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# Long-term exposure to β-hexachlorocyclohexane (β-HCH) promotes transformation and invasiveness of MCF-7 human breast cancer cells

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**Abstract**

Due to its lipophilicity and persistence, an organochlorine compound, β-hexachlorocyclohexane (β-HCH), is known to frequently accumulate in human adipose and breast tissues. An epidemiological study has indicated that exposure to β-HCH could be one of the significant environmental risk factors for the development of human breast cancers. Additionally, β-HCH has recently been identified as an environmental estrogen capable of activating estrogen receptor (ER) through a ligand-independent pathway. In the present investigation, we examined the impact of long-term *in vitro* exposure to β-HCH on cell transformation and the metastatic potentials of MCF-7 cells. We found that continuous exposure of MCF-7 cells to β-HCH at 100 nM and 1 μM or to 17β-estradiol ($E_2$) at 1 nM for up to 13 months (33 passages) not only enhanced their transformation tendencies but also promoted their invasiveness. Western blot analysis revealed that β-HCH induced transformation-related biochemical changes in MCF-7 cells, such as a decline in the levels of ERα and p44/42 MAP kinase and a significant increase in expression of c-ErbB2 and MMP-9 levels. In contrast, long-term $E_2$ treatment resulted in the downregulation of ERα and p44/42 MAP kinase and upregulation of MMP-9 only, but no changes in c-ErbB2. Together, these results indicate that these biochemical changes induced by β-HCH are consistent with the events taking place in these cells to promote the phenotypical expression of transformed cells. Our results provide the *in vitro* mechanistic basis supporting the hypothesis that β-HCH is one of the epigenetic risk factors assisting the progression of breast cancer cells to an advanced state of malignancy.


*Keywords:* MCF-7 breast cancer cells; β-Hexachlorocyclohexane (β-HCH); Long-term exposure; Environmental risk factor; c-ErbB2 overexpression

## 1. Introduction

It is well known that the etiology of human breast cancer is significantly affected by environmental factors. One of the factors frequently discussed is a class of pollutants known as organochlorine compounds, which include some pesticides and industrial chemicals such as DDT, HCH, and PCB. So far, several epidemiological studies have been undertaken to investigate the link between organochlorines and breast cancers. The results of some studies [1,2] suggest a positive correlation between the presence of these chemicals in human tissues and the genesis of human breast cancers, while other investigations have failed to demonstrate any relationship between organochlorine compounds [3] and breast cancers [4–8]. Thus, from an epidemiological point of view the question of whether organochlorine chemicals comprise a risk factor for the development of breast cancer remains equivocal. Since epidemiological studies can only give empirical correlations, long-term laboratory experiments are urgently needed to show a causal relationship exists between organochlorines and breast cancers, based on mechanistic evidence.

β-HCH is a contaminant of the pesticide formulation of HCH for which the active insecticidal ingredient is actually γ-hexachlorocyclohexane (γ-HCH) or lindane, which is still used in North America. The residues of β-HCH are

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*Abbreviations:* β-HCH, β-hexachlorocyclohexane; CDCS, charcoal dextran-treated calf serum; DMEM, Dulbecco's modified Eagle's medium; $E_2$, 17β-estradiol; ER, estrogen receptor; MAPK, mitogen activated protein kinase; MMP-9, matrix metalloproteinase 9; PMSF, phenylmethylsulfonylfluoride; PVDF, polyvinylidene diflouride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis; TBST, tris-buffered saline plus Tween 20.

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found in many places. For instance, β-HCH is found in mothers' milk [9] at a similar level as $p'p'$-DDT (32 and 39 ppb, respectively) among samples from Arkansas. The high lipophilicity of β-HCH, together with its extreme resistance to metabolism, makes it an extremely bioaccumulating and persistent pollutant in fatty tissues such as breast tissues with an estimated half-life in humans of 7–10 years. A Finnish epidemiological study has revealed that the average concentration of β-HCH in the breast tissues of cancer patients is 130 μg/kg fat, compared to 80 μg/kg fat for controls (equivalent of 130 and 80 ppb). The authors concluded that this organochlorine compound is a significant risk factor for the development of breast cancers [10].

β-HCH has no agonistic affinity to the ER [11,12]. Nevertheless, it can act estrogenically through a mechanism other than the classic pathway of ER binding and activation. Steinmetz *et al*. [12] first observed the estrogenic actions of β-HCH in MCF-7 cells and proposed that this chemical may activate ER through an unconventional pathway without involving ER binding. Recently, it was reported from this laboratory that β-HCH as well as other organochlorine compounds are capable of activating c-ErbB2 *in vitro*, and thereby act estrogenic [13–15]. A further study has delineated the pathway for the estrogenic actions of β-HCH, in which activation of c-ErbB2 in the presence of β-HCH can lead to phosphorylation of p44/42 MAP kinase, and ultimately activation of ER through a mechanism known as ''ligand-independent activation'' of ER [16].

During the course of the above studies, we found that one of the most reliable markers for the estrogenic actions of β-HCH and other related organochlorine compounds in MCF-7 cells was the increase in foci formation [14,15], indicating the early sign of cellular transformation and thereby a possible link between their estrogenic actions and the potential to eventually promote the etiology of breast cancer. Since the chemically-induced increase in foci formation is only a short-term cellular response (14–21 days), we posed the question of whether methodologies can be developed to address longer term effects of these pollutants with respect to their carcinogenic potentials. In the present investigation, we exposed MCF-7 human breast cancer cells to β-HCH at environmentally realistic concentrations, along with cells exposed to $E_2$, which were used as a positive control for up to 13 months. In addition, cells were exposed only to the carrier solvent during this period to serve as a negative control. We found that long-term exposure to β-HCH not only promoted cellular transformation but also increased the invasiveness of MCF-7 cells. Molecular analysis revealed that the increased aggressiveness of MCF-7 cells treated by β-HCH was accompanied by downregulation of ERα and p44/42 MAP kinase and overexpression of c-ErbB2, while $E_2$-induced cellular alteration was only associated with downregulation of ER and p44/42 MAP kinase. The different profiles of molecular changes induced by β-HCH and $E_2$ suggest that the molecular mechanisms underlying increased aggressiveness of MCF-7 cells treated with these two chemicals may not be identical. These observations also provide *in vitro* mechanistic evidence for those scientists supporting the epidemiologically derived hypothesis that β-HCH is an environmental promoter of human breast cancers.

