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Functional integration of the Ah receptor with RelB and IL-8 confers the pro-survival advantage to mammary epithelial cells of inflammatory breast tumors

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Running Title: RelB and IL-8 overexpression in breast tumor regulated by AhR

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Abstract

The Ah receptor (AhR) has been best known for its role in mediating the toxicity of dioxin. However, the carcinogenic meaning of AhR in breast cancer has not been fully addressed yet. Here we show that AhR overexpression is found among estrogen receptor (ER) α -negative human breast tumors and that its overexpression is positively correlated to that of NF- κ B subunit RelB and Interleukin (IL)-8. Increased binding activity of the AhR/RelB complex in the nucleus, coupled to IL-8 overexpression, has been found in primary breast cancer tissue, which was also supported by in situ hybridization evidence. To assess the carcinogenic mechanism of AhR/RelB collaboration we searched in vitro cell models and found similar genotype in transformed MDA-MB 231, MDA-MB 436, MCF10AT1- and MCF-7-derived mammary epithelial cell lines. Activation of AhR function by dioxin (TCDD) promoted the formation of AhR/RelB complex in the nucleus and increased IL-8 expression in MDA-MB 436 and MCF-7 cells. Consistently, downregulation of RelB or AhR by small interfering RNAs decreased the level of IL-8 in vitro in MCF-7 cells. The existence of this unique genotype of breast tumors, overexpressing these three pro-survival factors, helps to explain the process of the genesis of this type of environmentally-induced type of breast cancers.

Keywords: AhR, NF- κ B RelB, IL-8, Inflammation, breast cancer, TCDD

Introduction

It was initially reported by David H. Sherr's group that high levels of AhR expression is detected in DMBA-induced mammary tumors *in vivo* in rats (e.g. Trombino et al., 2000) as well as in several mouse and human cell lines exhibiting malignant phenotypes (Shin et al., 2006). It was pointed out by the same group of scientists (Schlessinger et al. 2006) that there is likelihood of the AhR contributing greatly to ongoing mammary tumor cell growth and apoptosis resistance while promoting transition to an invasive, metastatic phenotype in these cases. The above observations at the same time have raised a new set of important questions: such as, how the overexpressed AhR contributes to those cellular changes, even in the absence of its ligands, how it coordinates its actions with signaling of growth factors and hormones, how it causes inflammatory responses in those cells, and how it contributes to malignant progression of those cells? Meanwhile there have been a number of recent discoveries on the physiological roles of the AhR itself (i.e. its natural biological activities that is not induced by exogenous ligands) mostly based on studies on AhR knockout mice (Gonzales and Fernandez-Salguero, 1998; McMillan and Bradfield, 2007; Kawajiri and Fujii-Kuriyama, 2007) as well as the roles of AhR as one of the major coordinators of cellular stress responses, particularly inflammatory responses (Matsumura, 2009). The role of AhR as a pro-survival factor conferring cells to acquire apoptosis resistance, even in the absence of any exogenously added ligand of this receptor, has been reported in an ER α -negative MCF10AT1 breast cancer cells from our group (Wong et al., 2009). These recent developments point to the possibility that the overexpressed AhR could indeed play an important role in aiding the process of malignant transformation of those transformed mammary epithelial cells.

Despite the clear-cut demonstration of overexpression of AhR in DMBA-induced breast tumors in rats and mice, and findings on the presence of overexpressed AhR in a number of established human breast cancer cell lines, there has not been any documentation on the expression of AhR in human breast tumor samples. Accordingly our major objectives for this study have been set as: (a) to clearly establish the existence of human breast tumors, overexpressing the AhR, (b) to identify the basic type of breast tumors that overexpresses AhR, (c) to characterize the genotype of this type of tumors, and (d), if possible, to gain insights to the mechanistic causes of the AhR-dependent progression of those mammary epithelial cells to the malignant phenotype.

Results

Relative gene expression differences measured by real-time PCR for NF- κ B and AhR target genes in breast tumors versus normal breast tissue

First we investigated the mRNA expression level of RelB, AhR and IL-8 in tumor tissues of breast cancer patients (Table 1), by analyzing the mRNA level of normal breast tissue and tumor tissue from a total of 17 patients. Of the 17 samples, 7 tumor tissue samples were identified as ER α -negative and 10 tissue samples were identified as ER α -positive. The expression of the selected genes correlated with the ER α -status (Table 1) showed that a significantly elevated level of the AhR mRNA was detected only in breast tumor tissue identified as ER α -negative samples. This group also showed elevated expression of both IL-8 and RelB. Although the number of samples was not large enough to derive statistical significance of this set of mRNA expression

data, the results provided at least an indication on the possible genotypic characteristics of the sub-group of the ER α -negative type of breast tumors.

When these data were re-analyzed according their ER α expression differences, it became apparent that the ER α -negative tumors show consistently higher expression of all markers studied than ER α -negative ones (Fig. 1). The elevated levels of the expressions of RelB, AHR, IL-8 and cytochrome P450 (CYP)1B1 were statistically significant as compared to the corresponding normal tissues, each collected from the same patient, but not that of CYP1A1.

Western Blot analysis of AhR and RelB in primary breast tissues

To gain further insight to the inter-relationship among AhR, RelB and ErbB2 we carried out a western blot assay on 6 pairs of breast tumor samples from the genotype of ER α -negative samples. For this purpose we compared their protein expression of AhR, ErbB2 and RelB in both the tumor sample (designated by T in Fig. 2) and the matched normal breast tissue samples (designated as N) from each patient. In five of the six samples we found a significantly higher level of RelB protein in the tumor tissue compared to normal tissue. We observed a less pronounced but significant increase of AhR protein in 4 out of 6 patients compared to the corresponding normal tissue. In contrast, the pattern of expression of ErbB2 protein does not appear to be closely correlated to either AhR or RelB protein.

