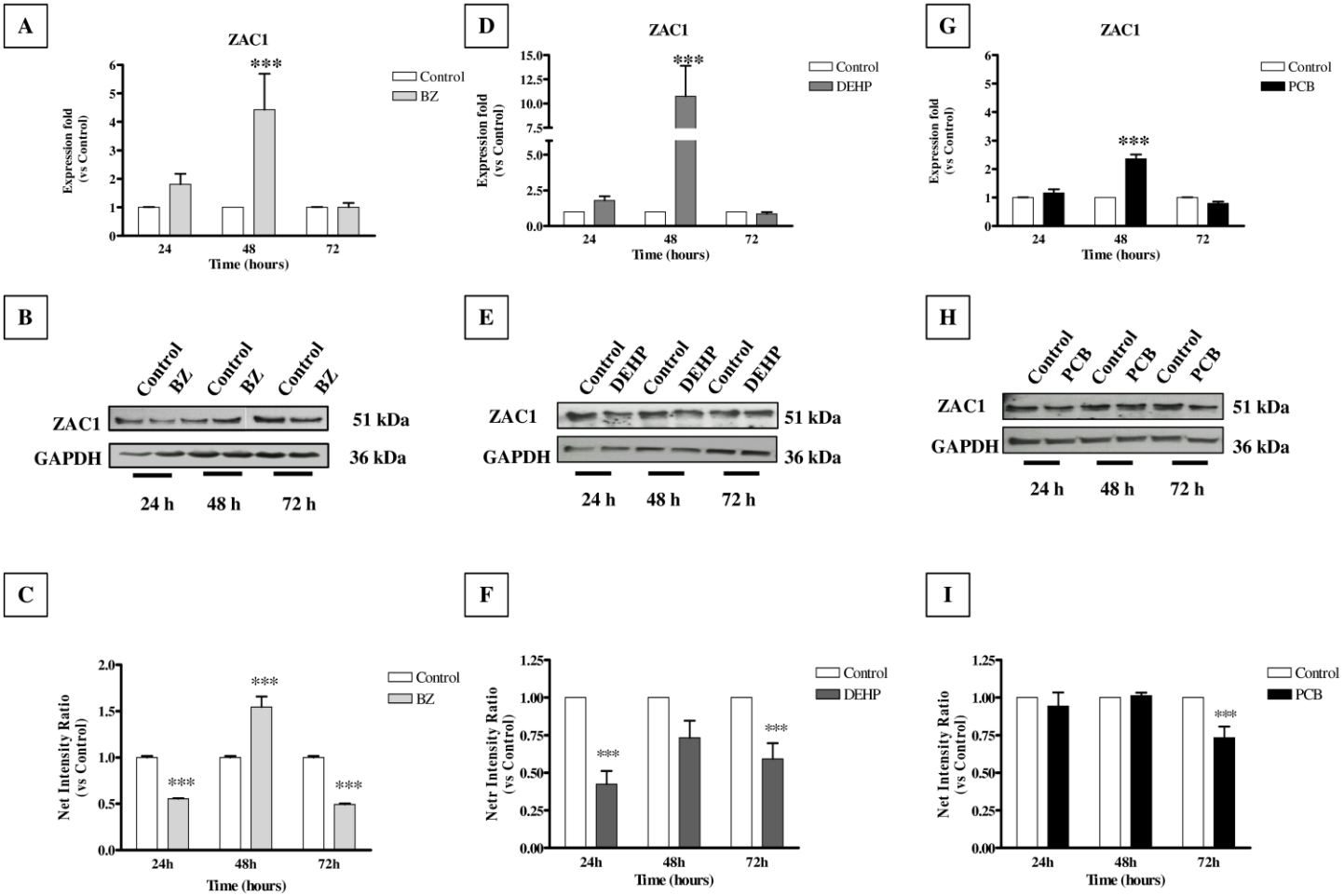

A
ZAC1
***
Control
BZ
Expression fold (vs Control)
Time (hours)
D
ZAC1
***
Control
DEHP
Expression fold (vs Control)
Time (hours)
G
ZAC1
***
Control
PCB
Expression fold (vs Control)
Time (hours)
B
Control
BZ
ZAC1
51 kDa
GAPDH
36 kDa
24 h
48 h
72 h
E
Control
DEHP
ZAC1
51 kDa
GAPDH
36 kDa
24 h
48 h
72 h
H
Control
PCB
ZAC1
51 kDa
GAPDH
36 kDa
24 h
48 h
72 h
C
Net Intensity Ratio (vs Control)
***
Control
BZ
Time (hours)
F
Netr Intensity Ratio (vs Control)
***
Control
DEHP
Time (hours)
I
Net Intensity Ratio (vs Control)
***
Control
PCB
Time (hours)