## 2. Materials and methods

### 2.1. Cells, culturing conditions and treatments

MCF-7 cells were originally purchased from the American Type Culture Collection and routinely cultured in DMEM (GIBCO BRL) supplemented with 10% bovine calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37° and 5% $CO_2$. The long-term experiment was initiated with low passage MCF-7 cells. Cells were cultured in phenol red-free DMEM supplement with 5% heat-inactivated, CDCS (Sigma Chemical Co.), 100 U/mL penicillin and 100 μg/mL streptomycin at 37° and 5% $CO_2$. Culture medium contained 100 nM (29.1 ppb) β-HCH, 1 μM (291 ppb) β-HCH, 1 nM $E_2$ or 100 nM tamoxifen and the control received 0.1% ethanol, which was used as a carrier for β-HCH and $E_2$. The reason why two concentrations of β-HCH were chosen is to assess a rough idea of the concentration-dependency of its action. The medium, together with the chemicals, were renewed twice a week. After reaching 80% confluence, cells were trypsinized, and $5 \times 10^5$ to $8 \times 10^5$ cells were seeded for the next passage. After continuous exposure to these two chemicals (and to ethanol only in the case of control batches) for 7 months (17 passages), cells at the end of each selection week were analyzed for anchorage-independent growth, matrigel invasion, zymographic electrophoresis and Western blot of c-ErbB2, p44/42 MAP kinase and ERα, while several batches of $5 \times 10^5$ to $8 \times 10^5$ cells for each treatment and control groups continued to be subjected to β-HCH and $E_2$ exposure. After an additional 6 months of continuous exposure (16 additional passages), long-term exposure experiments were terminated and the same analyses as above were performed to examine whether the physiology and biochemistry of these cells had changed.

### 2.2. Anchorage-independent growth assay

This assay was conducted in 6-well plates. Three batches of cells were prepared for each treatment group and each batch was plated in 10 wells. The bottom layer was prepared by introducing 2 mL 1% agar in phenol red-free DMEM containing 10% CDCS to each well. After the basal layer hardened, a 1.5 mL 0.3% agar in 10% CDCS-DMEM solution containing $1 \times 10^5$ cells was cast on top of the bottom layer. After 7 days of incubation at 37° and 5% $CO_2$, cell colonies were counted and transformation

efficiency, in terms of the number of colonies (cluster of several cells), was determined.

### 2.3. Cell invasion assay

*In vitro* invasion assays were performed according to the method of Moon *et al*. [17], with slight modifications. The experiments were carried out in 24-well Transwell plates fitted with polycarbonate filters, 6.5 mm in diameter and 8.0 μm in pore size. Three batches of cells were prepared for each treatment group and each batch was seeded in 10 separate wells. The lower side of the filter was first coated with 12 μL matrigel (BD Biosciences) and air-dried for 45 min. Then, the upper side of the filter was coated with 10 μL matrigel and allowed to air-dry for another 45 min. The lower compartment was filled with 600 μL phenol red-free DMEM containing 0.1% CDCS while a 100 μL phenol red- and serum-free DMEM solution containing $1 \times 10^5$ cells was introduced into the upper chamber. After 40 hr incubation at 37° and 5% $CO_2$, cells were fixed with 5% glutaraldehyde for 15 min. The matrigel on the upper side of the filter was wiped off with tissue paper and invasive cells on the lower side of the filter were counted with the aid of a compound microscope.

### 2.4. Zymographic electrophoresis assay

This test was carried out essentially as described by Moon *et al*. [17]. When cells reached 80% confluence, serum-free and phenol red-free DMEM without antibiotics or antimycotics was added to the cell culture. Four batches of cells were prepared for each treatment group. After 48 hr incubation at 37° and 5% $CO_2$, the conditioned medium was collected and centrifuged to remove debris of the dead cells. A 25 μL of conditioned medium for each treatment and control was incubated with 15 μL sample buffer without the reducing agent mercaptoethanol at room temperature for 15 min, and resolved on 10% SDS–PAGE. The gel contained 1 mg/mL gelatin (Sigma). To recover enzymatic activity, SDS in the gel was washed off with 2.5% Triton X-100 for $2 \times 15$ min, followed by rinsing $3 \times 10$ min with a 50 mM, pH 7.6 Tris–HCl buffer containing 5 mM $CaCl_2$, 0.02% Brij-35 and 0.2% sodium azide. After 96 hr incubation at 37°, the gel was stained with Coomassie brilliant blue R-250 and destained with a solution containing 7.5% acetic acid and 10% methanol. The clear areas against the blue-stained gelatin represent where the gelatin-degrading enzymes were present.