In situ hybridization of AhR, RelB and IL-8 proteins

To visualize the localization of AhR, RelB and IL-8 proteins within breast tissues, we have conducted in situ hybridization experiments on one pair of normal versus tumor samples from one patient. This tissue sample (ID#4061367) was identified to express high mRNA levels of

RelB and IL-8 in tumor tissue compared to the corresponding normal tissue. The result of AhR hybridization (Fig 3A) showed that AhR protein is expressed abundantly in the tumor tissue examined. AhR overexpressing cells in tumor samples were spread all over the tumor tissue, and not confined to the organized ductal tubular structures. In contrast the corresponding normal tissue showed well organized ductal structures along with stroma cells. While there were sporadic dark spots in both celltypes, the overall expression of AhR appears to be very low as compare to the corresponding tumor tissue. Regarding RelB hybridization (Fig. 3B), it appears that in the normal tissue sample the RelB protein is found only around the cells lining the ductal structures. In contrast, a much higher level of expression of this protein in the tumor sample as a whole, than the normal tissue, was found again. Upon close examination the main characteristic of the tumor sample was found to be the tremendous number of cancer cells overexpressing RelB protein packed tightly. A similar pattern of high expression of IL-8 in the tumor tissue was also observed (Fig. 3C) as compared to that in normal tissue. Again the areas with dark staining are found to consist of many cells that have spread in the tumor tissue.

DNA-binding activity of nuclear transcription factors in tumor versus normal breast tissue

To assess the nuclear protein binding activities to the specific response elements on the promoter region of the selected genes, we have carried out electromobility shift assay (EMSA) test on nuclear protein preparations from the tumor sample in comparison to the normal tissue counterpart (ID#4061367). For this purpose we selected several oligonucleotide probes which could be involved in the regulation of IL-8 as follows: a) an RelB and AhR binding sequence (designated as “RelBAhRE”) in the -50 to+1 base pair region of the human IL-8 promoter (Vogel et al., 2007a) consensus sequences for NF- κ B, c) AP-1, d) DRE (dioxin response element), or e)

CEBP. The most outstanding feature of the data shown in Fig. 4A is that nuclear protein binding to the RelBAhRE oligonucleotide is clearly higher in the tumor tissue than in the normal tissue (lanes 1 and 2). This band contains the heterodimer of RelB and AhR (Vogel et al., 2007a), which was confirmed in the super-shift assay. It is worthy to point out that the same band also exists in the EMSA visualization of nuclear protein binding to the labeled consensus NF- κ B-response element (lanes 3 and 4, Fig. 4A) of which increase in tumor sample appears to be accompanied by a simultaneous increase in the intensity of the higher complex compared to the normal sample. Since the latter proteins are expected to include RelA (p65), the above results appear to support the notion that the increase in the titer of RelB and AhR represents the negative cellular feedback response to the hyper-inflammatory status of those transformed cells (see Vogel and Matsumura, 2009). Interestingly the patterns of nuclear protein binding to the AP-1 and C/EBP consensus oligonucleotides were opposite; the former showing less, whereas the latter showed increased DNA-binding activity in the tumor sample compared to the normal counter-part. Just to make sure that the protein band showing a conspicuous increase in binding to the labeled RelBAhRE probe (Fig. 4A left two lanes) indeed contains AhR and RelB proteins, we have run an EMSA super-shift assay. The result shown in Fig. 4B demonstrates that indeed the intensity of this band decreases significantly when the nuclear protein preparation has been pre-treated with either AhR antibody or RelB antibody. As for the meaning of the increased protein binding to the consensus C/EBP response element (Fig. 4C right two lanes), we hypothesized that this is largely due to the increase in the C/EBP β isoform based on our previous experience in studying a sub-line of AhR expressing MCF10AT1 cells (=P20E) (Wong et al., 2009). Judging by the result of a super-shift assay with a specific antibody against the C/EBP β

protein, it was concluded that the increased protein binding of the C/EBP response element in tumor tissue is due mostly to that of C/EBP β protein (Fig. 4C).

Regulation of IL-8 by AhR and RelB in human breast cancer cells

An important item, still requiring confirmation is the functional correlation between the expression of IL-8 and that of RelB as well as that of AHR in mammary epithelial cells. This was tested first in ER α -negative MDA-MB-231 and MDA-MB-436 cells in comparison to ER α -positive MCF-7 cells (Fig. 5A). Both ER α -negative cells showed much higher levels of constitutive expression of IL-8 mRNA than MCF-7 cells. When cells were treated with TCDD (to functionally activate AhR) IL-8 expression increased significantly in all 3 cell lines as expected. We also assessed the levels of their mRNA expressions of AhR and RelB (Fig. 5B), which showed clearly that these two markers are highly expressed in MDA-MB-231 and -436 in comparison to MCF-7. Then we studied the effects of siRNA against AhR and RelB as well as their ectopic overexpression in ER α -positive MCF-7 cells. Transfection experiments with siRNA to target AhR and RelB suggest that IL-8 expression in MCF-7 largely depends on AhR and RelB, particularly the latter, both suppressing constitutive as well as TCDD-induced expression of this marker (Fig. 6A). On the other hand, the mRNA level of ER α was significantly increased in MCF-7 cells transfected with siRNA specific for AhR and RelB, confirming the inverse relationship between RelB (and to a lesser extent AhR) and ER α , as reported earlier (Wang et al., 2007). These results are further supported by ectopic overexpression of AhR and RelB, which further increased the level of IL-8 in TCDD-stimulated MCF-7 cells. RelB overexpression also increased the constitutive level of IL-8 mRNA in MCF-7 cells (Fig. 6B).

