Toxicology and Applied Pharmacology 334 (2017) 192–206

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

# A dioxin-like compound induces hyperplasia and branching morphogenesis in mouse mammary gland, through alterations in TGF-β1 and aryl hydrocarbon receptor signaling

Noelia Miret[a], Eva Rico-Leo[b], Carolina Pontillo[a], Elsa Zotta[c], Pedro Fernández-Salguero[b], Andrea Randi[a,*]

[a] Universidad de Buenos Aires, Facultad de Medicina, Departamento de Bioquímica Humana, Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Paraguay 2155, 5° piso, CP1121, Buenos Aires, Argentina
[b] Universidad de Extremadura, Facultad de Ciencias, Departamento de Bioquímica y Biología Molecular y Genética, Laboratorio de Biología Molecular del Cáncer, Avenida de Elvas s/n, CP06080 Badajoz, Spain
[c] Universidad de Buenos Aires, Facultad de Medicina, Departamento de Ciencias Fisiológicas, Sección Patología, Laboratorio de Fisiopatogenia, Paraguay 2155, 7° piso, CP1121, Buenos Aires, Argentina

ARTICLE INFO

*Keywords:*
Hexachlorobenzene
Endocrine Disruptor
Transforming Growth Factor β1
Aryl Hydrocarbon Receptor
Mouse Mammary Gland

ABSTRACT

Hexachlorobenzene (HCB) is a widespread environmental pollutant and a dioxin-like compound that binds weakly to the aryl hydrocarbon receptor (AhR). Because AhR and transforming growth factor β1 (TGF-β1) converge to regulate common signaling pathways, alterations in this crosstalk might contribute to developing preneoplastic lesions. The aim of this study was to evaluate HCB action on TGF-β1 and AhR signaling in mouse mammary gland, through AhR +/+ and AhR −/− models. Results showed a differential effect in mouse mammary epithelial cells (NMuMG), depending on the dose: 0.05 μM HCB induced cell migration and TGF-β1 signaling, whereas 5 μM HCB reduced cell migration, promoted cell cycle arrest and stimulated the dioxin response element (DRE) -dependent pathway. HCB (5 μM) enhanced α-smooth muscle actin expression and decreased TGF-β receptor II mRNA levels in immortalized mouse mammary fibroblasts AhR +/+, resembling the phenotype of transformed cells. Accordingly, their conditioned medium was able to enhance NMuMG cell migration. Assays in C57/Bl6 mice showed HCB (3 mg/kg body weight) to enhance ductal hyperplasia, cell proliferation, estrogen receptor α nuclear localization, branch density, and the number of terminal end buds in mammary gland from AhR +/+ mice. Primary culture of mammary epithelial cells from AhR +/+ mice showed reduced AhR mRNA levels after HCB exposure (0.05 and 5 μM). Interestingly, AhR −/− mice exhibited an increase in ductal hyperplasia and mammary growth in the absence of HCB treatment, thus revealing the importance of AhR in mammary development. Our findings show that environmental HCB concentrations modulate AhR and TGF-β1 signaling, which could contribute to altered mammary branching morphogenesis, likely leading to preneoplastic lesions and retaining terminal end buds.

## 1. Introduction

Environmental exposure can alter mammary gland (MG) development, impair lactation and increase susceptibility to cancer (Rudel et al., 2011). Hexachlorobenzene (HCB) is an environmental pollutant which was used as a fungicide in the past and is still released into the environment as a byproduct from several industrial processes. The International Agency for Research on Cancer has classified HCB as a possible human carcinogen (ATSDR, 2015) and several studies demonstrate the presence of this pollutant in human milk, baby formula, and cow milk for human consumption (Chen et al., 2014; Der Parsehian, 2008). We have previously reported that HCB activates estrogen receptor α (ERα) and induces estrogen-like responses in human breast cancer cells acting as an endocrine disruptor (ED) (García et al., 2010), while other authors have revealed that some EDs affect MG development (Fenton, 2006).

HCB is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR) (Hahn et al., 1989). The AhR is a ligand-dependent transcription factor that binds to its DNA consensus sequence known as the dioxin response element (DRE) and regulates the expression of many detoxification genes such as cytochrome P450 1A1 (CYP1A1). Furthermore, after dioxin activated-AhR, c-Src is released

* Corresponding author.
*E-mail addresses:* evaricoleo@unex.es (E. Rico-Leo), pmfersal@unex.es (P. Fernández-Salguero), arandi@fmed.uba.ar (A. Randi).

http://dx.doi.org/10.1016/j.taap.2017.09.012
Received 31 January 2017; Received in revised form 12 September 2017; Accepted 14 September 2017
Available online 18 September 2017
0041-008X/ 

from its cytosolic complex and stimulates growth factor receptors, including the transforming growth factor -β (TGF-β) receptor II (TβRII) (Galliher and Schiemann, 2006) and human epidermal growth factor receptor (HER1) (Park et al., 2007). AhR has been studied because of its role in the toxic effects caused by environmental pollutants, including dioxins, organochlorine compounds, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. Evidence has shown that this receptor plays an important role in normal physiologic functions such as development, cell cycle regulation, and immune response (Murray et al., 2014; Safe et al., 2013). Moreover, Hushka et al. (1998) have demonstrated that the inactivation of AhR results in impaired mammary development and lactation. However, the role of the AhR in cancer is complex, with clear discrepancies between pro- and anti-tumorigenic activities (Bekki et al., 2015; Jin et al., 2012; Pontillo et al., 2013).

TGF-β1 is a pluripotent cytokine critically important in mammary morphogenesis and significantly activated during breast cancer development (Moses and Barcellos-Hoff, 2011). It has been reported that TGF-β1 plays a dual role: it limits proliferation in epithelial cells but induces epithelial-mesenchymal transition (EMT) in carcinoma cells, accelerating cancer progression and metastasis (Lebrun, 2012). In the canonical pathway, TGF-β1 engagement of TβRII leads to the phosphorylation and activation of TGF-β receptor I (TβRI), which subsequently phosphorylates Smad2/3. Once activated, Smad2/3 rapidly interacts with Smad4 and this complex translocates to the nucleus to regulate the expression of TGF-β-responsive genes. In addition, TGF-β1 also stimulates an ever expanding array of molecules, including ERK1/2, p38, JNK, and PI3K/Akt (Parvani et al., 2011). Recently, we have reported that HCB activates TGF-β1 and enhances Smad3, p38, and JNK phosphorylation levels in the ERα-negative MDA-MB-231 breast cancer cell line (Miret et al., 2016). TGF-β1 and AhR signaling interact both in reciprocal regulation and toward common targets such as cell cycle control (Haarmann-Stemmann et al., 2009). Wolff et al. (2001) have demonstrated that TGF-β1 triggers cell type-specific effects on AhR by inhibiting receptor expression and activation in lung cancer cells, while it enhances receptor function in hepatoma cells. Furthermore, analyses in cell systems and mouse models lacking AhR expression have demonstrated that AhR is relevant to maintain TGF-β1 activity. The upregulation of this cytokine could have a role in producing some of the phenotypes identified in AhR-depleted mice (Gómez-Duran et al., 2009).

Understanding the mechanisms regulating normal MG development may contribute to explaining how tumors promote their own growth and invasion, changing these normal mechanisms. Given our previous observations in mammary cells and animal models, and considering epidemiological data available, we hypothesize that HCB could act as a risk factor in breast cancer. Unlike its well characterized mechanisms of action in breast cancer (García et al., 2010; Miret et al., 2016; Peña et al., 2012; Pontillo et al., 2011, 2013; Randi et al., 2006), little is known about HCB effects on normal MG. Therefore, the aim of the present study was to investigate HCB effects on mouse normal MG by means of in vitro and in vivo assays. To examine the role of AhR, we worked with wild-type (AhR +/+) and AhR-null (AhR −/−) mice, as well as AhR +/+ and AhR −/− mammary epithelial and fibroblast cells. HCB action was analyzed in terms of cell proliferation, cell cycle, cell migration, and AhR and TGF-β1 signaling pathways, as well as MG structure and histology. Because stromal cells dictate the different mammary side-branching patterns (Sternlicht, 2006), conditioned medium (CM) assays were conducted to determine whether different factors secreted by HCB-treated fibroblasts have impact on epithelial cells.

## 2. Materials and methods

### 2.1. Chemicals

HCB (> 99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). Anti-Smad3, anti-phospho-Smad3, anti-phospho T202/Y204 ERK1/2, anti-p38, and anti-phospho-p38 antibodies were purchased from Cell Signaling Technology, Inc. (MA, USA). Anti-ERα antibody was obtained from Chemicon International Inc. (CA, USA). Anti-progesterone receptor (PR) antibody was purchased from Santa Cruz Biotechnology, Inc. (TX, USA). Anti-AhR antibody was obtained from Abcam Ltd. (Cambridge, UK). Anti-α-smooth muscle Actin (α-SMA), anti-β-Actin and anti-Vimentin (V9) antibodies, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), diaminobenzidine tetrahydrochloride (DAB) tablets, dimethyl sulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), epidermal growth factor (EGF), insulin, bovine serum albumin (BSA), α-naphthoflavone (ANF) inhibitor, SB431542 inhibitor and carmine alum were obtained from Sigma-Aldrich Chemical Co. (MO, USA). Anti-ERK1/2 antibody was purchased from Upstate (NY, USA). Anti-Keratin 14 (AF64) antibody was obtained from Covance (NJ, USA). Anti-proliferative cell nuclear antigen (PCNA) antibody was from Dako Laboratories (CA, USA). The enhanced chemiluminescence kit (ECL) was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). The precision plus protein dual color standard and the Iscript reverse transcription supermix were obtained from Bio-Rad Laboratories (CA, USA). The high pure RNA isolation kit and collagen were purchased from Roche (IN, USA). The collagenase III was obtained from Gibco, Invitrogen Life Technology (MA, USA). The specific oligonucleotides for mouse AhR, CYP1A1, HER1, insulin-like growth factor-1 (IGF-I), TGF-β1, TβRI, and TβRII were purchased from Integrated DNA Technologies Inc. (IA, USA). The UltraTek HRP (Anti-Polyvalent) was obtained from Scytek Laboratories (UT, USA). The anti-E-cadherin antibody was graciously provided by Dr. Ibarra (Universidad de Buenos Aires), and anti-P-cadherin antibody by Dr. Vazquez-Levin (IBYME, CONICET). All other reagents used were of analytical grade.

### 2.2. Mice and treatment

Wild-type (AhR +/+) and AhR-null (AhR −/−) C57BL/6 N mice were produced by homologous recombination in embryonic stem cells as previously described (Fernandez-Salguero et al., 1995). Mice were maintained in a controlled environment: 24 ± 2 °C, 50 ± 10% relative humidity, and a 12–12 h light/dark cycle. Five-week-old virgin female AhR +/+ and AhR −/− mice were randomly separated in four groups (control AhR +/+, HCB-treated AhR +/+, control AhR −/− and HCB-treated AhR −/−) with 5 animals each one. Animals were treated humanely and with regard for alleviation of suffering. All the experiments involving animals were performed following the guidelines established by the Animal Care and Use Committee of the University of Extremadura. HCB (3 mg/kg body weight) was dissolved in corn oil and mice were treated by intraperitoneal injection (i.p.) (0.1 ml) four times a week for 21 days. Control animals were injected i.p. corn oil (0.1 ml) as vehicle. Treatment with HCB had no effect on the general health of the animals, as shown by the healthy appearance of mice and no effect on body weight or water and food consumption. The stages of the estrous cycle were determined using vaginal smears and the animals were euthanized on the morning of the first estrous phase. The fourth MG both right and left were removed under sterile conditions and processed for whole mounts and immunohistochemical studies. The HCB doses used in our experiments were chosen after reviewing the relevant literature. HCB (3 mg/kg b.w.) was assayed previously in other toxicological studies and had an androgenic effect in mice (Ralph et al., 2003). Besides, HCB (3 mg/kg b.w.) was assayed previously in our laboratory and found to promote tumor growth and metastatic focus in mice lungs (Pontillo et al., 2013). Although in this study, the HCB

internal dose in mice was not measured, we think that this should be similar those found in human populations. This is because when HCB (500 mg/kg b.w. in water solution) was administered to rats by gavage, the serum HCB concentration was 600 ng/ml (Chiappini et al., 2009). Assuming that HCB distribution is similar in rats and mice, we hypothesized that herein, the HCB serum concentration in mice could be at least 3 ng/ml. In this respect, To Figueras et al. (1997) have found 32 ng/ml of HCB in human serum, in a highly contaminated population in Spain. In addition, the HCB dose used in the current study is similar to that found in human serum samples from different populations (Guo et al., 2014; Mrema et al., 2013). Van Birgelen (1998) has reported that the no-observed-effect level (NOEL) is estimated to be 0.38 mg/kg/day for neoplastic liver nodules and adrenal pheochromocytomas in female rats in a two-generation study.

### 2.3. Primary culture and treatment

The primary cultures for each experiment were obtained from the same mouse and at least three independent experiments were performed using mice from different litters. Epithelial cells of MG (EMG) were isolated from 8 to 9-week-old female AhR +/+ and AhR −/− mice and called EMG AhR +/+ and EMG AhR −/−, respectively. The third, fourth, and fifth MG were removed under sterile conditions, finely minced and sequentially digested with 2 mg/ml collagenase Type-III at 37 °C, with constant shaking for 3 h at 200 rpm. Samples were then centrifuged at 1000 rpm for 5 min. The pellets were resuspended in Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DMEM-F12) and centrifuged at 800 rpm for 5 min. The pellets were incubated with DMEM-F12 containing 2 U/ml DNAse for 2 min and the reaction was stopped by addition of DMEM-F12 with 4% fetal bovine serum (FBS). The samples were then centrifuged at 1000 rpm for 10 min and organoids were purified by differential centrifugation in phosphate buffer saline (PBS) with 2% FBS. Finally, organoids formed monolayers when plated in dishes coated with collagen in DMEM-F12 medium containing 10% FBS, 5 ng/ml EGF, 10 mg/ml insulin, 5 mg/ml BSA and 50 mg/ml penicillin/streptomycin (growth medium), and cultured at 37 °C in a 5% $CO_2$ incubator. When detected, growing fibroblasts were removed by differential trypsinization. To confirm the phenotypic identity, living cells were observed under a transmitted light microscope for morphology and immunostaining analyses was performed. For RT-qPCR assays, cells at 70–80% confluence were incubated in FBS-free medium and 24 h later treated with HCB (0.05 and 5 μM) dissolved in absolute ethanol (EtOH) in growth medium containing 5% FBS for 24 h. The treatment with EtOH was used as vehicle control. The highest HCB dose used (5 μM) was similar to that found in human serum samples from a highly contaminated population (To-Figueras et al., 1997). In addition, the HCB dose of 0.5 μM is comparable to that observed in human serum samples from general population in France (Saoudi et al., 2014). The MLR (maximum limit residue) established by the European Commission for fruits and vegetables is 0.01 μM HCB, which is similar to 0.05 μM used in this study. Besides, it has been reported that HCB daily intake in lipid-rich foods in humans is 0.2 μg/day (Burton and Bennett, 1987).