### 2.5. Western blot analysis

For each treatment group, three to five batches (see figure captions) of cells were independently prepared. From those, the whole cell lysate was obtained according to the protocol provided by Santa Cruz Biotechnology, Inc. Approximately, 80% confluent MCF-7 cells in culture on ice were scraped off in 0.9 mL of 50 mM, pH 7.5 Tris–HCl buffer containing 0.15 M NaCl, 1 mM $Na_3VO_4$, 1% NP-40, 0.1% SDS, 0.1 mg/mL PMSF and 0.5% deoxycholate. Scraped cells were passed through a 21-gauge needle to shear the DNA, and incubated 45 min on ice. The cell lysate was centrifuged at 10,000 *g* for 10 min at 4°. The supernatant was collected and protein concentrations determined according to the method of Bradford [18].

For the Western blot analysis, carefully matched, equal amounts (20 μg) of proteins of the whole cell lysate were resolved by SDS–PAGE (3.75% stacking gel and 10% separating gel). Proteins were transferred to a PVDF membrane. The membrane was blocked in 5% nonfat milk prepared in TBST solution (10 mM, pH 7.8 Tris–HCl buffer containing 0.15 M NaCl and 0.09% Tween 20) for $2 \times 30$ min, and then incubated overnight with the following antibodies in 5% nonfat milk-TBST at 4°: c-ErbB2 antibody (Santa Cruz Biotechnology, Inc.), p44/42 MAP kinase antibody (Upstate Biotechnology),

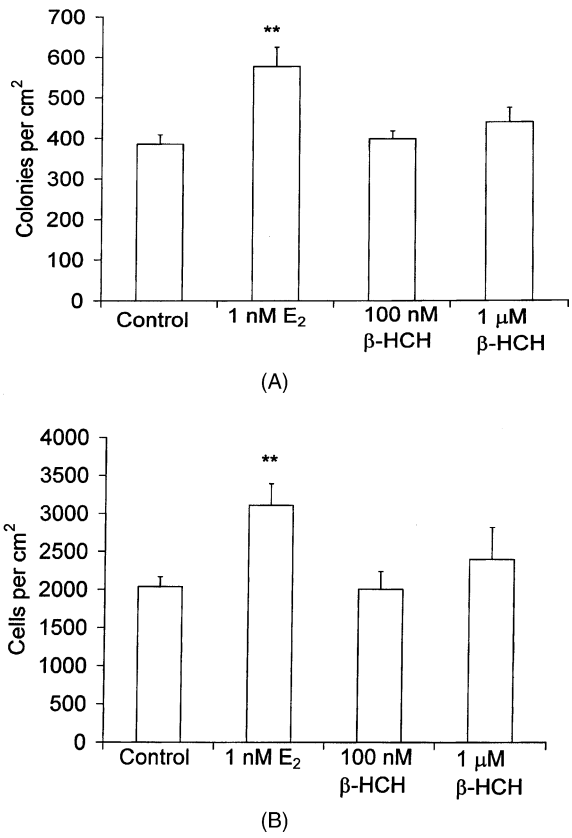


Fig. 1. (A) Anchorage-independent growth of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 7 months. Error bars represent standard deviations; N = 3. $^{**}P < 0.01$ vs. control. N represents the number of independently prepared batches of cells for each treatment group. Each batch consisted of 10 wells. (B) Matrigel invasion of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 7 months. Error bars represent standard deviations; N = 3. $^{**}P < 0.01$ vs. control (see Section 2 for statistical treatments).

and ERα antibody (Santa Cruz Biotechnology, Inc.). The membrane was washed 4 × 10 min in TBST and incubated with a horse radish peroxidase-conjugated second antibody in 5% nonfat milk-TBST at room temperature for 2 hr. After extensive washing, the membrane was immersed in a Super Signal West Dura Substrate working solution (Pierce) and chemiluminescence was visualized with a ChemImager 440 low light imaging system. Densitometry analysis was performed with ChemImager v5.5 (San Leandro, CA). Each plate was scanned three times, the band intensities were normalized to the matched control value (=100) to obtain the averaged value for each batch and mean ± SD were calculated from variation among three to five independently prepared batches for each treatment group.

### 2.6. Statistical analysis

In all cases, several independently prepared batches of cells for each treatment group were assessed simultaneously to obtain means and standard deviations among different batches (i.e. "N" values shown in figure captions). ANOVA and Fisher's least-significant differences test (SYSTAT 10 Computer Software) were used to determine the significance of difference between control and chemically treated groups. A probability value of less than 0.05 was regarded as significant.

## 3. Results

### 3.1. Characterization of phenotypic changes of cells exposed to chemicals for 7 months

After 7 months of continuous exposure (17 passages), we examined whether β-HCH and $E_2$ had induced phenotypical changes of MCF-7 cells as compared to control cells which were mock treated under the identical scheme for 17 passages with 0.1% ethanol only. Anchorage-independent growth in soft substrate is generally considered a marker of transformation and eventual tumorigenicity. As expected, after the 7-month exposure, MCF-7 cells treated with 1 nM $E_2$ produced significantly more cell colonies in soft agarose substrate than control ($P < 0.01$, Fig. 1A). However, MCF-7 cells exposed to β-HCH at 100 nM ($P > 0.05$, Fig. 1A) and 1 μM ($P > 0.05$) for 7 months did not appear to be more transformed than control.