Establishment of in vitro cellular models that exhibit essentially the same genotype as those found in this sub-type of breast tumors

Knowing that the lack of ER α function is likely the basic requirement for the etiology of this sub-type of AhR overexpressing breast cancer cells, we expanded the list of ER-negative cells to cover other cell lines generated from different lineages. Two of ER-negative cell lines we have already developed through long-term exposure to 17 β -estradiol (E2) (for 20 passages and 35 passages, respectively), P20E from MCF10AT1 (Wong et al., 2009) and P35E from MCF-7 (Zou and Matsumura, 2003) turned out to be also excellent in vitro models for this sub-type of tumors (Table 2). Their mRNA marker expressions, each being compared to a critically matched ER α -positive control, which was subjected to the identical process of multi-passage exposure to only the vehicle as compared to P20E or P35E (i.e. no E2), showed that the ER function of P20E or P35E is very low (see PR expression) and that their expression of both AhR, RelB and IL-8 are clearly elevated as compare to their respective control sub-lines. These E2-selected lines, in addition, also showed modestly increased expression of ErbB2 and heregulin (HRG). When their protein expression levels were compared through western blotting (Fig. 7), it was found that both AhR, RelB expressions were higher in each E2-selected cell line as compared to the matched control line. Furthermore, when one of these two pairs of cell lines was examined through the same EMSA approach used for Fig. 4 B experiments, it was found that the band representing the AhR:RelB heterodimer does indeed exist in the nuclear protein preparation from AhR overexpressing P20E cells as judged by nuclear protein binding to the labeled DRE probe (Fig. 8A) or by the labeled NF- κ B probe (Fig. 8B), but not in control P20C cells. The functional significance of the AhR-RelB interaction in P20E cells in comparison to P20C cells was studied next by using the IL-8 reporter plasmid containing the RelBAhRE binding site. The results

showed unambiguously that activation of the IL-8 promoter activity through nuclear protein binding to this RelBAhRE site is clearly susceptible to inhibition either by siAhR or by siRelB (Fig. 9).

To assess the functional significance of the AhR/RelB collaboration at the intact cell level, we further examined the sensitivity of these two lines derived from MCF10AT1 cells to UV-induced apoptosis (Fig. 10). The results have unambiguously illustrated that P20E cells are naturally resistant to UV-induced apoptosis compared to P20C cells. Treatment with either MNF (a specific AhR antagonist), siRNA targeting AhR (siAhR) or RelB (siRNA), restores the apoptotic response toward UV in P20E, which underlines the critical role of AhR and RelB for the anti-apoptotic effect in P20E cells.

Finally we compared selected mRNA marker expressions that are designed to assess the degree of cell transition from the epithelial characteristics to those of mesenchymal cell type in both P20E and P35E, compared to P20C and P35C, respectively (Table 3). The results indicate that both P20E and P35E show advanced levels of mesenchymal transition based on their expression of positive marker genes like vimentin, bcl-2 and fibronectin. P35E cells show also the appropriate direction of change compared to P35C of its negative marker (E-cadherin), and hence may be viewed as a line with a higher level of mesenchymal transition than P20E cells.

Discussion

The first question we should address is how this unique genotype of breast tumors is developed. For this purpose, the initial observation that the AhR overexpressing breast tumors are found

only among the ER α -negative type of tumors (Fig. 1) has already provided an important clue, although the number of samples available was not sufficient to unequivocally confirm this possibility. Additionally, a search in literature for a similar sub-type of breast cancer overexpressing RelB showed that the case of inflammatory breast cancers (IBC) (see Van Laere et al., 2006) appears to fit best to this sub-type. In that work an extensive search has been made to ascertain that the RelB overexpressing IBC cases are found only among the ER α -negative tumors.

There are additional pieces of supportive evidence for the importance of the loss of ER α function leading to the genesis of this sub-type in vitro. First, both AhR/RelB overexpressing P20E and P35E lines of transformed mammary epithelial cells were obtained through their long-term exposure to E2 (to down-regulate ER α , Wong et al., 2009; Zou and Matsumura, 2003, respectively). In this regard it may be pertinent to briefly describe here the past history of the development of P20E line. MCF10AT1 cells were originally developed by Dr. Fred Miller's group at Wayne State University by stably transfecting MCF10A cells with a mutant c-Ha-ras (codon 12 valine) (Shekhar et al., 1998; Miller, 2000). While this transfection treatment alone made those cells to express a modest level of ER α (about 1/10 of MCF-7 cells), it did not give them the ability to spontaneously form pre-neoplastic lesions in nude mice (Miller et al., 1996). After xenografting them into nude/beige mice, hosts treated with E2 and waiting for over one year, however, some colonies sporadically progressed to a proliferative state. The MCF10AT1 line was isolated (Dawson et al., 1996; Miller, 2000) from one of those simple duct lesions. Upon exposure to 1 nM E2 for multiple passages MCF10AT1 cells gradually lost ER α functions, and gained overexpression of AhR after 17 to 20 passages (Wong et al., 2009). It must be noted that no other artificial alteration than the E2 exposure for 20 passages was necessary to produce

this unique genotype. On the other hand, Wang et al. (2009) could obtain a similar genotype by artificially overexpressing RelB in MCF-7 breast cancer cells, which showed the repressed state of ER α expression, which was also confirmed in the current study (Fig. 6C), although such a short term treatment did not cause AhR overexpression. Thus it appears likely that in order to develop the AhR overexpressing genotype continuous suppression of ER α functions is required.

ER α -negative type of breast tumors has been known to be associated with high production of inflammatory cytokines that include IL-8 (Chavy et al., 2007). IL-8 has been known to be also associated with Cox-2 overexpressing type of breast tumors (Singh et al., 2006), which is involved in metastasis. Thus, this sub-type of breast tumors overexpressing RelB and IL-8 has been known for some time. The most important aspect of the current study is then the discovery of a tight association of the overexpressed AhR with this inflammatory type of breast tumors, which has not been considered by any of those previous investigations.

AhR has been known mostly for its role in mediating the toxicity of dioxin. However, in recent years enough evidence has been accumulated to shed light on its multiple biological roles. One of the major functions of AhR being proposed is its role as a mediator of “stress response” and regulator of inflammatory responses to promote cell survival (e.g. Matsumura, 2009).

For instance, data from the literature show the potential role of the AhR to prevent apoptosis, which is associated with mammary tumor development (Schlezingner et al. 2006). A number of reports, including one from our laboratory, have shown that activation of AhR by dioxin leads to a significant inhibition of apoptosis in breast epithelial cells (Park and Matsumura 2006) as well as in lymphoma cells (Vogel et al. 2007c). According to our recent report the AhR forms a complex with RelB in the nucleus in U937 macrophages, which is stimulated through activation of AhR and regulates gene expression of certain cytokines and chemokines (Vogel et al. 2007b).