### 2.4. Cell culture and treatment

Mouse mammary epithelial cell line NMuMG (Sigma-Aldrich) was cultured in DMEM supplemented with 10% FBS, 10 mg/ml insulin, 50 mg/ml penicillin/streptomycin, and 1% glutamine (complete growth medium). Cells were seeded in 6-well plates in DMEM complete growth medium and 24 h later changed to Roswell Park Memorial Institute (RPMI) medium without phenol red and FBS. The next day, cells at 70–80% confluence were exposed to HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle in phenol red free-RPMI 5% FBS. The treatment with EtOH was used as vehicle control. For the inhibitor treatments, the cells were pretreated for 1 h with 2 μM SB431542, which is an inhibitor of TβRI that prevents Smad phosphorylation, and 1 μM ANF for AhR. Because both inhibitors were dissolved in DMSO, a control with DMSO was performed. Then, the HCB or EtOH was added to the media in the presence or absence of the inhibitors. FGM AhR +/+ and FGM AhR −/− cell lines are derived from breast fibroblasts of AhR +/+ and AhR −/− C57BL/6 N mice (Mulero-Navarro et al., 2005). These cells were cultured in DMEM-F12 supplemented with 10% FBS, 50 ng/ml penicillin/streptomycin and 1% glutamine. After 24 h of starvation, FGM cells were exposed to HCB (0.005, 0.05, 0.5, and 5 μM) or EtOH in DMEM-F12 with 5% FBS. For assays with CM, FGM AhR +/+ and FGM AhR −/− were exposed to HCB (0.05 and 5 μM) or EtOH for 48 h and the CM was collected, centrifuged at 500 x g and supplemented with 100 μg/μl BSA and 100 μg/μl PMSF for conservation. The CM was added to fresh NMuMG culture media for up to two thirds of the final volume and this was used to grow NMuMG cells.

### 2.5. Whole mount evaluation

Each whole MG specimen was spread on a glass slide and fixed in Carnoy's solution (100% ethanol:chloroform:glacial acetic acid, 6:3:1) for 2 h at room temperature. Then, glands were washed with 70% ethanol for 15 min, followed by a wash with distilled water for 5 min. Gland staining was performed in Carmine Alum (1 g carmine and 2.5 g aluminum potassium sulfate in 500 ml water) at 4 °C overnight. Tissues were then dehydrated and mounted in mounting medium. Images of MG whole mounts were recorded using an Olympus BX50 F-3 microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The distance from the lymph node to the end of the growing edge of the MG was measured between parallel lines drawn in each image and results were expressed in relation to fat pad total area. Branch density was calculated as the number of branch points of the whole MG divided by fat pad total area. All teardrop-shaped ductal end structures measuring 0.03 $mm^2$ or more in area were counted as terminal end buds (TEBs) in relation to fat pad total area in each sample, as previously established (Fenton, 2009). All these parameters were analyzed with ImageJ software (National Institute of Health, MD, USA).

### 2.6. Histology and immunohistochemistry

MG were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5-μm sections for histochemical evaluation. Sections were stained with hematoxylin-eosin for morphological studies. The leading edge and TEBs were localized and the ducts located at 400 μm from the most proximal TEB were counted as described Murray et al. (2007). The breast ducts were classified into: normal, when the linings of the breast ducts contained no more than three layers of cells; or hyperplastic, if there was an increase in the number of cells that constituted this ductal lining. For immunohistochemical analysis, sections were deparaffinized in xylene and rehydrated through graded alcohols, followed by microwaving in 10 mM sodium citrate buffer for antigen retrieval. Non-specific staining was blocked by incubation of the sections with Super Block (UltraTek HRP Anti-Polyvalent Lab Pack, ScyTek Laboratories, Logan, USA) for 10 min at room temperature. Tissue sections were incubated with ERα (dilution 1:50), PR (dilution 1:50), PCNA (dilution 1:50), E-cadherin (dilution 1:50), and P-cadherin (dilution 1:50) antibodies overnight at 4 °C. Negative control slides consisted of sections incubated with the vehicle antibody (1% BSA in PBS 1 ×). The samples were then incubated with Ultra Tek Anti-Polyvalent (UltraTek HRP Anti-Polyvalent Lab Pack, ScyTek Laboratories) for 10 min at room temperature; subsequently, they were incubated with UltraTek HRP (UltraTek HRP Anti-Polyvalent Lab Pack, ScyTek Laboratories) at room temperature for 10 min. Signal detection was carried out using DAB substrate kit and lightly counterstaining with hematoxylin. Three slides for each animal were analyzed and all tissue sections were examined by two blinded observers using an × 60 objective lens on a light microscope (Nikon, Tokyo, Japan) connected to a digital camera. Ten fields

of immunostained sections were randomly chosen and captured from each specimen, and the percentage of positive cells was established.

### 2.7. Immunofluorescense assay

EMG and FGM cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 24 h. After washing, cells were fixed with paraformaldehyde for 10 min and incubated with blocking buffer (2% BSA, 0.05% Triton X-100 in PBS) for 30 min at room temperature. FGM cells were incubated with anti-α-SMA (1:200) mouse monoclonal antibody, whereas EMG cells were incubated with anti-Keratin 14 (1:500) rabbit polyclonal antibody and anti-Vimentin (1:100) mouse monoclonal antibody, in blocking buffer for 24 h at 4 °C in a humidified chamber. Finally, cells were incubated with secondary antibodies Alexa 633 anti-rabbit IgG (1:250) or Alexa 488 anti-mouse IgG (1:250) for 1 h at room temperature in the dark. DAPI was used to stain cell nuclei. Five non-overlapping, randomly chosen images were obtained using FV1000 Confocal microscope (Olympus). Quantification of the positive cells/total cells ratio was normalized to arbitrary units, and at least 2500 cells were counted per treatment.

### 2.8. Flow cytometry analysis

NMuMG and FGM cells were starved and then exposed to HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 12, 24, and 36 h. Cells were released from the plates by the addition of 0.25% trypsin, fixed at 4 °C in 70% cold ethanol, centrifuged and treated with RNase (10 μg/ml) for 30 min at 37 °C. DNA content per cell was determined in a Cyan flow cytometer (DAKO Cytomation) after staining with propidium iodide (50 μg/ml) for 15 min at room temperature in the dark. Cell cycle distribution was calculated from DNA histograms with Cylchred 1.0.2 software (Cardiff University, UK). For cell cycle analysis, only signals from single cells were considered (10,000 cells/sample).

### 2.9. Migration assay

NMuMG and FGM cell migration was evaluated by the scratch motility assay as previously described (Miret et al., 2016). The scratched area was photographed at 0 and 12 h for NMuMG and at 0 and 9 h for FGM, taken into account the doubling time and the migration ability of each cell line. The distance of wound healing in each well was evaluated and the migration rate was calculated by D t0 − D tf / (D t0 × 100). D t0 = distance at 0 h; D tf = distance at 12 or 9 h, respectively.

### 2.10. MTT assay

NMuMG and FGM cell viability were analyzed after 24 h of HCB (0.005, 0.05, 0.5, and 5 μM) treatment. Cells were incubated at 37 °C for 1 h with 150 μg/ml MTT in basic saline solution [137 mM NaCl, 3.5 mM KCl, 0.4 mM $KH_2PO_4$, 0.33 mM $Na_2HPO_4{\cdot}7H_2O$, 5 mM *N*-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid pH 7.4, 10 mM D-glucose]. Formazan, as a precipitate generated by mitochondrial dehydrogenase activity in living cells, was dissolved in DMSO and measured using spectroscopy as the difference in absorbance at 490 and 650 nm. Data are shown as a percentage of control cultures, which was regarded as 100%.

### 2.11. RNA preparation and reverse transcription (RT)-quantitative PCR (qPCR)

Total RNA was isolated with the High Pure RNA Isolation kit, following the manufacturer's instructions. Aliquots of 1 μg RNA were reverse-transcribed at 42 °C for 30 min using Reverse Transcription Supermix for RT-qPCR. Expression levels of AhR, CYP1A1, HER1, IGF-I, TGF-β1, TβRI, and TβRII were analyzed using specific primers (Table 1). SYBR-Green I/QTaq DNA polymerase mix was used on the iCycler equipment (Bio-Rad). Cycling conditions were as follows: denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s (50 cycles). GAPDH mRNA was used to normalize gene expression (Ct) and $2^{-\Delta\Delta Ct}$ to calculate variations with respect to control (fold change).

### 2.12. Western blotting

Total cell protein lysates were prepared, the protein concentration was determined according to Bradford (1976), and 40 μg of protein was resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were then blotted for P-Smad3 (1:250), P-ERK1/2 (1:500), and P-p38 (1:500) and then reblotted for Smad3 (1:500), ERK1/2 (1:500), p38 (1:500), AhR (1:500), and β-Actin (1:1000) as previously described (Miret et al., 2016).

### 2.13. Statistical analysis

Data were evaluated by one-way ANOVA. For the experiments with NMuMG cells, the results were analyzed using Dunnett's post-hoc test to identify significant differences between controls and treatments. For assays with FGM cells, epithelial primary cultures and mice, we used Tukey post-hoc test to identify not only the effect of treatment but also differences between AhR +/+ and AhR −/−. Differences were considered significant when p values were $< 0.05$. Results represent the mean ± SD of at least three independent experiments.

## 3. Results

### 3.1. HCB reduces epithelial cell viability and induces cell cycle arrest

We have previously reported that 0.5 and 5 μM HCB induce a significant loss in cell survival in rat thyroid cells (Chiappini et al., 2013). In this work, MTT colorimetric assay was performed to evaluate HCB (0.005, 0.05, 0.5, and 5 μM) action on the viability of epithelial NMuMG cells and fibroblasts FGM AhR +/+ and FGM AhR −/−. In the MTT assay, the lack of mitochondrial integrity and activity (MTT uptake) was interpreted to represent low cell viability. HCB significantly decreased cell viability at 0.05, 0.5, and 5 μM in NMuMG cells (Fig. 1A). However, no cytotoxicity was observed in FGM AhR +/+ or FGM AhR −/− cells after pesticide exposure (Fig. S1A–B).

Because AhR is involved in cell signaling pathways critical to cell cycle regulation and apoptosis (Gasiewicz et al., 2008), we next studied whether pesticide exposure affected cell cycle progression. Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) for 12, 24, and 36 h, and the number of cells in each part of the cell cycle was analyzed by flow cytometry assays. The pesticide increased the number of epithelial cells in G0/G1 at 0.5 μM after 36 h of exposure and at 5 μM at all times assayed (Fig. 1B–D). Furthermore, the number of cells in the S phase was reduced at 5 μM HCB after 12 h of exposure (Fig. 1B) and at all doses assayed after 36 h (Fig. 1D). These data suggest that HCB exposure arrested epithelial cells in the G0/G1 phase, and thus inhibited entry into the S phase. No changes were observed in the cell cycle of FGM AhR +/+ and FGM AhR −/− exposed to HCB (Fig. S1C–H), which might indicate that pesticide actions are cell context-dependent.

### 3.2. HCB alters NMuMG cell migration involving the AhR and TGF-β1 signaling pathways

Increasing evidence demonstrates that AhR can stimulate cell migration, and we have reported that HCB induces cell migration in the MDA-MB-231 breast cancer cell line (Pontillo et al., 2011). These facts prompted us to evaluate epithelial and fibroblast cell migration after HCB (0.005, 0.05, 0.5, and 5 μM) exposure. Results from wound-

**Table 1**
Sequences of primers used in quantitative PCR assay.

| | Forward | Reverse |
|---|---|---|
| AhR | 5′AGCCGGTGCAGAAAACAGTAA′3 | 5′AGGCGGTCTAACTCTGTGTTC′3 |
| CYP1A1 | 5′ACAGACCTCATTGAGCACAG′3 | 5′GGCTCCACGAGATAGCAGTT′3 |
| TGF-β1 | 5′GGACTCTCCACCTGCAAGAC′3 | 5′GACTGGCGAGCCTTAGTTTG′3 |
| TβRI | 5′GGCGAAGGCATTACAGTGTT′3 | 5′TGCACATACAAATCGCCTGT′3 |
| TβRII | 5′GCTTGGCCAGAAAGACAGAC′3 | 5′CACTCCACAAGCTGTCTCCA′3 |
| HER1 | 5′TCTTCAAGGATGTGAAGTGTG′3 | 5′TGTACGCTTTCGAACAATGT′3 |
| IGF-I | 5′GATACACATCATGTCGTCTTCACA′3 | 5′CAGTACATCTCCAGTCTCCTCAGA′3 |
| GADPH | 5′TGAAGCAGGCATCTCAGGG′3 | 5′CGAAGGTGCAAGAGTGGGAG′3 |

healing assays showed that NMuMG cell migration was enhanced at 0.05 μM HCB but decreased at 5 μM HCB (Fig. 2A–B). In contrast, no alterations were observed in FGM AhR +/+ and FGM AhR −/− migration at any dose assayed (Fig. S2).