Although transformation and metastasis may develop in the same cell, they are not necessarily regulated by the same molecular mechanisms [19,20]. Indeed, increased metastatic potential or invasiveness could occur without enhancing transformation abilities in some breast cancer cells [21]. Therefore, we additionally examined the invasiveness of those chemically treated cells. The Matrigel invasiveness assay is one of the standard tools for determining the invasiveness of cells. Results of matrigel

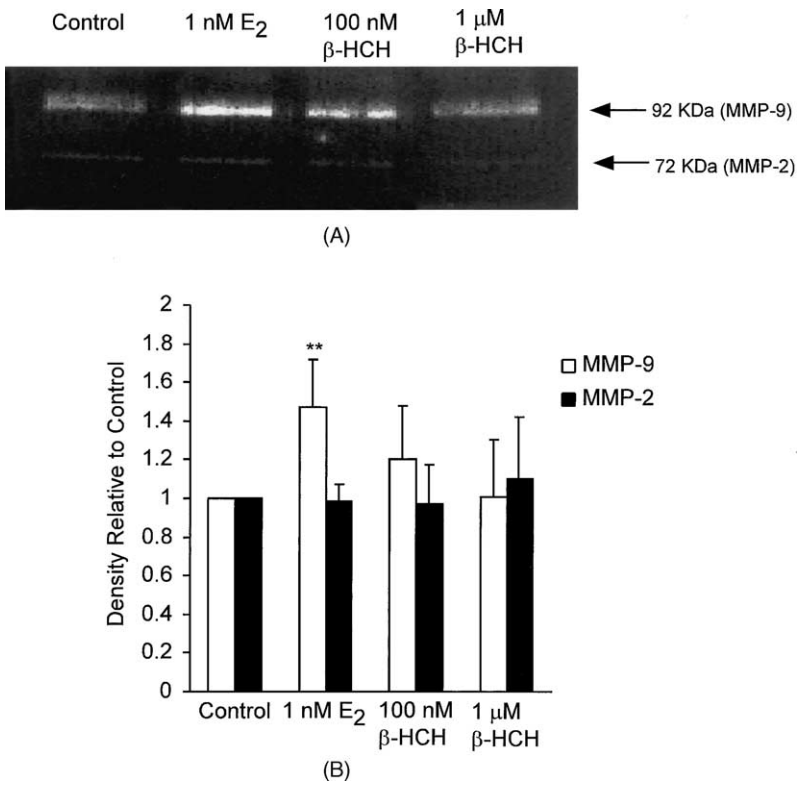


Fig. 2. Zymographic electrophoresis of conditioned media of MCF-7 cell cultures treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 7 months. (A) A representative zymographic gel where the clear bands represent collagenases MMP-2 and -9. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 5. $^{**}P < 0.01$ vs. control.

invasiveness assay (Fig. 1B) showed that 1 nM $E_2$-treated cells were significantly more invasive than control ($P < 0.01$), while metastatic potential of MCF-7 cells treated with 100 nM ($P > 0.05$) or 1 μM β-HCH ($P > 0.05$) did not appear to be significantly higher than that of control cells. When cells become invasive, they often produce proteolytic enzymes such as 92 kDa type IV collagenase (also called matrix metalloproteinase 9 or MMP-9), and 72 kDa type IV collagenase (also called matrix metalloproteinase 2 or MMP-2). These enzymes help cells to migrate through the matrigel, which is very similar to the basement membrane in chemical composition and texture. According to the zymographic electrophoresis profiles, the collagenase (MMP-9) activity of conditioned medium from $E_2$-treated cells was 47% more than that for control cells ($P < 0.01$, Fig. 2A and B), while MCF-7 cells exposed to β-HCH at 100 nM ($P > 0.05$, Fig. 2A and B) or 1 μM ($P > 0.05$, Fig. 2A and B) did not appear to produce significantly more MMP-9 than control cells. MMP-2 activity was not affected by either $E_2$ or β-HCH ($P > 0.05$ in all cases, Fig. 2A and B). These results suggest that after 7-month exposure only 1 nM $E_2$ rendered MCF-7 cells more transformed and more invasive than control cells. In all cases, control cells treated with vehicle only for 7 months did not exhibit any noticeable changes from the low-passaged, original strain of MCF-7 cells in any of the categories studied so far.

### 3.2. Characterization of phenotypic changes of cells exposed to chemicals for 13 months

After additional 6 months (additional 16 passages) of continuous exposure (altogether 13 months or 33 passages), MCF-7 cells were analyzed for their state of transformation and invasiveness. Results in Fig. 3A show that as judged by anchorage-independent growth, exposure of MCF-7 cells to 1 nM $E_2$ ($P < 0.01$), 100 nM ($P < 0.01$) or 1 μM β-HCH ($P < 0.01$) resulted in a significantly advanced state of transformation compared to the control. As judged by the matrigel assay, 13-month exposure to 1 nM $E_2$ ($P < 0.05$, Fig. 3B), or β-HCH at 100 nM ($P < 0.01$, Fig. 3B) and 1 μM ($P < 0.01$, Fig. 3B) produced cells showing significantly enhanced invasiveness compared to control. Similarly, cells selected with 100 nM tamoxifen for 33 passages (used here as a positive control) showed an increased level of invasiveness ($4738 \pm 656$ as compared to $2282 \pm 188$ cells per $cm^2$ for control, N = 3) similar to those treated with 1 nM $E_2$ (Fig. 3B), indicating that a tamoxifen resistant phenotype has probably been selected by this treatment [22]. Such increased invasiveness of chemically treated cells was also manifested by their ability to produce more collagenase MMP-9. Zymographic electrophoresis results demonstrate that 13-month exposure to 1 nM $E_2$ led to a 3-fold increase in MMP-9 activity ($P < 0.01$, Fig. 4A and B) while the cells treated with β-HCH at 100 nM and 1 μM ($P < 0.01$ in both cases,