Independently others have shown that overexpression of the NF- κ B subunit RelB causes prevention of apoptosis which is associated with tumorigenesis including lymphoma, mammary, and prostate tumor development (Lwin et al. 2007, Demicco et al. 2005, Xu et al. 2009), although none of those considered the possible involvement of AhR in any of their studies. For instance, activation of the alternative NF- κ B signaling pathway via the NF- κ B subunits RelB and p52 has been associated with up-regulation of anti-apoptotic proteins in Non-Hodgkin lymphoma cells (Lwin et al. 2007). Ectopic expression of relB, or RelB knockdown using small interfering RNA, demonstrated the important role of this subunit in control of tumor cell survival and implicated activation of the anti-apoptotic factors survivin and manganese superoxide dismutase (Mineva et al. 2007). An aberrant constitutive expression NF- κ B subunits including RelB has been reported in over 90% of breast cancers. RelB complexes were observed in mouse mammary tumors induced by either ectopic c-Rel expression or carcinogen exposure (Demicco et al. 2005) and a constitutive de novo RelB synthesis is selectively active in invasive ER α -negative breast cancer cells. Induction of Bcl-2 by RelB promoted the more invasive phenotype of ER α -negative cancer cells. Indeed the inhibition of de novo RelB synthesis may represents a new mechanism whereby ER α prevents epithelial to mesenchymal transition (Wang et al. 2007).

Recent reports show that development of certain types of breast tumor is associated with inflammation and an inappropriate production of chemokines especially IL-8 which is a critical phase in breast tumor metastasis (Ali and Lazennec 2007, Freund et al. 2004, Bieche et al. 2007). It must be pointed out that despite these recent findings only little had been known about the role of RelB interacting with the AhR in human breast diseases. Together, these data indicate that the anti-apoptotic effect mediated through an activated AhR and the promotion of tumor development involves the formation of AhR/RelB complexes associated with overexpression of

chemokines like IL-8. In this regard, it is interesting to note that, in addition to AhR, both IL-8 (Benoy et al., 2004) and RelB (Baud and Jacque, 2008) are regarded as pro-survival factors contributing to carcinogenesis. When viewed in this way, the participation of AhR, a known environmental sensor and a powerful pro-survival factor, in the formation of this sub-type of ER-negative breast tumors may provide a valuable hint to the selective advantages of this sub-type of breast cancer cells in surviving in the hostile cellular environments.

Materials and methods

Reagents and Antibodies

Dimethylsulfoxide (Me₂SO) and Phorbol-12-myristate-13-acetate (TPA) were obtained from SIGMA (St. Louis, MO). [³²P] ATP (6000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). TCDD (>99% purity) was originally obtained from Dow Chemicals Co (Midland, MI). Other molecular biological reagents were purchased from Qiagen (Valencia, CA) and Roche (Indianapolis, IN). Polyclonal RelA, CEBPβ (Santa Cruz Biotechnology, Santa Cruz, CA), NF-κB member RelB, p50 (Active Motif, Carlsbad, CA) and polyclonal AhR (Novus Biologicals, Littleton, CO) antibodies were used for supershift in EMSA. Actin AC-15 antibody was purchased from Sigma and anti-ErbB2 Ab2 from EMD BioSciences.

Breast tissue samples

Frozen human breast tissues with pathology reports were obtained from the National Cancer Institute Cooperative Human Tissue Network and the University of California at Davis Cancer Center Specimen Repository. All of the samples were approved for laboratory use by the institutional review board at the University of California at Davis School of Medicine. Samples were homogenized and analyzed via western blot analysis as described (Miller et al, 2008).

Cell Culture and transient transfection experiments

Human breast cancer cell lines were obtained from A.T.C.C. (Manassas, VA) and maintained in DMEM medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). For transient transfection MCF-7 cells were plated in DMEM with 10% FBS. Transfection of short interfering RNA (siRNA) or plasmid DNA was performed using jetPEI™ (PolyTransfection, Qbiogene, Irvine, CA), according to the manufacturer's instructions. The transfection was allowed to proceed for 24 h and cells were treated with 10 nM TCDD for another 24 h where indicated. Cells transfected with the IL-8 luciferase reporter construct were then washed twice with PBS and lysed with 100 µl passive lyses buffer. Luciferase activities were measured with the Luciferase Reporter Assay System (Promega, Madison, WI) using a luminometer (Berthold Lumat LB 9501/16, Pittsburgh, PA). In case of siRNA transfection, the reduction of the target RNA and protein was detected by quantitative real-time RT-PCR and Western blot. siRNA to target human AhR (catalog no. M-004990) was designed and synthesized by Dharmacon (Lafayette, CO). siRNA to target human RelB (5'-GGAUUUGCCGAAUUAACAA-3') and a negative control siRNA (catalog no. 10272280) were synthesized by Qiagen (Valencia, CA) as described earlier (Vogel et al. 2007a).

RNA isolation, cDNA synthesis and real time PCR

The basic methods of RT-PCR assays have already been described (Vogel et al., 2007a). All PCR assays were performed in triplicate. The intra-assay variability was <7%.

Nuclear protein extraction and EMSA

Preparation of nuclear proteins and EMSA has been described earlier (Vogel et al., 2007a).

Immunohistochemistry

Immunohistochemistry with paraffin sections of breast tissue was performed with Vectastain Kits (Burlingame, CA) according to the manufacturer's instructions. In brief, sections were deparaffinized and incubated with primary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Novus Biologicals (Littleton, CO) for immuno-histochemical staining of RelB (clone C-19), and AhR (NB100-39027), respectively. After 18h at 4 °C sections were incubated with a biotinylated Anti-Ig and avidin-biotin-HRP for 30 min each. Tissue sections from tumors and non-tumors were stained with DAB used for visualization of the antibody binding. In each tissue section, hotspots with nuclear staining were searched.

Antibodies and Western blotting

A polyclonal anti-human ErbB2, RelB, AhR, a polyclonal antibody against human ACTIN (SC-1616), a horseradish peroxidase conjugated secondary antibody, and pre-stained standard markers (SC-2361) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Whole cell lysates (30 µg) were separated on a 10% SDS–polyacrylamide gel and blotted onto a PVDF

membrane (Bio-Rad, Hercules, CA). The antigen–antibody complexes were visualized using the chemiluminescence substrate SuperSignal®, West Pico (Pierce, Rockford, IL) as recommended by the manufacturer. For quantitative analysis, respective bands were quantified using a ChemiImager™4400 (Alpha Innotech Corporation, San Leandro, CA).