Considering that HCB stimulates MDA-MB-231 cell migration through AhR (Pontillo et al., 2011) and TGF-β1 (Miret et al., 2016), we assessed their possible involvement in the HCB effects on NMuMG cell migration. NMuMG cells were pretreated with specific inhibitors (ANF for AhR and SB431542 for TβRI), and then exposed to HCB in the presence or absence of the inhibitors. Taking into account that HCB exerted a biphasic effect on NMuMG, inducing cell migration at low dose and cell cycle arrest at high dose, we chose 0.05 and 5 μM HCB for further assays. Our results show that pretreatment with the inhibitors in the absence of HCB had no effect on cell migration (Fig. 2C–D). As shown in Fig. 2C–D, the increase in cell migration at 0.05 μM HCB was prevented by the AhR and TGF-β1 inhibitors, indicating that the HCB action requires the activation of both signaling pathways. Similar results were obtained at 5 μM, where the reduction of migration was blocked by the inhibitors (Fig. 2C–D), showing that NMuMG cell migration modulated by HCB is AhR and TGF-β1-dependent.

### 3.3. HCB mediates changes in AhR and TGF-β1 signaling pathways in NMuMG cells

We have previously shown that HCB alters the interaction between AhR and TGF-β1 in MDA-MB-231 cells (Miret et al., 2016), and other authors have described the involvement of these signaling pathways in cell cycle regulation (Dietrich and Kaina, 2010) and epithelial-mesenchymal plasticity (Contador-Troca et al., 2013; Rico-Leo et al., 2013; Zu et al., 2012). Besides, herein we observed that HCB modulates NMuMG cell migration involving the AhR and TGF-β1 pathways. These observations led us to investigate whether HCB (0.05 and 5 μM) could modify TGF-β1 and AhR pathways in NMuMG cells. RT-qPCR results showed 5 μM HCB to significantly upregulate AhR mRNA content and its target gene CYP1A1, whereas 0.05 μM HCB enhanced TGF-β1 and TβRII mRNA levels. In addition, 5 μM HCB increased TβRI mRNA content but decreased TβRII mRNA levels (Fig. 3). Given that we have previously reported that the pesticide may have different effects on AhR mRNA and protein levels (Miret et al., 2016), a dose-response study was conducted to analyze the HCB (0.005, 0.05, 0.5, and 5 μM) action on AhR protein expression by Western blot. We found that the pesticide reduced AhR protein levels in a dose-dependent manner (Fig. 4A).

Because 0.05 μM HCB treatment increased TGF-β1 mRNA levels, we next examined whether the pesticide could activate its downstream signaling pathways. A time-course study (5, 15, and 30 min, and 2 h)

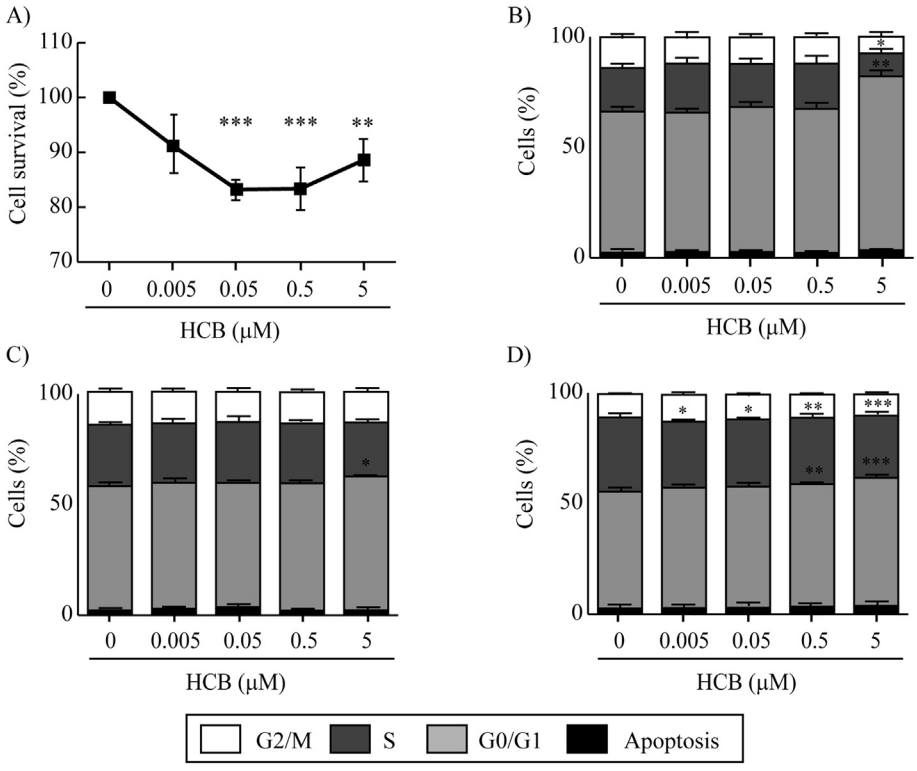


**Fig. 1.** HCB effects on NMuMG cell survival and cell cycle progression. A) The viability of NMuMG cells was evaluated using the MTT assay. Cells were incubated for 24 h with HCB (0.005, 0.05, 0.5, and 5 μM) or EtOH and then treated with MTT (150 μg/ml) for 1 h at 37 °C. Dehydrogenase activity was determined as the difference between absorbance at 490 nm and 650 nm, and the percentage of cell survival was expressed as treated cells relative to EtOH cells. (B–D) Cell cycle distribution was determined by propidium iodide staining and flow cytometric analysis. Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or EtOH for (B) 12 h, (C) 24 h, and (D) 36 h. Data are expressed as means ± SD of at least three independent experiments performed in triplicate. Asterisks indicate significant differences versus control (EtOH) (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). ANOVA and Dunnett's post-hoc test.

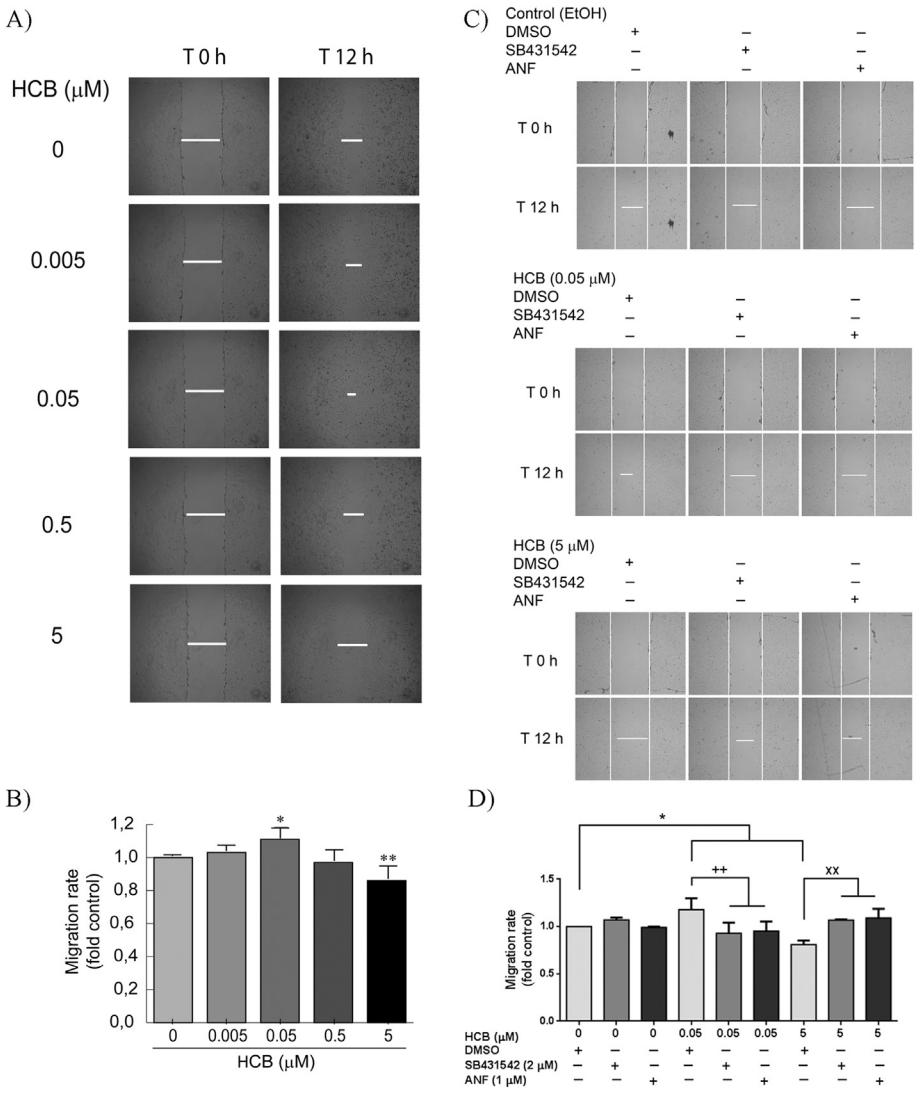


**Fig. 2.** HCB actions on NMuMG cell migration. Role of AhR and TGF-β1 signaling. (A–B) The serum-starved cell monolayer was scratched with a pipette tip and then treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle. (C–D) The serum-starved NMuMG cells were pretreated with specific inhibitors (2 μM SB431542 for TβRI and 1 μM ANF for AhR) for 1 h. The monolayer was scratched with a pipette tip, and the cells were treated with 0.05 and 5 μM HCB in the presence or absence of the inhibitors. (A, C) Representative experiment of wound healing. Relative wound closure was observed at 0 and 12 h under a microscope and photographed. (B, D) Quantification of migration rate of at least three independent experiments performed in triplicate. Data are expressed as means ± SD. Asterisks indicate significant differences vs. control without inhibitors (* $p < 0.05$, **$p < 0.01$). Plus symbols indicate significant differences vs. HCB 0.05 μM without inhibitors (++ $p < 0.01$). Crosses indicate significant differences vs. HCB 5 μM without inhibitors (xx $p < 0.01$). ANOVA and Tukey post-hoc test.

was performed to evaluate Smad3, ERK1/2, and p38 phosphorylation levels by Western blot. HCB-induced Smad3 phosphorylation was readily observed at 15 min, 30 min, and 2 h (Fig. 4B). Furthermore, the pesticide activated ERK1/2 at 15 min (Fig. 4C), but no changes were observed in phospho-p38 levels (Fig. 4D).

Considering that HCB activates HER1 in MDA-MB-231 cells (Pontillo et al., 2011), and that this might lead to ERK1/2 phosphorylation (Biscardi et al., 1999), we investigated whether the pesticide could alter HER1 expression levels. Results showed 0.05 μM HCB to increase HER1 mRNA content (Fig. 3).

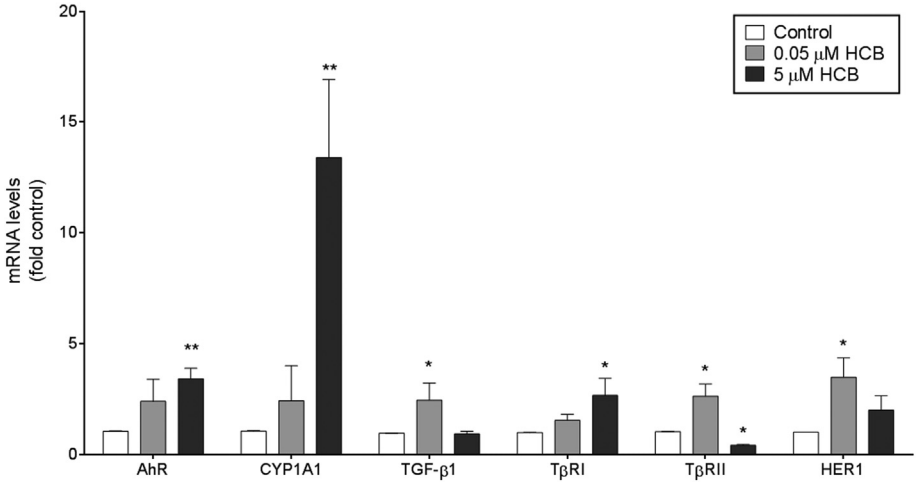


**Fig. 3.** HCB alters mRNA expression in NMuMG cells. Cells were exposed to HCB (0.05 and 5 μM) or vehicle for 24 h, and mRNA expression levels were evaluated by RT-qPCR. GAPDH expression was used as a control to normalize the data. Data are expressed as means ± SD of at least three independent experiments performed in triplicate. Asterisks indicate significant differences vs control (*$p < 0.05$ and **$p < 0.01$). ANOVA and Dunnett's post-hoc test.