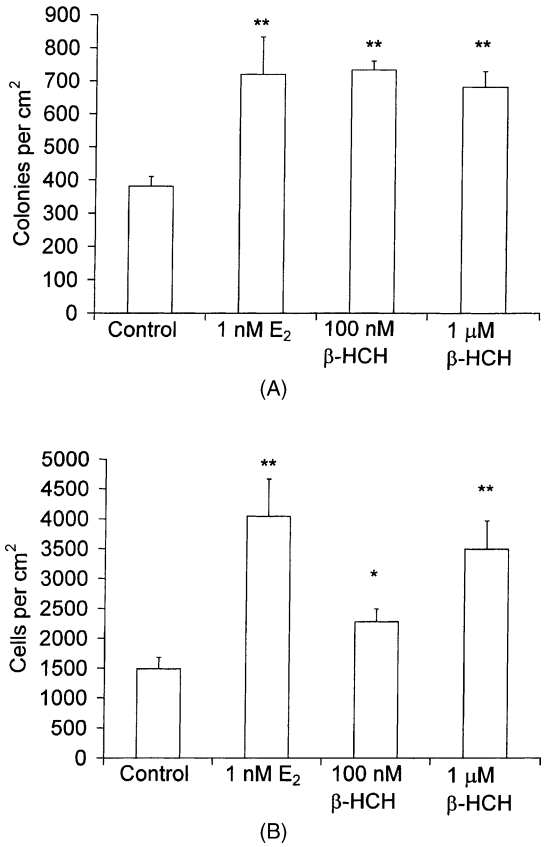


Fig. 3. (A) Anchorage-independent growth of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 13 months. Error bars represent standard deviations; N = 3. $^{**}P < 0.01$ vs. control. (B) Matrigel invasion of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 13 months. Error bars represent standard deviations; N = 3. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

Fig. 4A and B) resulted in a 2-fold elevation in MMP-9 activity. Again, neither chemical appeared to affect MMP-2 activity after 13-month exposure ($P > 0.05$ in all cases, Fig. 4A and B). It is apparent from the above results that after 13 months of continuous exposure, some phenotypical changes occurred in that MCF-7 cells treated with $E_2$ or β-HCH exhibited characteristics of more transformed and more invasive cells than control cells.

### 3.3. Studies on changes in biochemical markers

To gain insight into the molecular mechanisms underlying these phenotypical changes, we examined the profiles of three key biochemical components of the β-HCH signaling pathway, i.e. c-ErbB2, p44/42 MAP kinase and ERα [16]. After 7-month exposure, there was a 66% increase ($P < 0.05$, Fig. 5A and B) in c-ErbB2 level in the cells treated with 1 μM β-HCH, while in the cells exposed to 1 nM $E_2$ or 100 nM β-HCH there was no change in the c-ErbB2 level as compared to control cells ($P > 0.05$ in both cases, Fig. 5A and B). After an additional 6 months of continuous exposure, a significant, 15-fold elevation in c-ErbB2 level relative to control was recorded in the cells

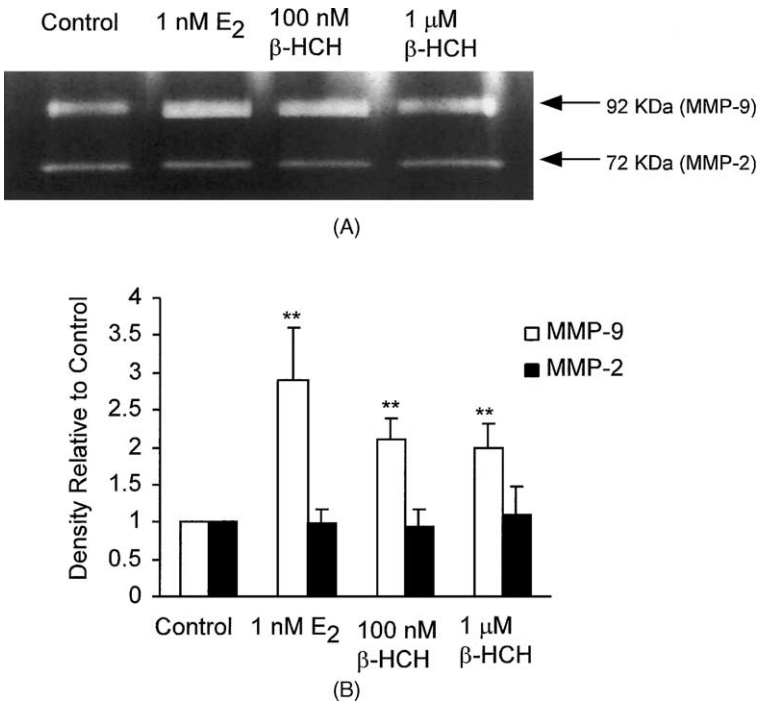


Fig. 4. Zymographic electrophoresis of conditioned media of MCF-7 cell cultures treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 13 months. (A) A representative zymographic gel where the clear bands represent collagenases MMP-2 and -9. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 4. $^{**}P < 0.01$ vs. control.

treated with β-HCH both at 100 nM and 1 μM ($P < 0.01$ in both cases, Fig. 6A and B). Interestingly, c-ErbB2 levels of the cells having undergone 13 months of $E_2$ exposure did not change significantly compared to that of control cells ($P > 0.05$, Fig. 6A and B). These results indicate that the action pathway of β-HCH may be different from that of $E_2$, the former elevating c-ErB2 at the initial 7-month time point when those phenotypical changes have not taken place in contrast to the latter showing those changes without the involvement of c-ErbB2.