Figure 3

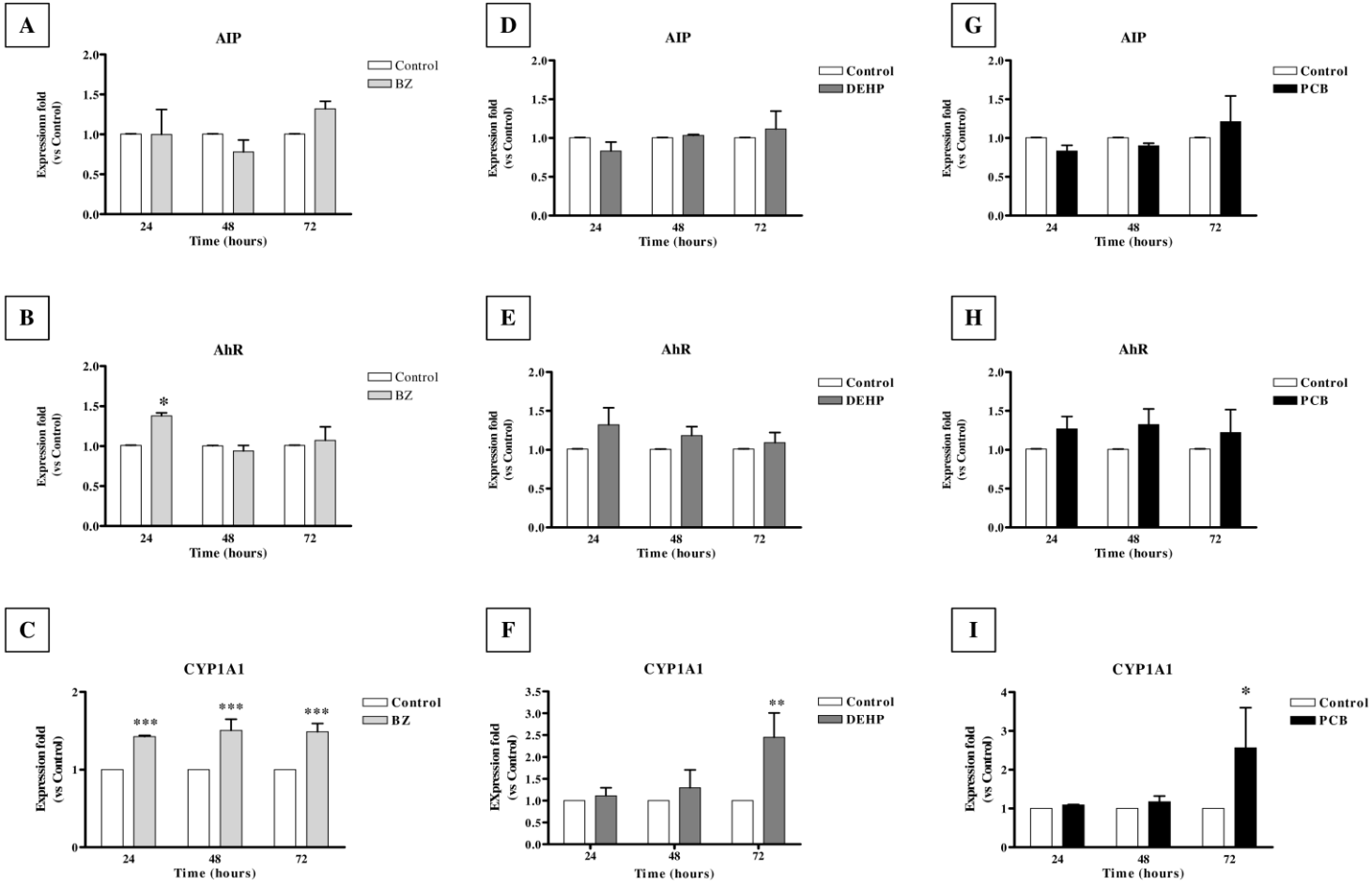

A
AIP
Control
BZ
Expressionn fold (vs Control)
Time (hours)
D
AIP
Control
DEHP
Expression fold (vs Control)
Time (hours)
G
AIP
Control
PCB
Expression fold (vs Control)
Time (hours)
B
AhR
Control
BZ
Expression fold (vs Control)
Time (hours)
E
AhR
Control
DEHP
Expression fold (vs Control)
Time (hours)
H
AhR
Control
PCB
Expression fold (vs Control)
Time (hours)
C
CYP1A1
Control
BZ
Expression fold (vs Control)
Time (hours)
F
CYP1A1
Control
DEHP
EXpression fold (vs Control)
Time (hours)
I
CYP1A1
Control
PCB
Expression fold (vs Control)
Time (hours)