Apoptosis Detection by Annexin V Staining

Cells were seeded at 1×10^5 cells in 60mm culture dishes in 2 ml medium after 24 hrs media was refreshed. After 21 hrs, cells were treated with various inhibitors and incubated for an additional 3 hrs. Apoptosis was induced by UV light ($100 \mu\text{J}/\text{cm}^2$) for 100 seconds and incubated at 37°C for 4 hrs. Afterwards, $2 \mu\text{l}$ CaCl_2 (20 mM), $2 \mu\text{l}$ annexin V-fluorescein isothiocyanate ($50 \mu\text{g}/\text{ml}$, Sigma) and $2 \mu\text{l}$ of propidium iodide (PI) solution ($50 \mu\text{g}/\text{ml}$) were added directly to each plate. Apoptotic cells were counted directly by using the fluorescence microscope (Leitz, Wetzlar, Germany). Both annexin V-positive and annexin V-PI-double-positive cells were considered to be apoptotic.

Conclusion

It is well known that breast cancer is a disease that is tremendously affected by the environment. While genetic factors are known to contribute, the contemporary view emerging is that carcinogenesis is the result of accumulation of many cellular changes -- even when each contributing a small change -- occurring during long time periods. This appears to be particularly applicable to the cases of hormonal carcinogenesis (Henderson and Feigelson, 2001). While most of experts consider that those cellular changes are caused by mutations including those occurring

DNA repair (e.g. Albert, 2009), the findings made in the current study point to the possibility that long periods of accumulation of a number of pro-survival factors and their functional integration, without involving any known mutations, may contribute to the process of malignant transformation of mammary epithelial cells as well. Particularly intriguing is the role of AhR interacting with RelB to express this inflammatory type of breast cancers.

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Figure legends

Figure 1 mRNA expression of IL-8, RelB, AhR, CYP1B1 and CYP1A1 in ER positive and ER negative breast tumors. Total RNA was extracted from breast tumor and the corresponding normal tissue of 8 breast cancer patients as described under Material and Methods. The mRNA expression of the above marker genes was analyzed by real time PCR. *, significantly different from mRNA expression in corresponding normal tissue ($p < 0.01$)

Figure 2 Western Blot analysis of AhR, RelB and ErbB2 in primary breast tissue. Whole tissue protein was used to analyze expression of AhR, ErbB2 and RelB in both the tumor sample (designated by T) and the matched normal breast tissue samples (designated as N) from 6 different patients.

Figure 3 Nuclear and cytoplasmic staining of breast tumor tissue sections using antibodies against (A) AhR, (B) NF- κ B member RelB, and (C) IL-8. Digital images were acquired with an Olympus microscope (model BH-2, Olympus, New York) and digital image transfer software (Leica Application suite 2.7). Morphometric analysis was performed with image analysis software (Image Pro Plus 4.1; Media cybernetics Inc., Maryland). For each slide, images of random fields were taken at a magnification of 100 \times (for RelB and IL-8) or 200 \times (for AhR).

Figure 4 DNA-binding activity of RelB/AhR response element and other consensus elements relevant for the activity of IL-8 in tumor versus normal breast tissue. (A) Nuclear protein extracts of normal (N) or tumor- (T) breast tissue from a cancer patient was incubated with

oligonucleotides containing the AhR/RelB-binding site identified on the promoter of IL-8 as well as consensus elements of NF- κ B, AP-1, C/EBP or DRE. **(B)** Supershift analysis of nuclear protein extracts from normal and tumor tissue with AhR/RelB probe of IL-8. A possible binding of AhR, and NF- κ B subunits RelA, and RelB was tested by supershift analyses using specific antibodies for the corresponding proteins. To confirm specificity a 100-fold excess of the unlabeled oligonucleotides was added. **(C)** Supershift analysis of nuclear protein extracts from normal and tumor tissue with a C/EBP consensus probe. Binding of C/EBP β was identified by supershift analyses using specific antibody for C/EBP β protein.

Figure 5 Analysis of IL-8, AhR and RelB mRNA expression in ER α -negative MDA-MB-231 and MDA-MB-436 cells in comparison to ER α -positive MCF-7 cells. **(A)** Quantitative IL-8 mRNA expression analyses in MCF-7, MDA-MB-231 and MDA-MB-436 cells after treatment with TCDD for 24 h. **(B)** Relative expression of AhR and RelB mRNA in MCF-7, MDA-MB-231 and MDA-MB-436 cells. Expression of mRNA was analyzed by real-time PCR as described above.

*, significantly different from control cells ($p < 0.01$); **, significantly higher than MCF-7 control cells ($p < 0.01$)

Figure 6 Induction of mRNA expression of IL-8 by TCDD is RelB- and AhR-dependent. **(A)** Total RNA was prepared 48 h post-transfection with either a scrambled siRNA or a specific siRNA targeted against AhR or RelB. mRNA expression analyses after treatment with 10 nM TCDD for 24 h (shaded bars). *, significantly different from control ($p < 0.01$); **, significantly lower than TCDD-treated cells transfected with scrambled siRNA; ***, significantly lower than

TCDD-treated cells transfected with scrambled siRNA. **(B)** IL-8 is increased in AhR and RelB overexpressing MCF-7 cells. Cells were transiently transfected with AhR or RelB overexpression plasmids. Control cells were transfected with an empty control vector. After 24 h cells were treated with 10 nM TCDD for 24 h. *, significantly different from untreated control cells ($p<0.01$); **, significantly higher than TCDD-treated cells ($p<0.01$); ***, significantly higher than untreated control cells ($p<0.01$). **(C)** ER α mRNA was analyzed 48 h post-transfection with either a scrambled siRNA or a specific siRNA targeted against AhR or RelB in MCF-7 cells. *, significantly different from control ($p<0.01$)

Figure 7 Western blot analysis of AhR, ErbB2 and RelB proteins expressed in P20 versus P20E and P35C versus P35E. Total cell protein was prepared from untreated P20C/P20E cells derived from MCF10AT1 and P35C/P35E cells derived from MCF-7 cells to analyze protein level of AhR, ErbB2 and RelB.