At the 7-month mark, neither $E_2$ nor β-HCH significantly affected p44/42 MAP kinase levels ($P > 0.05$ in all

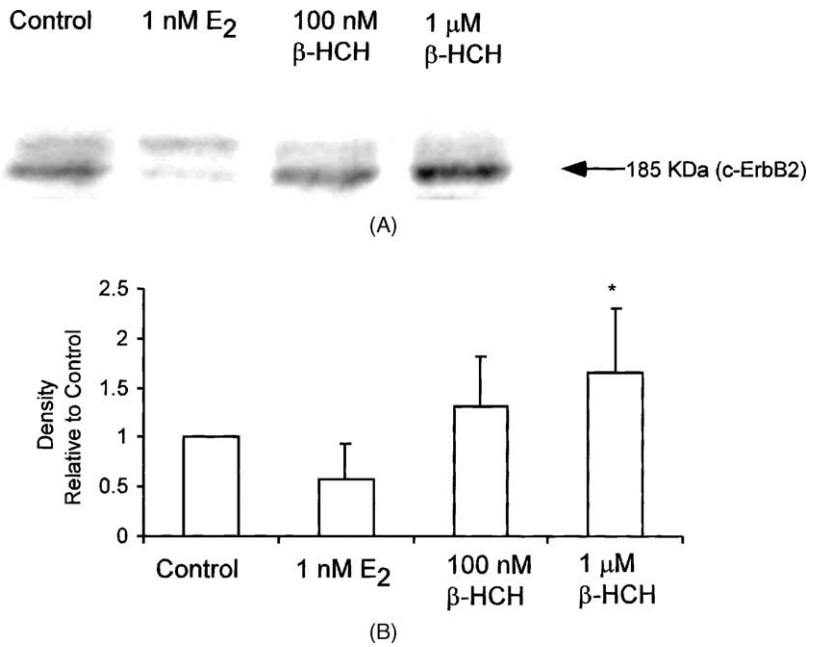


Fig. 5. Western blotting of c-ErbB2 from the whole-cell lysate of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 7 months. (A) A representative blot of c-ErbB2. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 5. $^{*}P < 0.05$ vs. control.

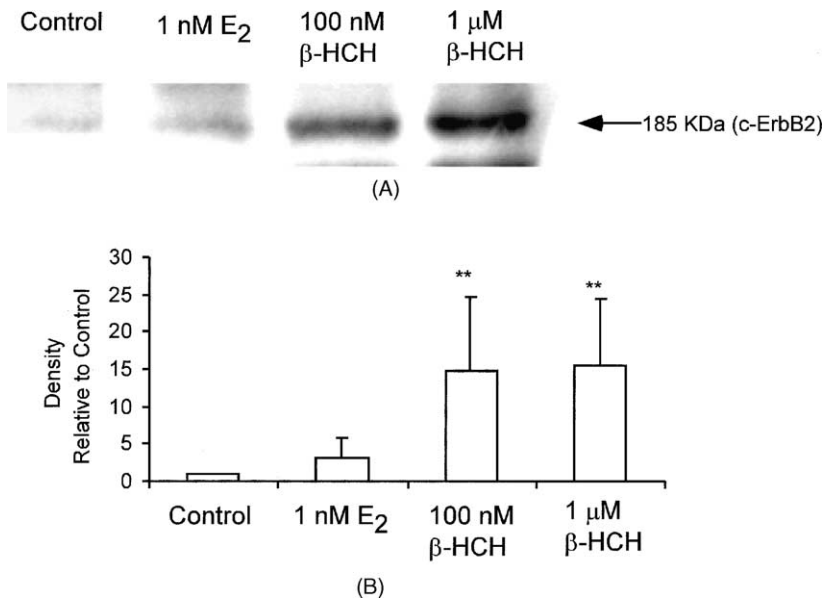


Fig. 6. Western blotting of c-ErbB2 from the whole-cell lysate of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 13 months. (A) A representative blot of c-ErbB2. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 5. $^{**}P < 0.01$ vs. control.

cases, Fig. 7A and B). However, an additional 6-month exposure led to a 41, 56 and 40% decline in p44/42 MAP kinase level of the cells treated with 1 nM $E_2$, 100 nM β-HCH and 1 μM β-HCH, respectively ($P < 0.01$ in all cases, Fig 8A and B). The level of p42 MAP kinase was also decreased by 33, 49 and 31% in 1 nM $E_2$-, 100 nM β-HCH-, and 1 μM β-HCH-treated cells, respectively ($P < 0.01$ in all cases, Fig. 8A and B). It is clear from the above data that long-term exposure to β-HCH and $E_2$ lead to a downregulation of p44/42 MAP kinase of MCF-7 cells.

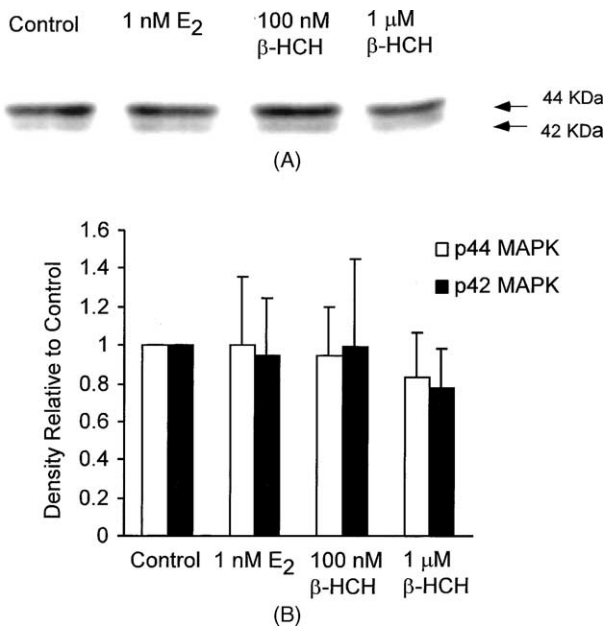


Fig. 7. Western blotting of p44/42 MAP kinase from the whole-cell lysate of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 7 months. (A) A representative blot of p44/42 MAP kinase. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 5.