Figure 4

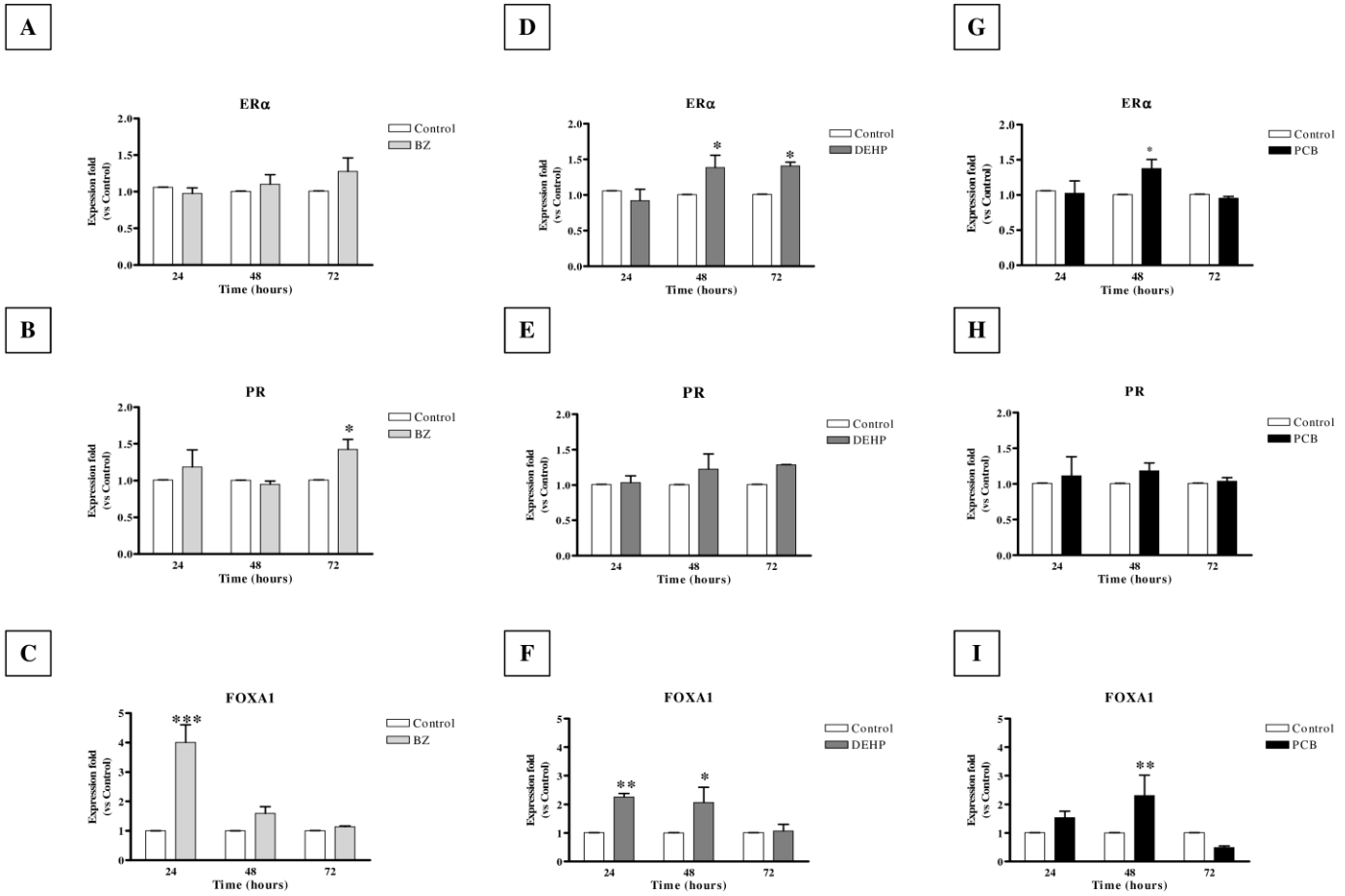