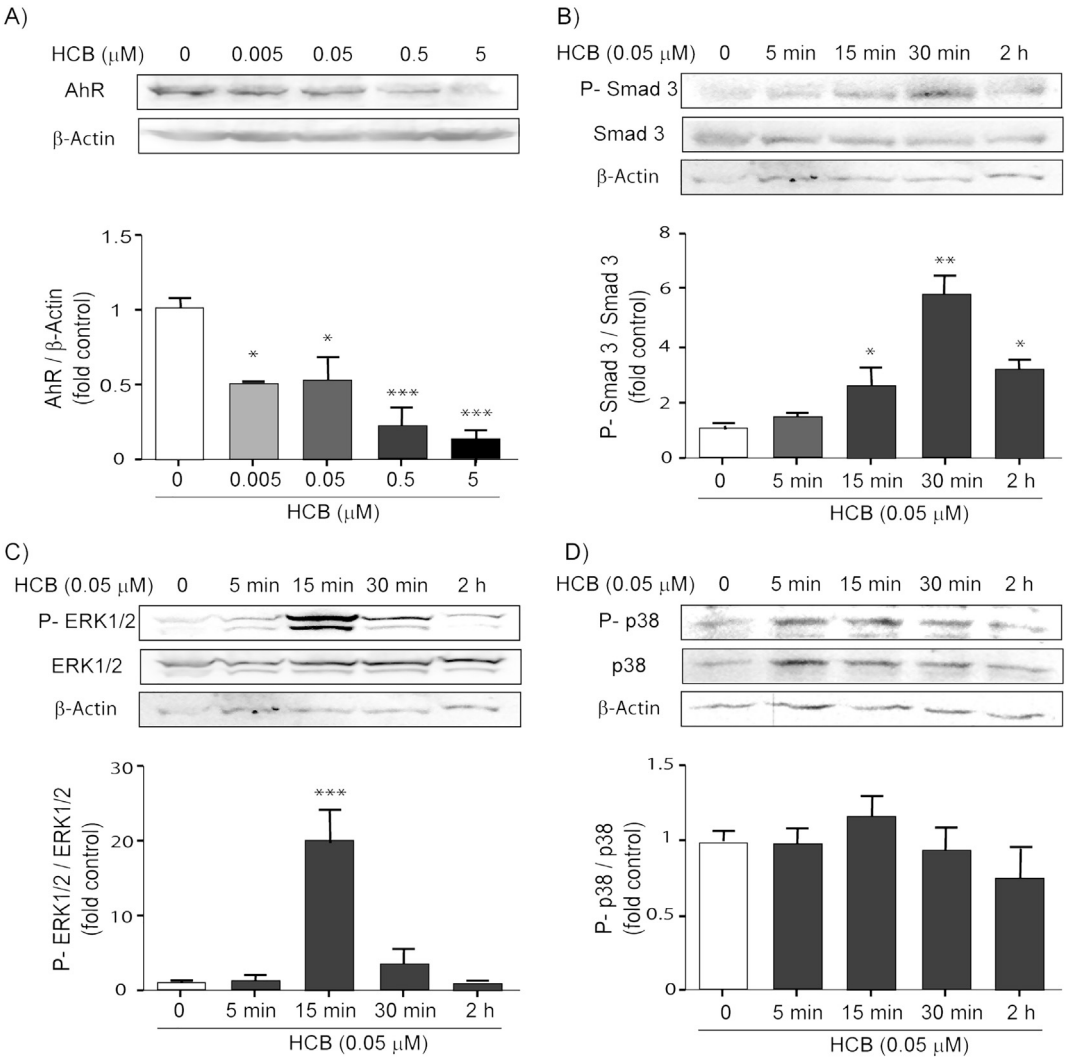


**Fig. 4.** AhR protein expression and TGF-β1 signaling pathways in NMuMG cells exposed to HCB. (A) AhR, (B) phospho and total-Smad3, (C) phospho and total-ERK1/2, and (D) phospho and total-p38 protein levels. (A) NMuMG cells were exposed to HCB (0.005, 0.05, 0.5, and 5 μM) or EtOH for 24 h. Whole-cell lysates were used to analyze the AhR protein levels by Western blot. Values were normalized by immunoblotting using anti-β-Actin antibody. (B–D) Cells were exposed to HCB (0.05 μM) or EtOH for 0, 5, 15, and 30 min and 2 h. The corresponding phosphorylated protein/total protein ratio was normalized to control. A representative Western blot from three independent experiments is shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Data are expressed as means ± SD of at least three independent experiments performed in triplicate. Asterisks indicate significant differences vs control (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). ANOVA and Dunnett's post-hoc test.

### *3.4. HCB alters TGF-β1 and AhR signaling in epithelial and fibroblast cells from AhR +/+ and AhR −/− mice*

Because HCB binds weakly to AhR and exerts some of its actions through this receptor, we studied whether AhR is involved in HCB-induced effects by means of primary cultures of epithelial cells from AhR +/+ and AhR −/− mouse MG (EMG AhR +/+ and EMG AhR −/−). Living cells observed in an optic microscope exhibited a typical cuboidal epithelial-like morphology in culture and formed monolayers with the cells in close contact with each other. To confirm the phenotypic identity of the isolated cells, Keratin-14 and Vimentin expression was evaluated through immunofluorescence assays. Results showed cells to express Keratin-14 but not Vimentin, thus confirming their epithelial identity (Fig. S3).

EMG AhR +/+ and EMG AhR −/−, as well as FGM AhR +/+ and FGM AhR −/−, were used to examine TGF-β1 and AhR signaling after 24 h of HCB exposure. Fig. 5 show that HCB decreased AhR mRNA levels in EMG AhR +/+ cultures at 0.05 and 5 μM, and in FGM AhR +/+ at 5 μM. Furthermore, the pesticide enhanced CYP1A1 mRNA expression in both EMG AhR +/+ (0.05 and 5 μM) and FGM AhR +/+ (5 μM). As expected, CYP1A1 mRNA levels were significantly lower in EMG AhR −/− than in EMG AhR +/+, and HCB treatment had no effect on this parameter (Fig. 5A).

Interestingly, in the absence of the pesticide, epithelial and fibroblast AhR −/− cells had increased TGF-β1 mRNA levels but reduced TβRII mRNA content compared to AhR +/+ cells (Fig. 5). After HCB treatment, TGF-β1 mRNA levels were increased at 0.05 μM in EMG AhR +/+ but not in EMG AhR −/− cells (Fig. 5A). In contrast, 0.05 μM HCB decreased TGF-β1 mRNA content in FGM AhR −/−, but produced no changes in FGM AhR +/+ (Fig. 5B), which suggests that HCB might exert AhR-dependent and -independent effects. Furthermore, 0.05 μM HCB increased TβRI mRNA expression in EMG AhR +/+ cells, but no alterations were observed in EMG AhR −/− cells or FGM at any dose assayed. Finally, results showed that TβRII expression was differentially altered after 5 μM HCB exposure, that is, TβRII mRNA was increased in EMG AhR +/+ but decreased in FGM AhR +/+. However, in both EMG AhR +/+ and FGM AhR +/+, these HCB actions were AhR-dependent (Fig. 5).

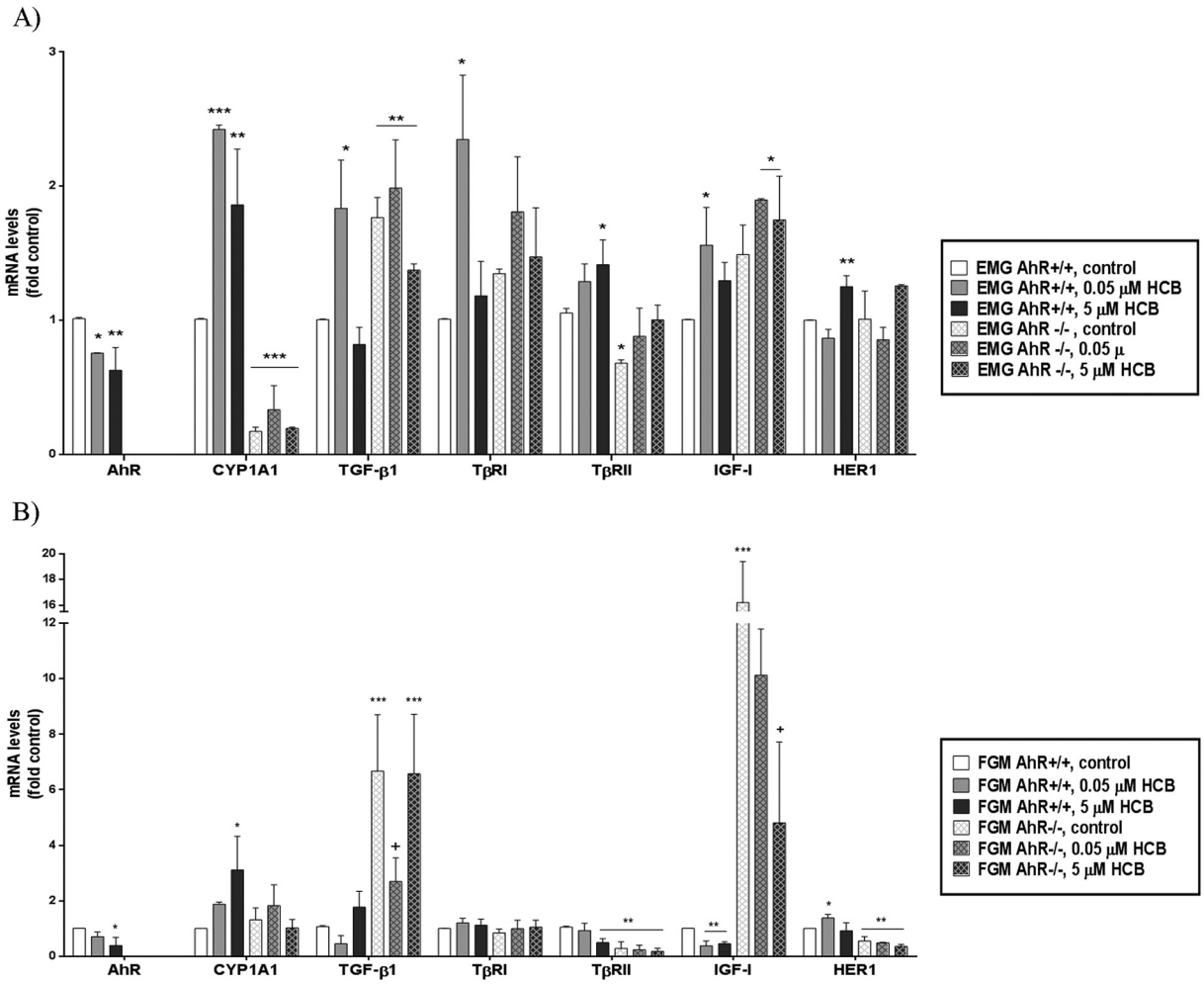


**Fig. 5.** HCB effects on mRNA expression levels in epithelial cells and fibroblast. A) EMG AhR +/+ and EMG AhR −/−. B) FGM AhR +/+ and FGM AhR −/−. Cells were exposed to HCB (0.05 and 5 μM) or vehicle for 24 h, and mRNA expression levels were evaluated by RT-qPCR. GAPDH expression was used as a control to normalize the data. The values are mean ± SD of at least three independent experiments performed in triplicate. Asterisks indicate significant differences vs control AhR +/+ cells (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Plus symbols indicate significant differences vs FGM AhR −/− control ($^{+}p < 0.05$). ANOVA and Tukey post-hoc test.

### 3.5. Changes in IGF-I and HER1 expression by HCB treatment in AhR +/+ and AhR −/− epithelial and fibroblast cells

Considering that IGF-I receptor and HER1 pathways are involved in MG development and breast cancer progression (Hynes and Watson, 2010), we studied whether HCB might modify IGF-I and HER1 mRNA content in EMG and FGM cells. RT-qPCR analyses revealed an increase in IGF-I mRNA expression in EMG AhR +/+ cells by 0.05 μM HCB, without changes in AhR −/−, for which, this effect depends on the AhR (Fig. 5A). Furthermore, IGF-I mRNA content in FGM AhR −/− was markedly higher than in FGM AhR +/+, although the pesticide down-modulated IGF-I mRNA expression levels in both genotypes. This HCB action was observed in FGM AhR +/+ at 0.05 and 5 μM, while in FGM AhR −/− at 5 μM (Fig. 5B). On the other hand, we observed HER1 mRNA content to be lower in FGM AhR −/− than in FGM AhR +/+ (Fig. 5B). In addition, HCB exposure enhanced HER1 mRNA expression in EMG AhR +/+ (5 μM) as well as in FGM AhR +/+ cells (0.05 μM) in an AhR-dependent manner (Fig. 5).

### 3.6. HCB alters α-smooth muscle actin expression in FGM cells

Fibroblasts in the tumor stroma acquire a perpetually activated phenotype and are called cancer-associated fibroblasts (CAFs); this subpopulation can be identified by the expression of α-smooth muscle actin (α-SMA) (Kalluri and Zeisberg, 2006). To investigate whether the pesticide is able to transform FGM fibroblasts toward a morphology resembling that of CAFs, α-SMA expression was analyzed by immunofluorescence after 24 h of HCB treatment (0.05 and 5 μM). Results show that α-SMA levels in FGM AhR −/− was significantly lower than in FGM AhR +/+ cells. Furthermore, 5 μM HCB increased α-SMA expression in an AhR-dependent fashion (Fig. 6).

### 3.7. Effect of fibroblast-derived CM on epithelial cells

Taking into account the former result, we hypothesize that these fibroblasts could affect epithelial cells, as they have turned into CAFs by the action of HCB. Therefore, we tested whether secreted factors by HCB-treated fibroblasts might alter NMuMG cell growth and migration. For this, we collected the supernatants from FGM AhR +/+ and FGM AhR −/− exposed to 0.05 and 5 μM HCB, which were processed and added to growing NMuMG cell cultures. Given that different culture media are used by NMuMG and FGM cells (RPMI and DMEM-F12, respectively), we studied the impact of DMEM-F12 on NMuMG cell viability. We compared the viability of NMuMG cells growing in RPMI with NMuMG cells growing in control CM through an MTT assay at 24 h. We found that control CM had no effect on the NMuMG cell growth (data not shown). However, results showed a reduction in cell survival when grown in CM from FGM AhR +/+ exposed to HCB (0.05 and 5 μM) (Fig. 7A). In a similar way, FGM AhR −/− CM decreased NMuMG viability, although HCB treatment produced no alterations

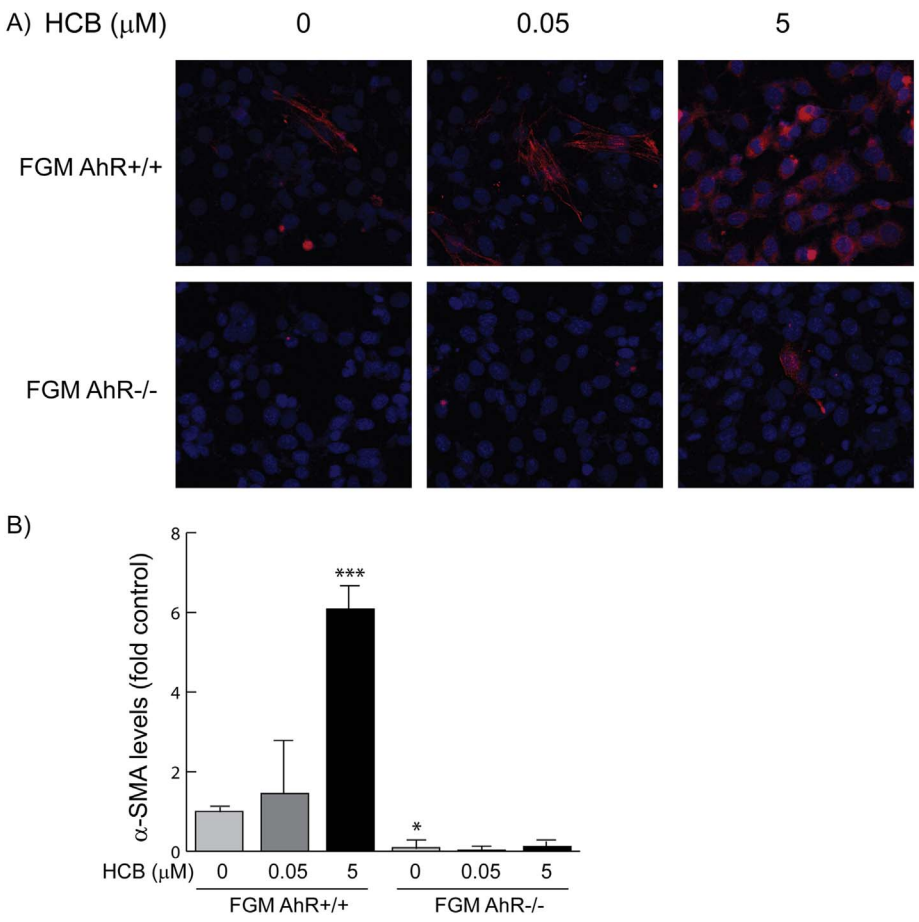


**Fig. 6.** HCB changes CAF specific biomarker levels. A) Merge images of α-SMA expression and DAPI nuclear staining in FGM AhR +/+ and FGM AhR −/−. Cells were exposed to HCB (0.05 and 5 μM) or vehicle for 24 h, and α-SMA expression was evaluated by immunofluorescence staining with specific antibodies. B) Quantification of positive cells. Data are expressed as means ± SD of at least three independent experiments performed in triplicate. Asterisks indicate significant differences vs FGM AhR +/+ control cells (*$p < 0.05$ and ***$p < 0.001$). ANOVA and Tukey post-hoc test.