As far as ERα levels are concerned, after a 7-month exposure only cells treated with 1 nM $E_2$ rendered a 52% drop in ER level ($P < 0.05$, Fig. 9A and B); β-HCH at 100 nM or 1 μM had no significant effect compared to control ($P > 0.05$ in both cases, Fig. 9A and B). After 13 months exposure, in contrast, a 70, 58 and 48% decrease in ERα level of the cells treated with 1 nM $E_2$, 100 nM β-HCH and 1 μM β-HCH, respectively, occurred after 13-month exposure ($P < 0.01$ in all cases, Fig. 10A and B), suggesting long-term exposure to $E_2$ and β-HCH can downregulate ERα levels of MCF-7 cells.

## 4. Discussion

A key question we must address immediately is whether the multiple passage selection process employed in this study could have resulted by chance in promoting the survival of specific cell clones that already existed in the original MCF-7 cell line. This is an important question, since MCF-7 cells are known to have subpopulations. In this regard, two observations appear to be relevant in indicating the unlikelihood of this possibility. First, we did not observe any shift in biochemical or phenotypical cell changes in any of the mock selected control cell batches throughout the entire period of selection. Second, during the initial period of 6 months (17 passages) selection through β-HCH treatments, we did not see any changes in the characteristics of MCF-7 cells. Instead, the changes became apparent only after 13 months (33 passages) of selection. The former provides evidence that these changes are not the artifactual result of selection itself, and the latter

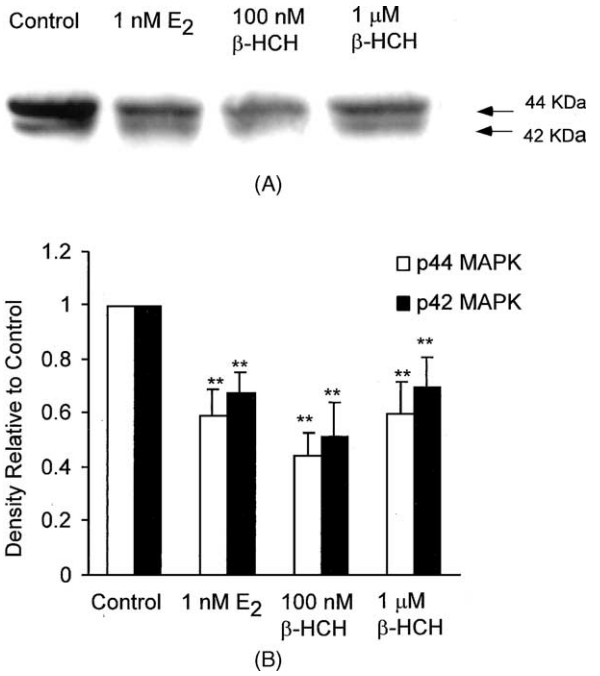


Fig. 8. Western blotting of p44/42 MAP kinase from the whole-cell lysate of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 13 months. (A) A representative blot of p44/42 MAP kinase. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 4. $^{**}P < 0.01$ vs. control.

indicates that the frequency of such cell populations (i.e. c-ErbB2 and MMP-9 overexpressing and ERα underexpressing cells), if any, is not likely to be high enough, and/or those specific cells do not have immediate survival advantages over the rest of the MCF-7 populations. Thus, while it is not possible to totally rule out the possibility of selecting pre-existing subpopulations of cells, the above

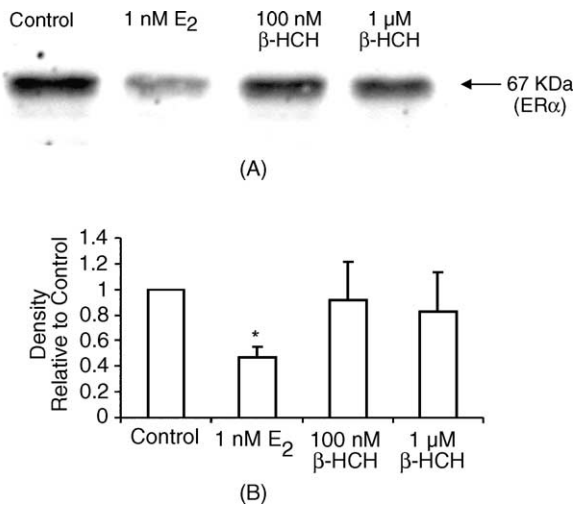


Fig. 9. Western blotting of estrogen receptor α (ERα) from the whole-cell lysate of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 7 months. (A) A representative blot of ERα. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 3. $^{*}P < 0.05$ vs. control.

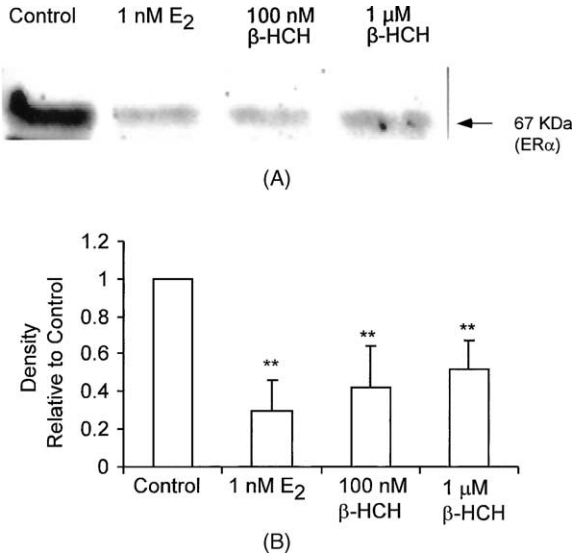


Fig. 10. Western blotting of estrogen receptor α (ERα) from the whole-cell lysate of MCF-7 cells treated with 1 nM $E_2$ and 100 nM and 1 μM β-HCH for 13 months. (A) A representative blot of ERα. (B) Results of densitometrical analysis. Error bars represent standard deviations; N = 5. $^{**}P < 0.01$ vs. control.

observations support the interpretation of gradual cellular transformation induced by $E_2$ and β-HCH. The observations that $E_2$ induced the expected rapid down-regulation of ERα [23] which was different from the changes produced by β-HCH selection, and that the effect of β-HCH was concentration-dependent also supports the above diagnosis.