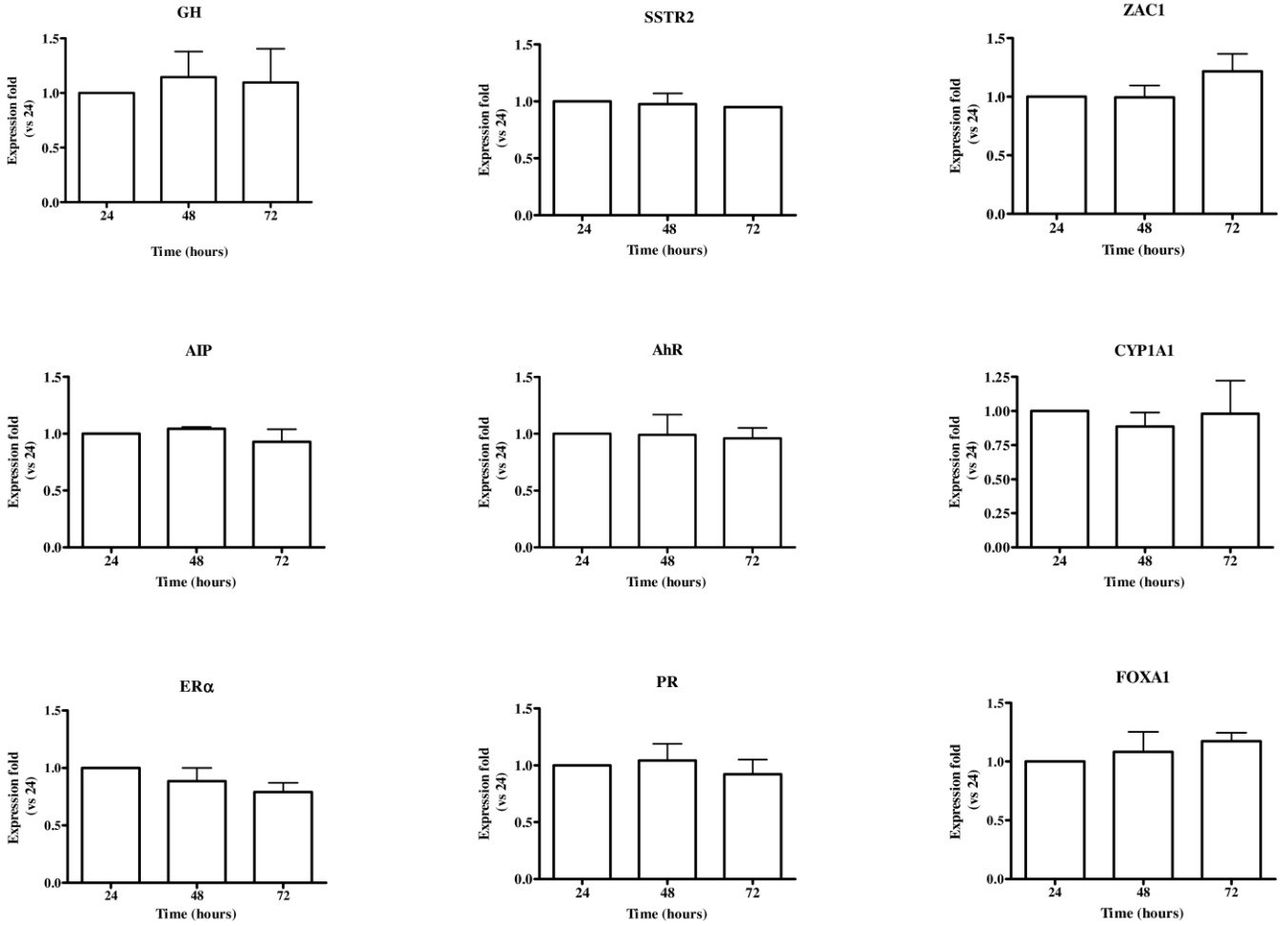
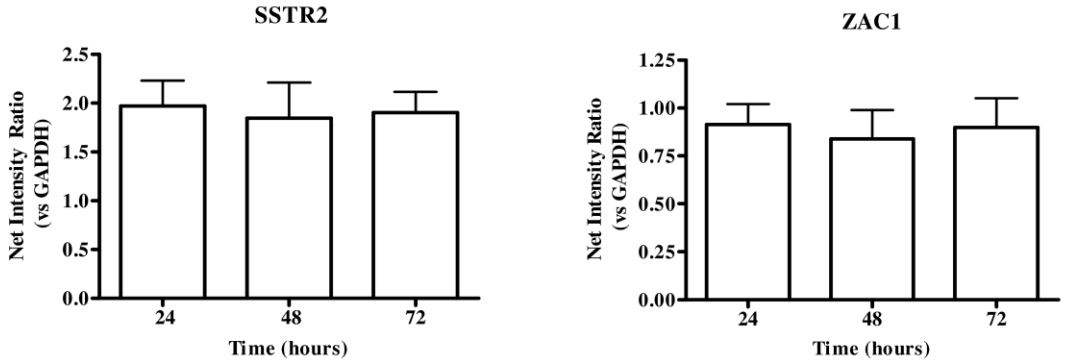