(Fig. 7A). Cell cycle analysis by flow cytometry revealed that CM from FGM AhR +/+ cells treated with HCB (0.05 and 5 μM) reduced the percentage of NMuMG cells in G0/G1, whereas it increased the proportion of cells in the S phase (Fig. 7B). Similar results were observed with FGM AhR −/− CM, regardless of whether fibroblasts were treated with the pesticide or not (Fig. 7B).

Next, NMuMG migratory ability was evaluated by the scratch motility assay. Results showed an increase in NMuMG cell migration when the supernatant derived from FGM AhR +/+ cells treated with 5 μM HCB, whereas no changes were found in NMuMG cells growing in CM from FGM AhR −/− exposed to HCB (Fig. 7C–D).

Finally, RT-qPCR results revealed NMuMG cells to express the lowest AhR mRNA levels when CM derived from FGM AhR −/− (Fig. 7E). In a similar way, CM from FGM AhR +/+ treated with HCB (0.05 μM) decreased AhR mRNA expression (Fig. 7E). However, no changes were observed in TGF-β1 mRNA levels in NMuMG cells treated with CM from either FGM AhR +/+ or FGM AhR −/− cells (Fig. 7F).

### 3.8. HCB induces morphological and histological alterations in mouse MG

Because branching morphogenesis occurs mostly in adolescence (Sternlicht, 2006), we examined HCB potential to alter mouse MG structure at this stage and the involvement of AhR. Female AhR +/+ and AhR −/− C57BL/6 N mice were exposed to HCB (3 mg/kg body weight) or vehicle for 21 days and MG whole mount analysis was performed in order to evaluate morphological changes. To assess ductal growth, we quantified the distance between the lymph node and MG final edge (Fig. 8A), as described in Materials and Methods. Results showed that MG from AhR −/− mice had a significant increase in ductal growth, the distance between the lymph node and the MG edge being at least 40% longer than in AhR +/+ mice. However, no changes in ductal growth were observed in mice exposed to HCB (Fig. 8B). In addition, we evaluated branch density and the number of TEBs, counting the number of branches of the gland and the number of TEBs per total area of MG. Our results showed that HCB-treatment significantly enhanced branch density and the number of TEBs in AhR +/+ mice in an AhR-dependent manner (Fig. 8C–E).

Given that preneoplastic lesions are considered premalignant structures and precursors of neoplastic lesions (Singh et al., 2000), we evaluated the presence of hyperplasia. Sections of MG were stained with hematoxylin-eosin and the breast ducts were counted and classified into normal, when the linings of the breast ducts contained no more than three layers of cells; or hyperplastic, if there was an increase in the number of cells that constituted this ductal lining. A review by Sternlicht (2006) shows that mammary ducts have stromal cells surrounding the ducts, while TEBs distal cap evidences absence of stroma, and this characteristic was taken into account. Furthermore, we analyzed the E-cadherin and P-cadherin expression by immunohistochemistry, as described Matos et al. (2016), which allows differentiate between ducts and TEBs (Daniel et al., 1995) (Fig. S4). In AhR +/+ mouse MG, HCB increased the percentage of hyperplastic ducts (Fig. 9A). Furthermore, we found a number of hyperplastic ducts in control AhR −/− mice as high as in HCB-treated AhR +/+ mice, although the pesticide did not produce changes in AhR −/− mice (Fig. 9A). Given these results, we next examined HCB action on cell proliferation by analyzing PCNA levels. As expected, MG sections which had hyperplastic ducts (HCB-treated mice and control AhR −/− mice) showed an increase in PCNA expression (Fig. 9B).

ERα and PR protein levels were evaluated to complement our studies, as these hormone receptors play a key role in MG development and function (Sternlicht, 2006). Immunohistochemistry results revealed an enhancement in nuclear ERα levels in ducts from HCB-treated AhR

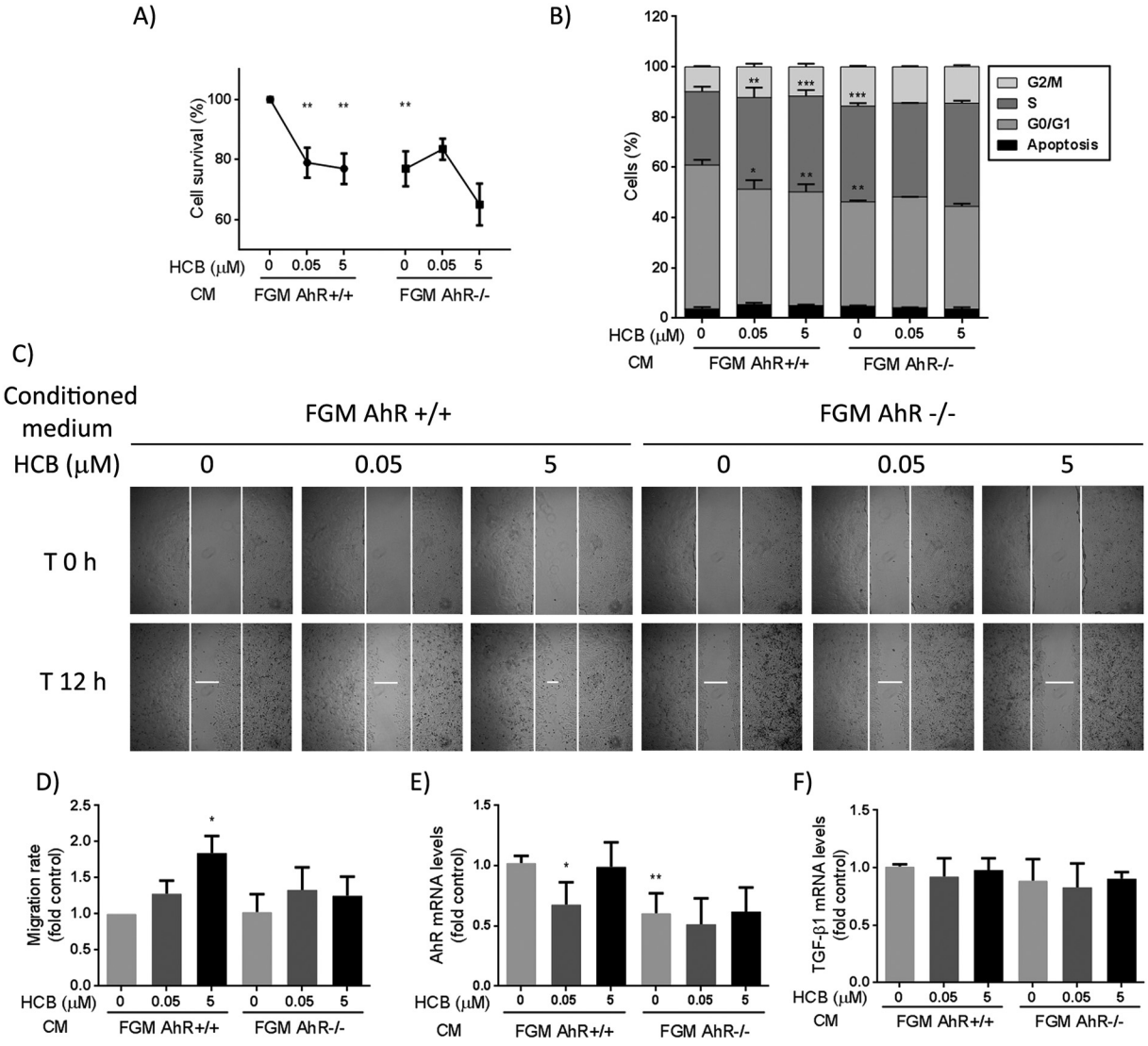


**Fig. 7.** Effect of CM from fibroblasts exposed to HCB on epithelial cells. FGM AhR +/+ and FGM AhR −/− were exposed to HCB (0.05 and 5 μM) or vehicle (EtOH) for 48 h, the supernatant was collected and this CM was used to grow NMuMG cells. A) NMuMG cell viability was determined by MTT assay after 24 h of treatment with the CM. (B) NMuMG cell cycle distribution was evaluated by flow cytometry after 24 h. (C) Migration ability of NMuMG was assessed through the scratch motility assay. Relative wound closure was observed at 12 h and one representative experiment is shown. (D) Quantification of migration rate. (E) AhR and (F) TGF-β1 mRNA expression levels were determined by RT-qPCR in NMuMG after 24 h of CM exposure. In all the graphs, data are expressed as means ± SD of at least three independent experiments performed in triplicate. Asterisks indicate significant differences vs NMuMG treated with CM from FGM AhR +/+ without HCB (*p < 0.05, **p < 0.01 and ***p < 0.001). ANOVA and Tukey post-hoc test.

+/+ mice and control AhR −/− mice; however, neither PR localization nor expression were altered (Fig. 9C–D).

## 4. Discussion

The incidence of breast cancer is rising in many countries (Bergman et al., 2012), and this evidence strengthens the biological plausibility that environmental pollutants such as organochlorine pesticides are also contributors to risk. The current study supports this hypothesis, as environmentally relevant HCB concentrations (Guo et al., 2014; Saoudi et al., 2014) produce alterations in normal mammary cells and mouse MG. Interestingly, the changes induced by HCB in mammary branching morphogenesis through AhR find correlation in other reports showing the implication of AhR in MG development and lactation. Lew et al. (2011) showed that activation of AhR by TCDD in both the epithelial parenchyma and associated stromal tissue are required for impairment in pregnancy-associated mammary development and lactogenesis. Furthermore, Basham et al. (2015) demonstrated that AhR agonists block transcription of the milk gene β-casein in mammary epithelial cells. The alterations observed in the present work include an enhancement in branch density and the number of TEBs, both parameters used as a measure of MG developmental progression, as previously established by Fenton (2009). This could be due to the slower pace of MG development, causing a permanence of the TEBs and preventing their differentiation. In agreement, other authors observed that perinatal exposure to Bisphenol A and Diethylstilbestrol impairs MG differentiation (Kass et al., 2012). Furthermore, the increase in undifferentiated and highly proliferative structures, such as TEBs, can enhance cancer risk, since they are sensitive to the effects of carcinogens and other chemicals (Macon and Fenton, 2013). Fenton et al. (2002) observed that TCDD-exposed rats retained undifferentiated terminal structures. ERα nuclear localization was increased in mammary ducts from HCB-treated AhR +/+ mice, in accordance with reports showing that ERα signaling is necessary for adolescent branching (Hewitt et al., 2000). In this respect, we have previously observed that HCB leads to ERα phosphorylation via c-Src and cell proliferation in MCF-7 cells (García et al., 2010). ERα activation in epithelial cells promotes HER1 signaling in the stroma, inducing mammary branching