Another technical point that merits a brief discussion is the reliability of our assay systems in detecting phenotypical changes induced by chemical treatments. In analyzing these changes we have always relied upon several batches of cells independently prepared for each treatment with sufficient replicates (10–12 wells) within each batch. Certainly, variations among replicates such as the number of cells per $cm^2$ are normal for this type of assay, but variations among batches were found to be reasonably reproducible in all cases. As for electrophoresis experiments, careful adjustments of protein quantities among samples in the same gel and comparison among different batches of cells gave us the reproducible data. The fact that in many assays, particularly at the 6-month period, we could not detect any differences among treatment groups indicated a very low probability of detecting false positives by chance in all of our assays.

Since the most conspicuous biochemical change we observed in the present investigation is the 15-fold increase in c-ErbB2 level of the cells treated with β-HCH for over a year, we must first discuss the possible implications of β-HCH-induced c-ErbB2 upregulation. According to Liu *et al.* [24], MCF-7 cells overexpressing transfected c-ErbB2 show growth advantages in estrogen-depleted conditions as well as reduced estrogen-dependency. Their observation agrees well with our finding in this study. Breast cancer

cells overexpressing c-ErbB2 are known to show an activated PI3K pathway which promotes anchorage independent growth [25]. Thus, these biochemical changes are consistent in general with the phenotypical changes observed in β-HCH selected MCF-7 cells. As for the cause of the increase in c-ErbB2 expression, we do not have enough solid evidence at this stage to support any of the possibilities we have examined so far to warrant in depth discussion. Possible causes include β-HCH induced ERα downregulation [26,27], synergism with heregulin [16] cyclin D activation [28] and anti-estrogen resistance [29]. Therefore, this question remains unanswered.

As for the possible significance of our findings, it is well acknowledged that breast cancer development is the result of multistage changes in cellular molecular biology. In this regard, MCF-7 cells are generally considered to be a cancer cell line representing an ''early'' phenotype (e.g. estrogen and progesterone receptor positive, steroid responsive with a low metastatic potential) [30]. Using MCF-7 cells, therefore, a number of scientists have selected more advanced forms of malignant progression to study the influence of epigenetic factors affecting critical phenotypic changes taking place in this cell lineage [31–36]. Typical phenotypic changes accompanying malignant progression include reduction and eventual loss of hormone-dependence and responsiveness [30], acquired resistance to therapeutic drugs [30,34], increased growth factors and growth factor receptors [37] and increased metastatic potential [32]. The results of our current study indicate that those phenotypic changes took place along with biochemical changes such as reduction in ERα, increased expression of MMP-9 and the increase in c-ErbB2 as a result of β-HCH selection, and that those changes are similar to those reported to occur in MCF-7 cells progressing to the direction of malignant transformation (see [32]). Judging by the fact that β-HCH-selected cells did not totally lose ERα and that the degree of c-ErbB2 upregulation was 15-fold, the extent of their progression to malignancy may not be as complete as some of the more advanced forms of MCF-7-derived malignant sublines (e.g. [30,37]). On the other hand, the 15-fold increase in c-Neu protein expression, at both concentrations of β-HCH, maybe quite significant; according to Carter *et al*. [38] stable transfection of MCF-7 cells with c-Neu overexpressing plasmid results in a 6.6-fold increase in c-Neu, and yet that was enough to convert MCF-7 cells to become more endothelial-transmigrating type of cancer cells in nude mice. In another study, Giani *et al.* [39] tranfected MCF-7 cells with another c-Neu expression vector and found that two of the most c-Neu overexpressing lines showed a 14.6- and 16.3-fold increase in c-Neu and found that those cells showed an acquired ability to grow in a hormone-deprived medium, as well as showing phenotypically different cell characteristics. An important point, in the end, is that these characteristics of the progressing phenotypes appear to have arisen in response to the selection pressure of β-HCH in a dose-dependent manner. Thus, the direction of β-HCH-induced phenotypical changes supports the view that β-HCH is likely to be at least one of the epigenetic promoters assisting malignant transformation process in this cell material.

Finally, it must be pointed out that the concentrations of β-HCH employed in the current study are realistic mainly based on actual residue levels found in human breast tissues. For instance, according to the Finnish study [10], the levels found were 180 ppb (fat basis) among cancer patients and 80 ppb in the control sample, indicating that an increase of 100 ppb is a risk factor. In the current study, the level we chose were 100 nM (29.1 ppb) and 1 μM (291 ppb). Moreover, the half-life value for β-HCH residues in humans has been estimated to be in the order of 7.2–7.6 years [40], and therefore, the continuous exposure regimen (to β-HCH for 13 months) employed in this study should not be viewed as an unrealistic treatment in view of many years of exposure expected to be experienced by human breast cells in the real world.

In conclusion, we have shown in the current study that long-term treatment of MCF-7 cells with an organochlorine compound, β-HCH, definitively results in directional phenotypic changes of MCF-7 cells to become more proliferative in an anchorage-independent manner and more invasive. All biochemical changes accompanying the above phenotypical shift are consistent with the diagnosis that these cells are moving to a more advanced state of carcinogenic progression.

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