A
ERα
Control
BZ
Expression fold (vs Control)
Time (hours)
B
PR
C
FOXA1
D
ERα
DEHP
E
PR
F
FOXA1
G
ERα
PCB
H
PR
I
FOXA1

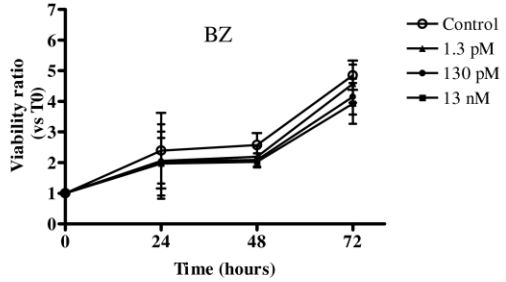
Figure 5

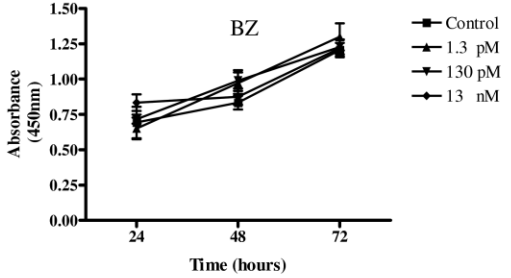


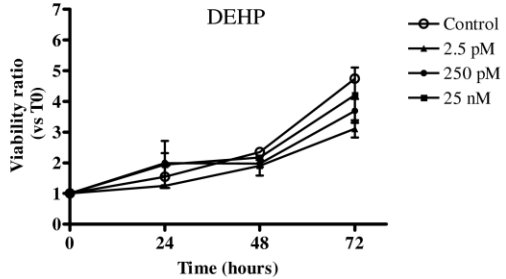
Supplementary Figure 1

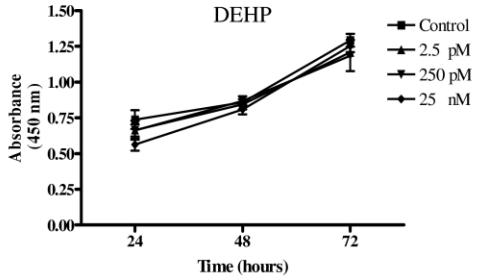


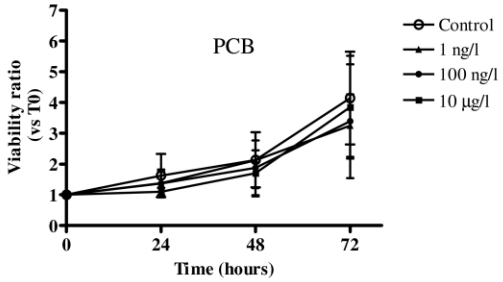
Supplementary Figure 2

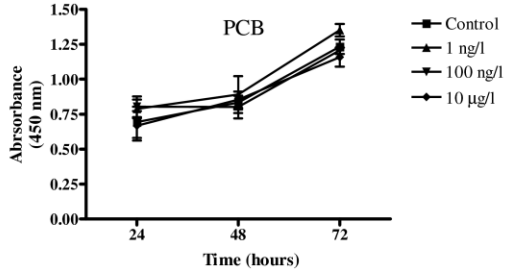












Supplementary Figure 3

Table 1. GH (ng/ml) secreted by GH3 cells after pollutant exposure for 72 hours. Significance *vs* control; $p<0.05$ *, $p<0.01$ **

	Control	BZ	DEHP	PCB
GH (ng/ml)	271 ± 17	440 ± 75 (**)	410 ± 6 (*)	513 ± 11 (**)

Supplementary Table 1- Primers for real time PCR

Primers		Sequences (5'-3')
GH	sense	TCGCTTCTCGCTGCTGCTC
	antisense	TGCTTGAGGATCTGCCCAATACG
PRL	sense	AAGACAAGGAACAAGCCCAGAAAG
	antisense	CAGAGGGTCATTCCAGGAGTGC
SSTR2	sense	TACTTCGTGGTGTGCGTGGTG