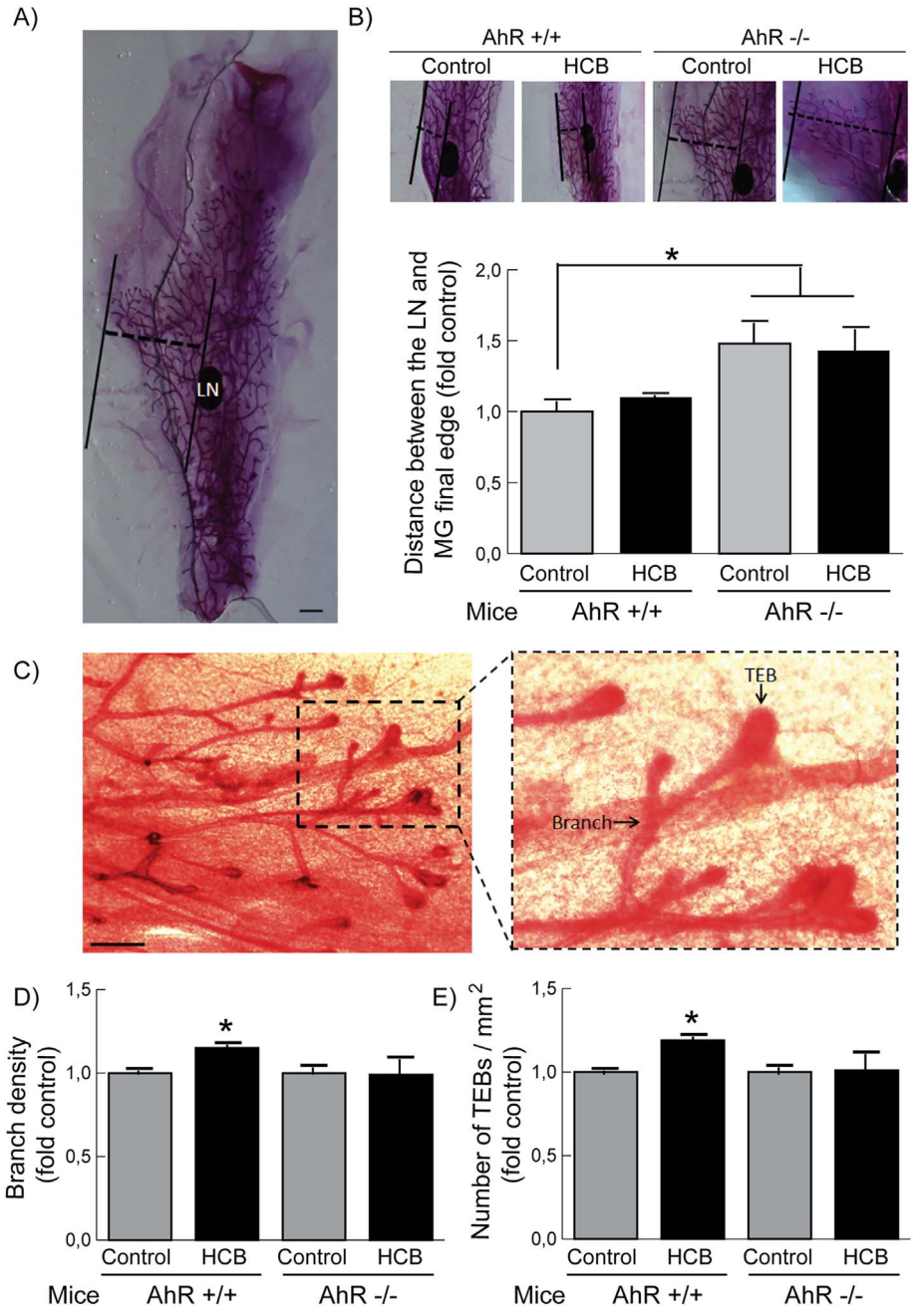


**Fig. 8.** HCB induces morphological changes in mouse MG. AhR +/+ and AhR −/− mice were exposed to HCB (3 mg/kg body weight) or vehicle four times a week for 21 days and the whole abdominal MG was evaluated. (A) Representative MG whole mount. The distance between the two parallel lines drawn on the image represents the longitudinal growth of the MG (dashed line). Scale bar is 1 mm. (B) The upper panel shows a representative photograph per group. The graph shows the quantification of the distance between the lymph node (LN) and MG final edge. (C) Representative image of MG whole mounts showing a TEB and a branch. Scale bar is 0.25 mm. Quantification of (D) branch density and (E) TEBs. Data are expressed as means ± SD of three independent experiments with n = 5 mice per group. Asterisks indicate significant differences vs control AhR +/+ mice (* $p < 0.05$). ANOVA and Tukey post-hoc test.

through metalloproteinase stimulation (Sternlicht, 2006). Our results have shown that HCB increases HER1 expression in FGM AhR +/+, which could be related to the enhancement in TEBs and branch density in AhR +/+ mice. Accordingly, it has been reported that the stroma plays a major role in the retarded development of the mammary gland following TCDD exposure (Fenton et al., 2002). Other authors observed that TGF-β1 inhibited DNA synthesis in the highly proliferative terminal end buds resulting in a reduction of ductal growth (Sun and Ingman, 2014). Despite EMG AhR −/− showed an increase in TGF-β1 mRNA levels, we found an increase in ductal growth in AhR −/− mice, the outgrowth of the mammary epithelium being bigger than in AhR +/+ mice. This discrepancy could be explained by the absence of the AhR, which modify other molecules such IGF-I and ERα. In this respect, the high levels of IGF-I in FGM AhR −/− cells might lead to proliferation and survival, as this growth factor could stimulate IGF-I receptor in mammary epithelial cells, as already described (Sternlicht, 2006). This behavior could induce ductal growth and hyperplasia in MG, explaining the phenotype observed in AhR −/− mice. Furthermore, ERα translocation to the nucleus in mammary ducts from AhR −/− mice is also likely to contribute to this phenotype.

Previous results have demonstrated that HCB is a tumor co-carcinogen in the rat MG (Randi et al., 2006), and Charlier and Dejardin (2007) found a strong association between the incidence of human breast cancer relapse and high serum levels of HCB. Our current study shows that HCB induces cell proliferation and ductal hyperplasia in MG from AhR +/+ mice, in agreement with another report showing an increase in preneoplastic lesions in rat MG after HCB treatment (Peña et al., 2012). As mice used for primary culture (EMG) and in vivo assays had the same age at the end of treatment, this increase in ductal hyperplasia could be related with a reduction in AhR mRNA levels in HCB-treated EMG. In a similar way, control AhR −/− mice also presented ductal hyperplasia, thus supporting a key role for AhR as a proliferation inhibitor in normal MG. In addition, these results could be partly explained by the enhancement in TGF-β1 mRNA content and the

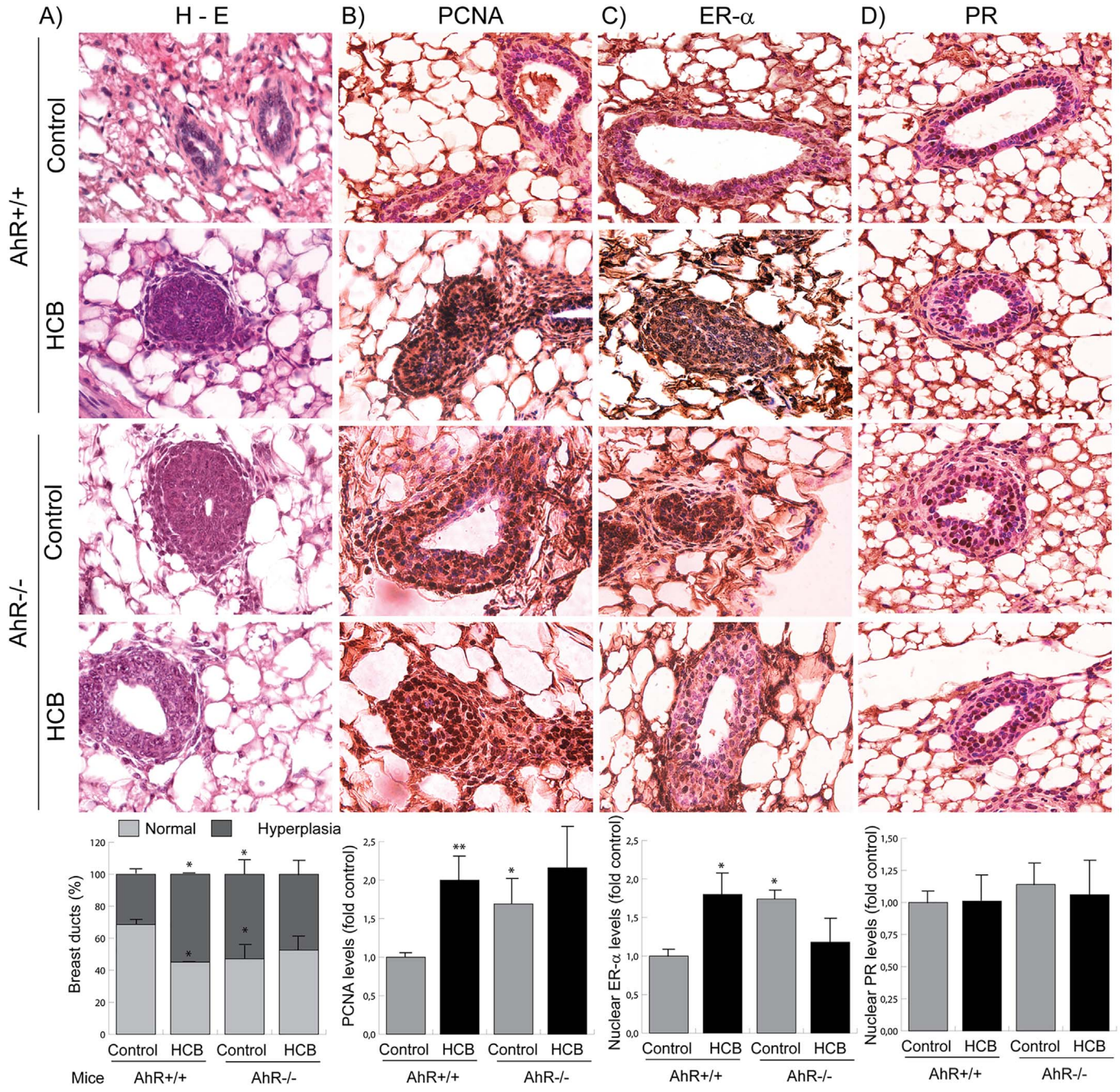


**Fig. 9.** HCB action on mouse MG histology. AhR +/+ and AhR −/− mice were exposed to HCB (3 mg/kg body weight) or vehicle four times a week for 21 days, MG was fixed and slices were prepared for histological evaluation. Representative images of MG sections stained with (A) hematoxylin-eosin, (B) PCNA, (C) nuclear estrogen receptor α (ERα), and (D) nuclear progesterone receptor (PR) are shown in the upper panels. For immunohistochemical studies, signal detection was carried out using DAB substrate kit and lightly counterstaining with hematoxylin. Quantification of (A) normal and hyperplastic ducts and (B-D) positive cells normalized to control is shown in the lower panels. Data are expressed as means ± SD of three independent experiments with n = 5 mice per group. Asterisks indicate significant differences vs control AhR +/+ mice (*$p < 0.05$, **$p < 0.01$). ANOVA and Tukey post-hoc test.

reduction in TβRII mRNA levels observed in control EMG AhR −/−. In this respect, Gorska et al. (1998) reported that mouse epithelial mammary cells expressing a dominant negative form of TβRII exhibited greater hyperplasia. Furthermore, high TGF-β1 levels but low TβRII content in human mammary tumors correlate with poor prognosis, which suggests that breast tumor cells have an ability to reduce their responsiveness to TGF-β1 (Chen et al., 2015).

In view of our results, HCB alters AhR expression in different ways, enhancing mRNA levels but decreasing protein expression in NMuMG cells. In this respect, we found in a previous investigation that HCB decreases AhR mRNA levels and enhances protein levels in MDA-MB-231 breast cancer cells (Miret et al., 2016). The present data suggest that HCB could be activating the proteasome pathway, as previously reported for other ligands of this receptor (Feng et al., 2013), and in consequence, mRNA expression is triggered to compensate the AhR levels in cells. On the other hand, it has been observed that the modulation of AhR expression (mRNA and protein levels) appear to be largely tissue- and cell-specific (Safe et al., 2013). Despite we have found that AhR mRNA levels is enhanced in NMuMG cells but reduced in EMG AhR +/+ cells, the remarkable result is that CYP1A1 mRNA levels is increased in both models, indicating AhR activation. These findings were previously observed by other authors, where AhR ligands

can mediate significant AhR transcriptional activity even in the presence of modest levels of AhR expression (Ma and Baldwin, 2000; Murray et al., 2014; Staršíchová et al., 2012). On the other hand, despite that HCB (0.05 μM) enhances TGF-β1 mRNA expression levels in both NMuMG and EMG cells, the regulation of TβRI and TβRII is different and dependent of the HCB dose. Further studies are necessary to evaluate the dose of HCB that could be activating TGF-β1 signaling pathways in EMG cells. Our interest in the role of AhR in cell proliferation, cell cycle control and migration lies in the fact that these processes become uncontrolled during tumor development. Moreover, the development, maintenance, chemoresistance and self-renewal of human breast cancer stem cells involve the activation of nuclear AhR pathway (Al-Dhfyan et al., 2017). The present report demonstrates that HCB slightly decreases NMuMG cell viability. In addition, 5 μM HCB induces G0/G1 cell cycle arrest, decreasing the number of cells in the S phase, and activates the genomic AhR pathway. Extensive evidence proves that, in the presence of exogenous ligand, AhR inhibits cell proliferation and induces cell cycle arrest in normally cycling cell populations (Puga et al., 2009). Accordingly, HCB lowers cell viability and induces apoptotic cell death in normal thyroid cells (Chiappini et al., 2013), while Bisphenol A reduces cell survival in epithelial mammary cells (Lee and Rhee, 2007). Barhoover et al. (2010) found that, upon activation with exogenous ligand, AhR induces G1 cell cycle arrest in human breast cancer cell lines. In addition, the decrease in NMuMG cell migration observed at 5 μM HCB could be related to cytotoxicity and cell cycle arrest induced by HCB. In contrast, HCB does not alter fibroblast viability or cell cycle, indicating that mammary epithelial cells are more sensitive to the pesticide exposure, in a single cell-based culture model.

We have previously shown that HCB induces cell migration in a dose-dependent manner in MDA-MB-231 human breast cancer cells (Pontillo et al., 2011). However, NMuMG cell migration was enhanced at a low HCB dose (0.05 μM) but reduced at a high dose (5 μM). Such biphasic effect has also been observed in previous studies from our laboratory in human breast cancer cells (García et al., 2010). This action in NMuMG migration could be explained by pesticide modulation of the crosstalk between AhR and TGF-β1, since HCB effects on cell migration are dependent on these signaling pathways. Herein, 5 μM HCB activates the nuclear AhR pathway in NMuMG cells, and Rico-Leo et al. (2013) have reported NMuMG cells expressing a constitutively active AhR to exhibit reduced migration. On the other hand, the same authors have demonstrated that exogenous TGF-β1 exacerbates NMuMG cell migration, while our work found 0.05 μM HCB to increase TGF-β1 mRNA expression and migration in this cell line. Bakiri et al. (2015) demonstrated that the modulation of TGF-β1 expression leads to the acquisition of mesenchymal, invasive and tumorigenic capacities by mammary epithelial cells. Furthermore, HCB activates Smad3 and ERK1/2 at low doses, which could contribute to cell migration and EMT of epithelial mammary cells, as other authors have previously reported (Dzwonek et al., 2009; Joslin et al., 2007). These effects on TGF-β1 signaling could be due to the activation of the non genomic AhR pathway, as we previously reported in MDA-MB-231 cells (Miret et al., 2016). This hypothesis is supported by the fact that the increase in NMuMG cell migration at 0.05 μM HCB not only depends on the TGF-β1 pathway, but also on AhR.