Figure 8 EMSA studies on the nature of the major nuclear protein band present only in P20E cells, not in P20C cells, which binds to **(A)** labeled consensus DRE and **(B)** consensus NF- κ B oligonucleotide probe as aided by super-shift assay with specific antibodies against AhR and RelB proteins to identify the major constituent nuclear transcription factor proteins.

Figure 9 Suppressive effect of siRNA against AhR as well as that against RelB on P20E expression of IL-8 gene activity in comparison to P20C as assessed by luciferase activity of the

reporter plasmid consisting of the short fragment of the IL-8 promoter containing the RelBAhRE site that is ligated to the luciferase (Luc) reporter.

Figure 10 Study on the contribution of AhR and RelB on the apoptotic response in P20E cells P20C cells. For this purpose cells were exposed to UV-irradiation after treatment with AhR antagonist (MNF), or siRNA transfection targeting AhR (siAhR) or RelB (siRelB), as described in Method and materials. Apoptotic cells were counted 4 hrs after UV-irradiation.

Table 1. Expression of AhR, CYP1B1, RelB, and IL-8 in several types of breast tumor samples, each compared to their matching normal tissue (=1.00) from the same patient.

Type	No.	AhR	IL-8	RelB
ER-, ErbB2+	4	6.0 ± 7.0	9.0 ± 10.0	4.0 ± 4.0
ER+,ErbB2+	3	1.8 ± 0.4	5.0 ± 8.0	3.0 ± 2.9
ER-, ErbB2-	3	0.8 ± 0.7	27 ± 30	2.9 ± 3.8
ER+, ErbB2-	7	0.7 ± 0.5	2.1 ± 2.0	2.0 ± 1.9

Table 2. Comparisons between P20E (MCF10AT1 origin) and P35E (MCF-7 origin) in terms of mRNA expressions of selected markers assessed under fresh serum condition as compared to respective controls. *significantly different from control cells ($p<0.005$).

**nd, not detectable

mRNA	P20E/P20C	P35E/P35C
CYP1A1	$3.80 \pm 0.75^*$	$5.71 \pm 1.31^*$
AhR	$3.92 \pm 0.50^*$	$7.35 \pm 4.20^*$
RelB	1.41 ± 0.60	2.94 ± 1.71
IL-8	$9.25 \pm 1.89^*$	1.78 ± 0.91
ErbB2	$1.53 \pm 0.30^*$	$2.60 \pm 1.00^*$
HRG	$5.20 \pm 0.15^*$	$2.20 \pm 0.34^*$
ER α	$1.53 \pm 0.30^*$	$0.15 \pm 0.06^*$
PR	nd**	nd**

Table 3. Expression of selected mRNA markers indicating EMT in P20E and P35E cells compared to respective control cells. Cells showing EMT are expected to exhibit up-regulation of all markers except E-cadherin, which is expected to be down-regulated.

*significantly different from control cells ($p<0.005$)

mRNA	P20E/P20C	P35E/P35C
Vimentin	$1.41 \pm 0.01^*$	$1.40 \pm 0.08^*$
Bcl-2	$1.83 \pm 0.18^*$	$1.26 \pm 0.18^*$
E-cadherin	$1.71 \pm 0.10^*$	$0.38 \pm 0.06^*$
Fibronectin	$3.75 \pm 0.25^*$	$1.33 \pm 0.07^*$