	antisense	CTTGGCGTAGCGGAGGATGAC
ZAC1	sense	GGCAAGAGGAGCGGAGAGC
	antisense	AATCAAGCAGGAACAACCACACG
AIP	sense	TCGTGCGTACCATGCGTGAG
	antisense	TGTTGCGGAGGCTCTTGGC
AhR	sense	ACCAGTGTAGAGCACAAGTCAGAG
	antisense	AGACGCATAGAAGACCAAGGCATC
CYP1A1	sense	GAGGAGTTAGACACAGTGATTGGC
	antisense	GGATGGTGAATGGGACAAAGGATG
ERα	sense	GCACATTCCTTCCTTCCGTCTTAC
	antisense	GCCGCCGAGGTACAGATTGG
PR	sense	CTGGATGAGCCTGATGGTGTTTG
	antisense	GGCACAGCGAGTAGAATGACAAC
FOXA1	sense	TTAGGGACTGTGAAGATGGAAGGG
	antisense	TCATGTTGCCGCTCGTGGTC
βACT	sense	CCACACCCGCCACCAGTTC
	antisense	GACCCATACCCACCATCACACC
β2–microglobulin	sense	TCTTTCTGGTGCTTGTCTCTCTGG
	antisense	CTATCTGAGGTGGGTGGAACTGAG
L13A	sense	AGGTGGTGGTTGTACGCTGTG
	antisense	GGTTGGTGTTCATCCGCTTTCG

Supplementary Table 2. Viable cells at 72 hours after treatment with different pollutants.

	Cells (10^6/ml)
Control	2.5 ± 0.7
BZ	2.7 ± 0.5
DEHP	2.3 ± 0.1
PCB	2.4 ± 0.4