Accumulating evidence has demonstrated that the epithelial-stromal dialogue is critical in the development of the MG (Sternlicht, 2006). Despite the growing number of studies focusing on the effects of ligands of AhR on mammary epithelial cells, the role played by fibroblasts is not yet fully understood. Herein, we have found that HCB (5 μM) can alter the characteristics of fibroblasts, which resemble the activated phenotype found in breast cancer stroma. The pesticide increased the number of α-SMA-positive cells, whereas it reduced TβRII mRNA levels in fibroblasts by a process likely to involve AhR. The presence of CAFs, which express α-SMA, is apparent in the stromal compartment of most invasive human breast cancers (Sappino et al., 1988). In accordance with this behavior, we found that CM from HCB-treated FGM AhR +/+ promotes NMuMG cell migration. In contrast, FGM AhR −/− showed the lowest α-SMA expression and its CM produced no alterations in NMuMG cell migration. On the other hand, co-culture assays have shown that lack of TβRII expression in CAFs is sufficient to boost breast cancer cell growth and survival (Busch et al., 2015). However, we report a reduction in cell viability of NMuMG cells growing in CM from FGM AhR +/+ exposed to HCB. This discrepancy could indicate that the effects of CAFs on epithelial cells need cell-cell contact, as *in vivo* assays demonstrate that the pesticide increases cell proliferation by enhancing PCNA levels. In CM assays, when FGM AhR +/+ were exposed to HCB, we observed a reduction in the percentage of NMuMG cells in G0/G1 and an increase in cells in the S phase. Given that no changes in the G2/M phase were observed and a decrease in cell survival was reported, NMuMG cells could be thought to become arrested in the S phase. In this respect, Sadlonova et al. (2005) have demonstrated that both normal breast-associated fibroblasts and CAFs have the ability to inhibit the growth of breast epithelial cells. Ventura et al. (2012) found that Chlorpyrifos, a weak ligand of AhR, induces intra-S phase arrest modifying checkpoint proteins in MCF-7 cells. It has been reported that TGF-β1 treatment leads both to inhibition of cell proliferation and induction of characteristic EMT markers in non-tumorigenic prostate epithelial cells (Slabáková et al., 2011). This suggests that some migrant cells have been selected, as we have observed in the present study with CM from FGM AhR +/+ exposed to HCB 5 μM. In addition, we could hypothesize that the enhancement in TGF-β1 mRNA content observed in FGM AhR −/− might be implicated in NMuMG cell cycle arrest. In agreement, TGF-β1 signaling is known to play a major role in epithelial MG as a potent inhibitor of proliferation (Macias and Hinck, 2012). Furthermore, CM from FGM AhR −/− decreases Mv1Lu cell proliferation due to its high TGF-β1 content (Gómez-Duran et al., 2009).

In conclusion, our results show for the first time that environmental HCB concentrations differentially alter normal mammary epithelial cells and fibroblasts, triggering an increase in hyperplasia and changes in mammary branching morphogenesis. Given that several molecules involved in normal MG development act in mammary tumor promotion (Lanigan et al., 2007), another possible implication of this investigation is a potential connection to breast cancer. It is clear that HCB alters AhR and TGF-β1 signaling and that its action depends on the dose of exposure, different pathways being thus activated. Disturbingly, a lower HCB dose than that found in a contaminated population can contribute to abnormal cell migration, potentially affecting the tumorigenic action of this pesticide on target cells.

## Funding

This work was supported by grants from the Ministerio de Economía y Competitividad [SAF2014-51813-R], España; Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICET [PIP0654], Argentina; and Universidad de Buenos Aires [PID 20020130100631BA], Argentina.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

Carolina Pontillo and Andrea Silvana Randi are established researchers of the National Council of Scientific and Technological Research (CONICET, Argentina). Boehringer Ingelheim Fonds awarded a travel grant to Noelia Miret.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2017.09.012.

## References


Agency for Toxic Substances and Disease Registry, ATSDR, 2015. Toxicological Profile for Hexachlorobenzene. http://dx.doi.org/10.1155/2013/286524.

Al-Dhfyan, A., Alhoshani, A., Korashy, H.M., 2017. Aryl hydrocarbon receptor/cytochrome P450 1A1 pathway mediates breast cancer stem cells expansion through PTEN inhibition and β-catenin and Akt activation. Mol. Cancer 16, 14. http://dx.doi.org/10.1186/s12943-016-0570-y.

Bakiri, L., Macho-Maschler, S., Custic, I., Niemiec, J., Guío-Carrión, A., Hasenfuss, S.C., Eger, A., Müller, M., Beug, H., Wagner, E.F., 2015. Fra-1/AP-1 induces EMT in mammary epithelial cells by modulating Zeb1/2 and TGFβ expression. Cell Death Differ. 22, 336–350. http://dx.doi.org/10.1038/cdd.2014.157.

Barhoover, M., Hall, J.M., Greenlee, W.F., Thomas, R.S., 2010. Aryl hydrocarbon receptor regulates cell cycle progression in human breast cancer cells via a functional interaction with Cyclin-Dependent Kinase 4. Mol. Pharmacol. 77, 195–201. http://dx.doi.org/10.1124/mol.109.059675.

Basham, K.J., Leonard, C.J., Kieffer, C., Shelton, D.N., McDowell, M.E., Bhonde, V.R., Looper, R.E., Welm, B.E., 2015. Dioxin exposure blocks lactation through a direct effect on mammary epithelial cells mediated by the aryl hydrocarbon receptor repressor. Toxicol. Sci. 143, 36–45. http://dx.doi.org/10.1093/toxsci/kfu203.

Bekki, K., Vogel, H., Li, W., Ito, T., Sweeney, C., Haarmann-Stemmann, T., Matsumura, F., Vogel, C.F.A., 2015. The aryl hydrocarbon receptor (ahr) mediates resistance to apoptosis induced in breast cancer cells. Pestic. Biochem. Physiol. 120, 5–13. http://dx.doi.org/10.1016/j.pestbp.2014.12.021.

Bergman, A., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T., 2012. State of the Science of Endocrine Disrupting Chemicals, United Nations Environment Programme and. World Health Organization, Geneva, Switzerland, pp. 12–13. http://dx.doi.org/10.1016/j.toxlet.2012.03.020.

Biscardi, J.S., Tice, D.A., Parsons, S.J., 1999. C-Src, receptor tyrosine kinases, and human cancer. Adv. Cancer Res. 76, 61–119 (doi:10.1101/cshperspect.a003186).

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burton, M.A., Bennett, B.G., 1987. Exposure of man to environmental hexachlorobenzene (HCB)-an exposure commitment assessment. Sci. Total Environ. 66, 137–146 (PMID: 3685945).

Busch, S., Acar, A., Magnusson, Y., Gregersson, P., Ryden, L., Landberg, G., 2015. TGF-beta receptor type-2 expression in cancer-associated fibroblasts regulates breast cancer cell growth and survival and is a prognostic marker in pre-menopausal breast cancer. Oncogene 34, 27–38. http://dx.doi.org/10.1038/onc.2013.527.

Charlier, C.J., Dejardin, M.T.C., 2007. Increased risk of relapse after breast cancer with exposure to organochlorine pollutants. Bull. Environ. Contam. Toxicol. 78, 1–4. http://dx.doi.org/10.1007/s00128-007-9010-4.

Chen, X., Panuwet, P., Hunter, R.E., Riederer, A.M., Bernoudy, G.C., Barr, D.B., Ryan, P.B., 2014. Method for the quantification of current use and persistent pesticides in cow milk, human milk and baby formula using gas chromatography tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 970, 121–130. http://dx.doi.org/10.1016/j.jchromb.2014.08.018.

Chen, C., Zhao, K.-N., Masci, P.P., Lakhani, S.R., Antonsson, A., Simpson, P.T., Vitetta, L., 2015. TGFβ isoforms and receptors mRNA expression in breast tumours: prognostic value and clinical implications. BMC Cancer 15, 1010. http://dx.doi.org/10.1186/s12885-015-1993-3.

Chiappini, F., Álvarez, L., Lux-Lantos, V., Randi, A.S., Kleiman de Pisarev, D.L., 2009. Hexachlorobenzene triggers apoptosis in rat thyroid follicular cells. Toxicol. Sci. 108, 301–310. http://dx.doi.org/10.1093/toxsci/kfp016.

Chiappini, F., Pontillo, C., Randi, A., Álvarez, L., Kleiman de Pisarev, D., 2013. Reactive oxygen species and extracellular signal-regulated kinase 1/2 mediate hexachlorobenzene-induced cell death in FRTL-5 rat thyroid cells. Toxicol. Sci. 134, 276–290. http://dx.doi.org/10.1093/toxsci/kft117.

Contador-Troca, M., Alvarez-Barrientos, A., Barrasa, E., Rico-Leo, E.M., Catalina-Fernández, I., Menacho-Márquez, M., Bustelo, X.R., García-Borrón, J.C., Gómez-Durán, A., Sáenz-Santamaría, J., Fernández-Salguero, P.M., 2013. The dioxin receptor has tumor suppressor activity in melanoma growth and metastasis. Carcinogenesis 34, 2683–2693. http://dx.doi.org/10.1093/carcin/bgt248.

Daniel, C.W., Strickland, P., Friedmann, Y., 1995. Expression and functional role of E- and P-cadherins in mouse mammary ductal morphogenesis and growth. Dev. Biol. 169, 511–519. http://dx.doi.org/10.1006/dbio.1995.1165.

Der Parsehian, S., 2008. Plaguicidas organoclorados en leche materna. Rev. Hosp. Materno Infant. Ramón Sardá 27, 70–78. http://www.redalyc.org/resumen.oa?id = 91227203\nhttp://www.redalyc.org/articulo.oa?id = 91227203\nhttp://www.redalyc.org/pdf/912/91227203.pdf.

Dietrich, C., Kaina, B., 2010. The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact and tumor growth. Carcinogenesis 31, 1319–1328. http://dx.doi.org/10.1093/carcin/bgq028.

Dzwonek, J., Preobrazhenska, O., Cazzola, S., Conidi, A., Schellens, A., van Dinther, M., Stubbs, A., Klippel, A., Huylebroeck, D., ten Dijke, P., Verschueren, K., 2009. Smad3 is a key nonredundant mediator of transforming growth factor beta signaling in nme mouse mammary epithelial cells. MCR 7, 1342–1353. http://dx.doi.org/10.1158/1541-7786.MCR-08-0558.

Feng, S., Cao, Z., Wang, X., 2013. Role of aryl hydrocarbon receptor in cancer. Biochim. Biophys. Acta 1836, 197–210. http://dx.doi.org/10.1016/j.bbcan.2013.05.001.

Fenton, S.E., 2006. Endocrine-disrupting compounds and mammary gland development: early exposure and later life consequences. Endocrinology 147, 18–24. http://dx.doi.org/10.1210/en.2005-1131.

Fenton, S., 2009. Mammary gland whole mount round robin summary. In: NIEHS, NTP, pp. 1–6. http://www.silentspring.org/sites/default/files/MammaryGlandWholeMountRoundRobinSummary.pdf.

Fenton, S.E., Hamm, J.T., Birnbaum, L.S., Youngblood, G.L., 2002. Persistent abnormalities in the rat mammary gland following gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Toxicol. Sci. 67, 63–74. http://dx.doi.org/10.1093/toxsci/67.1.63.

Fernandez-Salguero, P., Pineau, T., Hilbert, D.M., McPhail, T., Lee, S.S., Kimura, S., Nebert, D.W., Rudikoff, S., Ward, J.M., Gonzalez, F.J., 1995. Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding ah receptor. Science 268, 722–726. http://dx.doi.org/10.1126/science.7732381.

Galliher, A.J., Schiemann, W.P., 2006. Beta3 integrin and Src facilitate transforming growth factor-beta mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res. 8, R42. http://dx.doi.org/10.1186/bcr1524.

García, M.A., Peña, D., Álvarez, L., Cocca, C., Pontillo, C., Bergoc, R., Kleiman de Pisarev, D., Randi, A., 2010. Hexachlorobenzene induces cell proliferation and igf-i signaling pathway in an estrogen receptor alpha-dependent manner in MCF-7 breast cancer cell line. Toxicol. Lett. 192, 195–205. http://dx.doi.org/10.1016/j.toxlet.2009.10.026.

Gasiewicz, T.A., Henry, E.C., Collins, L.L., 2008. Expression and activity of aryl hydrocarbon receptors in development and cancer. Crit. Rev. Eukaryot. Gene Expr. 18, 279–321. http://dx.doi.org/10.1615/CritRevEukarGeneExpr.v18.i4.10.

Gómez-Duran, A., Carvajal-González, J.M., Mulero-Navarro, S., Santiago-Josefat, C., Puga, A., Fernández-Salguero, P.M., 2009. Fitting a xenobiotic receptor into cell homeostasis: how the dioxin receptor interacts with TGFb signaling. Biochem. Pharmacol. 77, 700–712. http://dx.doi.org/10.1016/j.bcp.2008.08.032.

Gorska, A.E., Joseph, H., Derynck, R., Moses, H.L., Serra, R., 1998. Dominant-negative interference of the transforming growth factor beta type ii receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. Cell Growth Differ. 9, 229–238.

Guo, H., Jin, Y., Jin, Y., Cheng, Y., Leaderer, B., Lin, S., Holford, T.R., Qiu, J., Zhang, Y., Shi, K., Zhu, Y., Niu, J., Bassig, B.A., Xu, S., Zhang, B., Li, Y., Hu, X., Chen, Q., Zheng, T., 2014. Prenatal exposure to organochlorine pesticides and infant birth weight in China. Chemosphere 110, 1–7. http://dx.doi.org/10.1016/j.chemosphere.

Haarmann-Stemmann, T., Hanno, B., Josef, A., 2009. Growth factors, cytokines and their receptors as downstream targets of arylhydrocarbon receptor (AhR) signaling pathways. Biochem. Pharmacol. 77, 508–520. http://dx.doi.org/10.1016/j.bcp.2008.09.013.