Fig. 1

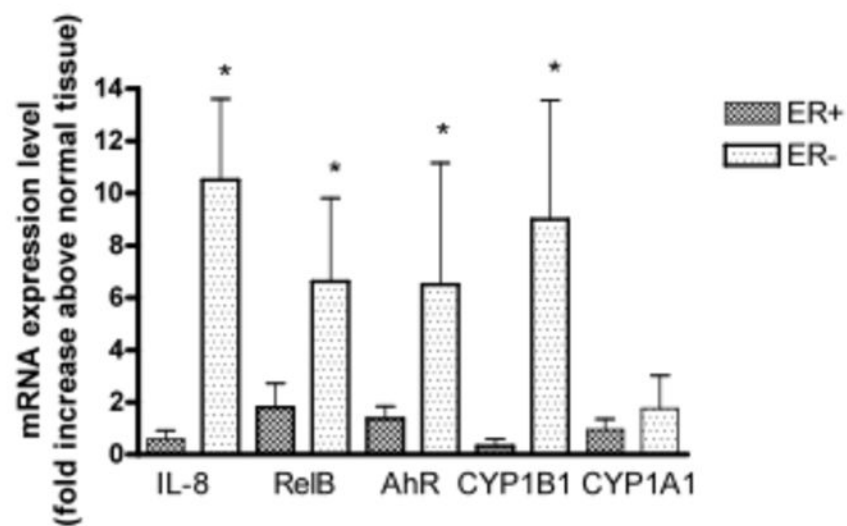


Fig. 2

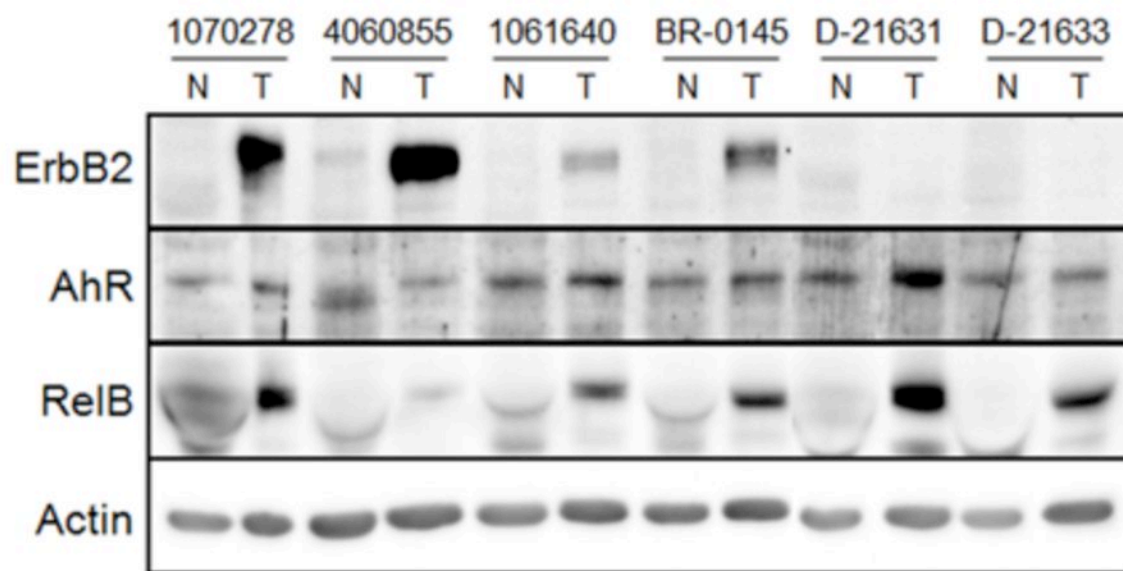


Fig. 3

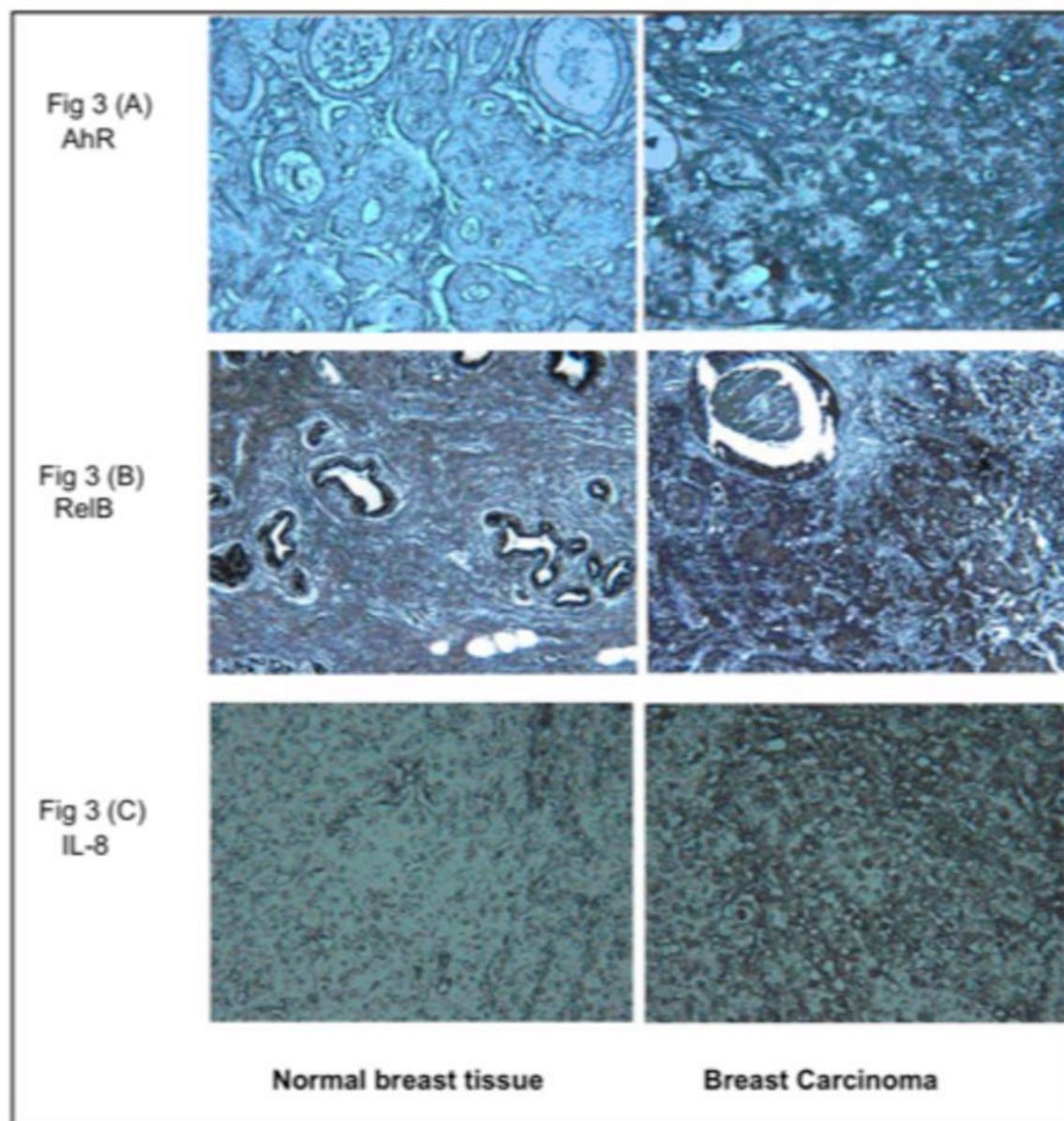