Hahn, M.E., Goldstein, J.A., Linko, P., Gasiewicz, T.A., 1989. Interaction of Hexachlorobenzene with the receptor for 2, 3, 7, 8-tetrachlorodicenzo-p-dioxin in vitro and in vivo. Arch. Biochem. Biophys. 270, 344–355. http://dx.doi.org/10.1016/0003-9861(89)90037-4.

Hewitt, S.C., Couse, J.F., Korach, K.S., 2000. Estrogen receptor transcription and transactivation estrogen receptor knockout mice: what their phenotypes reveal about mechanisms of estrogen action. Breast Cancer Res. 2, 345–352. http://dx.doi.org/10.1186/bcr79.

Hushka, L.J., Williams, J.S., Greenlee, W.F., 1998. Characterization of 2,3,7,8-tetrachlorodibenzofuran-dependent suppression and AH receptor pathway gene expression in the developing mouse mammary gland. Toxicol. Appl. Pharmacol. 152, 200–210 doi: S0041-008X(98)98508-8[pii]\r10.1006/taap.1998.8508.

Hynes, N.E., Watson, C.J., 2010. Mammary gland growth factors: roles in normal development and in cancer. Cold Spring Harb. Perspect. Biol. 2, a003186–a003188. http://dx.doi.org/10.1101/cshperspect.a003186.

Jin, U., Lee, S., Safe, S., 2012. Aryl hydrocarbon receptor (ahr) -active pharmaceuticals are selective ahr modulators in MDA-MB-468 and BT474 breast cancer cells. J. Pharmacol. Exp. Ther. 343, 333–341. http://dx.doi.org/10.1124/jpet.112.195339.

Joslin, E.J., Opresko, L.K., Wells, A., Wiley, H.S., Lauffenburger, D.A., 2007. EGF-receptor-mediated mammary epithelial cell migration is driven by sustained ERK signaling from autocrine stimulation. J. Cell Sci. 120, 3688–3699. http://dx.doi.org/10.1242/jcs.010488.

Kalluri, R., Zeisberg, M., 2006. Fibroblasts in cancer. Nat. Rev.Cancer 6, 392–401. http://dx.doi.org/10.1038/nrc1877.

Kass, L., Altamirano, G.A., Bosquiazzo, V.L., Luque, E.H., Muñoz de Toro, M., 2012. Perinatal exposure to xenoestrogens impairs mammary gland differentiation and modifies milk composition in wistar rats. Reprod. Toxicol. 33, 390–400. http://dx.doi.org/10.1016/j.reprotox.2012.02.002.

Lanigan, F., O'Connor, D., Martin, F., Gallagher, W.M., 2007. Molecular links between mammary gland development and breast cancer. Cell. Mol. Life Sci. 64, 3161–3184. http://dx.doi.org/10.1007/s00018-007-7386-2.

Lebrun, J.-J., 2012. The dual role of TGF beta in human cancer: from tumor supresor to cancer metastasis. ISRN Mol. Biol. 2012, 1–28. http://dx.doi.org/10.5402/2012/381428.

Lee, I.-K., Rhee, S.-K., 2007. Inhibitory effect of bisphenol A on gap junctional intercellular communication in an epithelial cell line of rat mammary tissue. Arch. Pharm. Res. 30, 337–343. http://dx.doi.org/10.1007/BF02977615.

Lew, B.J., Manickam, R., Lawrence, B.P., 2011. Activation of the aryl hydrocarbon receptor during pregnancy in the mouse alters mammary development through direct effects on stromal and epithelial tissues. Biol. Reprod. 84, 1094–1102. http://dx.doi.org/10.1095/biolreprod.110.087544.

Ma, Q., Baldwin, K.T., 2000. 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. J. Biol.

Chem. 275 (12), 8432–8438. http://dx.doi.org/10.1074/jbc.275.12.8432.
Macias, H., Hinck, L., 2012. Mammary gland development. Wiley Interdiscip. Rev. Dev. Biol. 1, 533–557. http://dx.doi.org/10.1002/wdev.35.Mammary.
Macon, M.B., Fenton, S.E., 2013. Endocrine disruptors and the breast: early life effects and later life disease. J. Mammary Gland Biol. Neoplasia 18, 43–61. http://dx.doi.org/10.1007/s10911-013-9275-7.
Matos, M.L., Lapyckyj, L., Rosso, M., Besso, M.J., Mencucci, M.V., Marín Briggiler, C.I., Giustina, S., Furlong, L.I., Vazquez-Levin, M.H., 2016. Identification of a novel human E-cadherin splice variant and assessment of its effects upon EMT-related events. J. Cell. Physiol. 232, 1368–1386. http://dx.doi.org/10.1002/jcp.25622.
Miret, N., Pontillo, C., Ventura, C., Carozo, A., Chiappini, F., Kleiman de Pisarev, D., Fernández, N., Cocca, C., Randi, A., 2016. Hexachlorobenzene modulates the cross-talk between the aryl hydrocarbon receptor and transforming growth factor-β1 signaling, enhancing human breast cancer cell migration and invasion. Toxicology 366, 20–31. http://dx.doi.org/10.1016/j.tox.2016.08.007.
Moses, H., Barcellos-Hoff, M.H., 2011. TGF-b biology in mammary development and breast cancer. Cold Spring Harb. Perspect. Biol. 3, 1–14. http://dx.doi.org/10.1101/cshperspect.a003277.
Mrema, E.J., Rubino, F.M., Mandic-Rajcevic, S., Sturchio, E., Turci, R., Osculati, A., Brambilla, G., Minoia, C., Colosio, C., 2013. Exposure to priority organochlorine contaminants in the Italian general population. Part 1. Eight priority organochlorinated pesticides in blood serum. Hum. Exp. Toxicol. 32, 1323–1339. http://dx.doi.org/10.1177/0960327113485255.
Mulero-Navarro, S., Pozo-Guisado, E., Pérez-Mancera, P.A., Álvarez-Barrientos, A., Catalina-Fernández, I., Hernández-Nieto, E., Sáenz-Santamaria, J., Martínez, I., Rojas, J.M., Sánchez-García, I., Fernández-Salguero, P.M., 2005. Immortalized mouse mammary fibroblasts lacking dioxin receptor have impaired tumorigenicity in a subcutaneous mouse xenograft model. JBC 280, 28731–28741. http://dx.doi.org/10.1074/jbc.M504538200.
Murray, T.J., Maffini, M.V., Ucci, A.A., Sonnenschein, C., Soto, A.N., 2007. Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. Reprod. Toxicol. 23, 383–390.
Murray, I.A., Patterson, A.D., Perdew, G.H., 2014. Ah receptor ligands in cancer: friend and foe. Nat. Rev. Cancer 14, 801–814. http://dx.doi.org/10.1038/nrc3846.
Park, S., Dong, B., Matsumura, F., 2007. Rapid activation of c-src kinase by dioxin is mediated by the Cdc37-HSP90 complex as part of ah receptor signaling in MCF10A cells. Biochemistry 46, 899–908. http://dx.doi.org/10.1021/bi061925f.
Parvani, J.G., Taylor, M.A., Schiemann, W.P., 2011. Noncanonical TGF-b signaling during mammary tumorigenesis. J. Mammary Gland Biol. Neoplasia 16, 127–146. http://dx.doi.org/10.1007/s10911-011-9207-3.
Peña, D., Pontillo, C., García, A., Cocca, C., Álvarez, L., Chiappini, F., Bourguignon, N., Frahm, I., Bergoc, R., Kleiman de Pisarev, D., Randi, A., 2012. Alterations in c-Src/HER1 and estrogen receptor α signaling pathways in mammary gland and tumors of hexachlorobenzene-treated rats. Toxicology 293, 68–77. http://dx.doi.org/10.1016/j.tox.2011.12.012.
Pontillo, C., García, A., Peña, D., Cocca, C., Chiappini, F., Álvarez, L., Kleiman de Pisarev, D., Randi, A., 2011. Activation of c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways and cell migration by hexachlorobenzene in MDA-MB-231 human breast cancer cell line. Toxicol. Sci. 120, 284–296. http://dx.doi.org/10.1093/toxsci/kfq390.
Pontillo, C., Rojas, P., Chiappini, F., Sequeira, G., Cocca, C., Cricci, M., Colombo, L., Lanari, C., Kleiman de Pisarev, D., Randi, A., 2013. Action of Hexachlorobenzene on tumor growth and metastasis in different experimental models. Toxicol. Appl. Pharmacol. 268, 331–342. http://dx.doi.org/10.1016/j.taap.2013.02.007.
Puga, A., Ci, M., Marlowe, J.L., 2009. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem. Pharmacol. 77, 713–722. http://dx.doi.org/10.1016/j.bcp.2008.08.031.
Ralph, J.L., Orgebin-Crist, M.C., Lareyre, J.J., Nelson, C.C., 2003. Disruption of androgen regulation in the prostate by the environmental contaminant hexachlorobenzene. Environ. Health Perspect. 111, 461–466. http://dx.doi.org/10.1289/ehp.5919.
Randi, A.S., Cocca, C., Carbone, V., Nuñez, M., Croci, M., Gutiérrez, A., Bergoc, R., Kleiman de Pisarev, D., 2006. Hexachlorobenzene is a tumor co-carcinogen and induces alterations in insulin-growth factors signaling pathway in the rat mammary gland. Toxicol. Sci. 89, 83–92. http://dx.doi.org/10.1093/toxsci/kfj023.
Rico-Leo, E.M., Alvarez-Barrientos, A., Fernández-Salguero, P.M., 2013. Dioxin receptor expression inhibits basal and transforming growth factor β-induced epithelial-to-mesenchymal transition. JBC 288, 7841–7856. http://dx.doi.org/10.1074/jbc.M112.425009.
Rudel, R.A., Fenton, S.E., Ackerman, J.M., Euling, S.Y., Makris, S.L., 2011. Environmental exposures and mammary gland development: state of the science, public health implications, and research recommendations. Environ. Health Perspect. 119, 1053–1561. http://dx.doi.org/10.1289/ehp.1002864.
Sadlonova, A., Novak, Z., Johnson, M.R., Bowe, D.B., Gault, S.R., Page, G.P., Thottassery, J.V., Welch, D.R., Frost, A.R., 2005. Breast fibroblasts modulate epithelial cell proliferation in three-dimensional in vitro co-culture. BCR 7, R46–59. http://dx.doi.org/10.1186/bcr949.
Safe, S., Lee, S., Jin, U., 2013. Review role of the aryl hydrocarbon receptor in carcinogenesis and potential as a drug target. Toxicol. Sci. 135, 1–16. http://dx.doi.org/10.1093/toxsci/kft128.
Saoudi, A., Fréry, N., Zeghnoun, A., Bidondo, M.-L., Deschamps, V., Göen, T., Garnier, R., Guldner, L., 2014. Serum levels of organochlorine pesticides in the french adult population: the French national nutrition and health study (ENNS), 2006–2007. Sci. Total Environ. 472, 1089–1099. http://dx.doi.org/10.1016/j.scitotenv.2013.11.044.
Sappino, A.P., Skalli, O., Jackson, B., Schürch, W., Gabbiani, G., 1988. Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. Int. J. Cancer 41, 707–712. http://dx.doi.org/10.1002/ijc.2910410512.
Singh, M., McGinley, J.N., Thompson, H.J., 2000. A comparison of the histopathology of premalignant and malignant mammary gland lesions induced in sexually immature rats with those occurring in the human. Lab. Investig. 80, 221–231. http://dx.doi.org/10.1038/labinvest.3780025.
Slabáková, E., Pernicová, Z., Slavíčková, E., Staršíchová, A., Kozubík, A., Souček, K., 2011. TGF-β1-induced EMT of non-transformed prostate hyperplasia cells is characterized by early induction of SNAI2/slug. Prostate 71, 1332–1343. http://dx.doi.org/10.1002/pros.21350.
Staršíchová, A., Hrubá, E., Slabáková, E., Pernicová, Z., Procházková, J., Pěnčíková, K., Seda, V., Kabátková, M., Vondráček, J., Kozubík, A., Machala, M., Souček, K., 2012. TGF-β1 signaling plays a dominant role in the crosstalk between TGF-β1 and the aryl hydrocarbon receptor ligand in prostate epithelial cells. Cell. Signal. 24, 1665–1676. http://dx.doi.org/10.1016/j.cellsig.2012.04.008.
Sternlicht, M.D., 2006. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. BCR 8, 201. http://dx.doi.org/10.1186/bcr1368.
Sun, X., Ingman, W.V., 2014. Cytokine networks that mediate epithelial cell-macrophage crosstalk in the mammary gland: implications for development and cancer. J. Mammary Gland Biol. Neoplasia 19, 191–201. http://dx.doi.org/10.1007/s10911-014-9319-7.
To- Figueras, J, Sala, M., Otero, R., Barrot, C., Santiago-Silva, M., Rodamilans, M., Herrero, C., Grimalt, J., Sunyer, J., 1997. Metabolism of hexachlorobenzene in humans: association between serum levels and urinary metabolites in a highly exposed population. Environ. Health Perspect. 105, 78–83. http://dx.doi.org/10.2307/3433066.
Van Birgelen, A.P., 1998. Hexachlorobenzene as a possible major contributor to the dioxin activity of human milk. Environ. Health Perspect. 106, 683–688 (PMCID: PMC1533492).
Ventura, C., Núñez, M., Miret, N., Martinel Lamas, D., Randi, A., Venturino, A., Rivera, E., Cocca, C., 2012. Differential mechanisms of action are involved in chlorpyrifos effects in estrogen-dependent or -independent breast cancer cells exposed to low or high concentrations of the pesticide. Toxicol. Lett. 213, 184–193. http://dx.doi.org/10.1016/j.toxlet.2012.06.017.
Wolff, S., Harper, P.A., Wong, J.M.Y., Mostert, V., Wang, Y.P., Abel, J., 2001. Cell-specific regulation of human aryl hydrocarbon receptor expression by transforming growth factor-beta(1). Mol. Pharmacol. 59, 716–724. http://dx.doi.org/10.1124/mol.59.4.716.
Zu, X., Zhang, Q., Cao, R., Liu, J., Zhong, J., Wen, G., Cao, D., 2012. Transforming growth factor-b signaling in tumor initiation, progression and therapy in breast cancer: an update. Cell Tissue Res. 347, 73–84. http://dx.doi.org/10.1007/s00441-011-1225-3.