Fig. 4

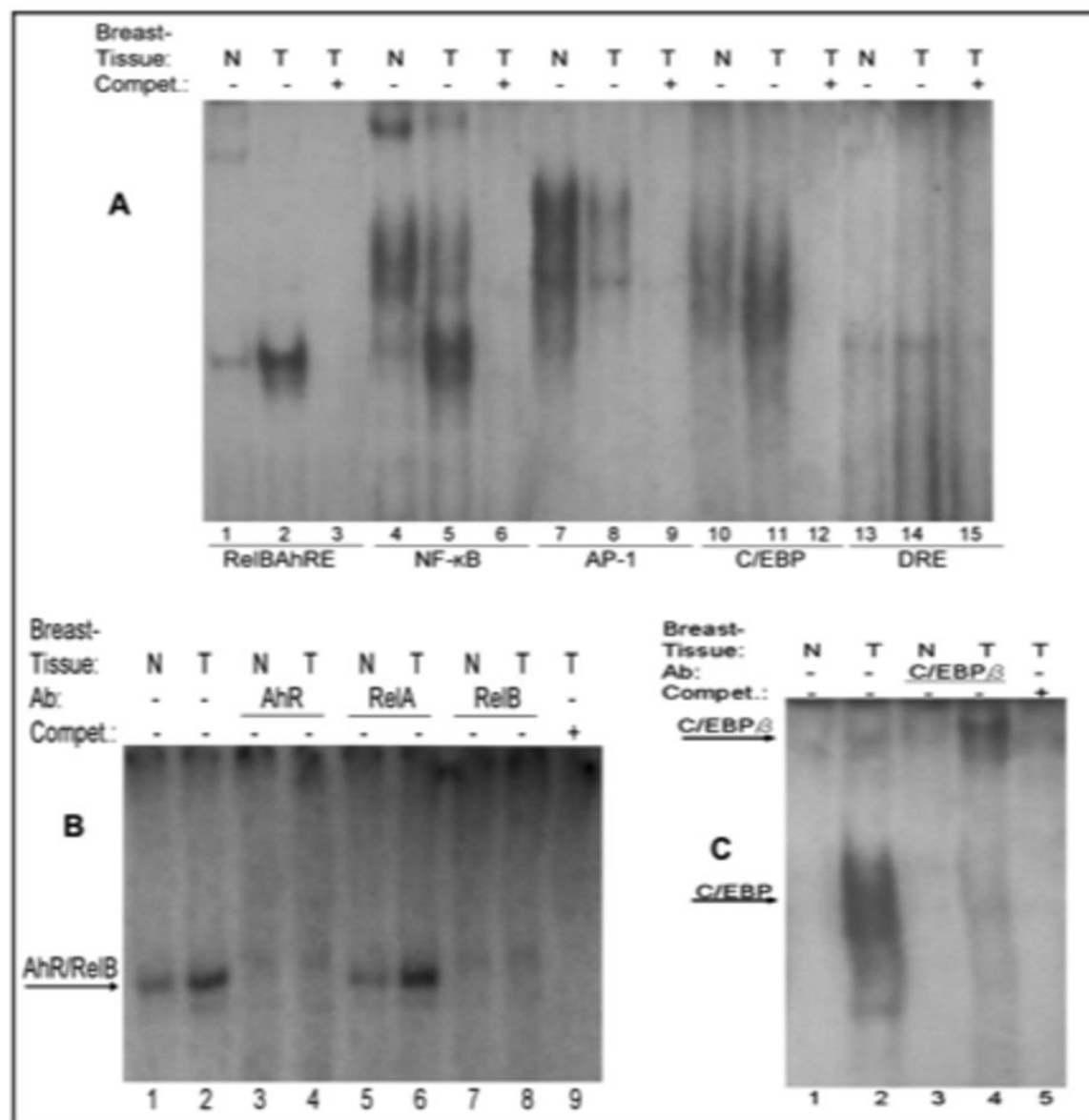


Fig. 5

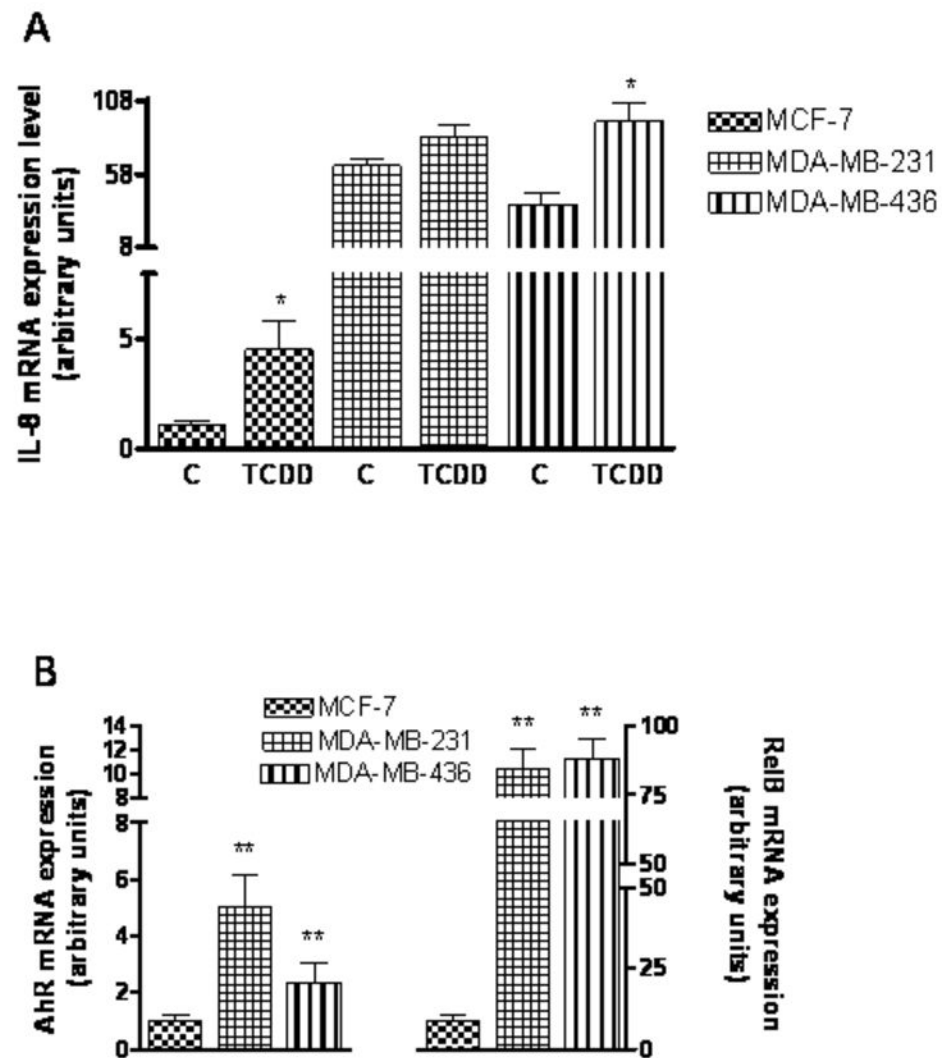


Fig. 6

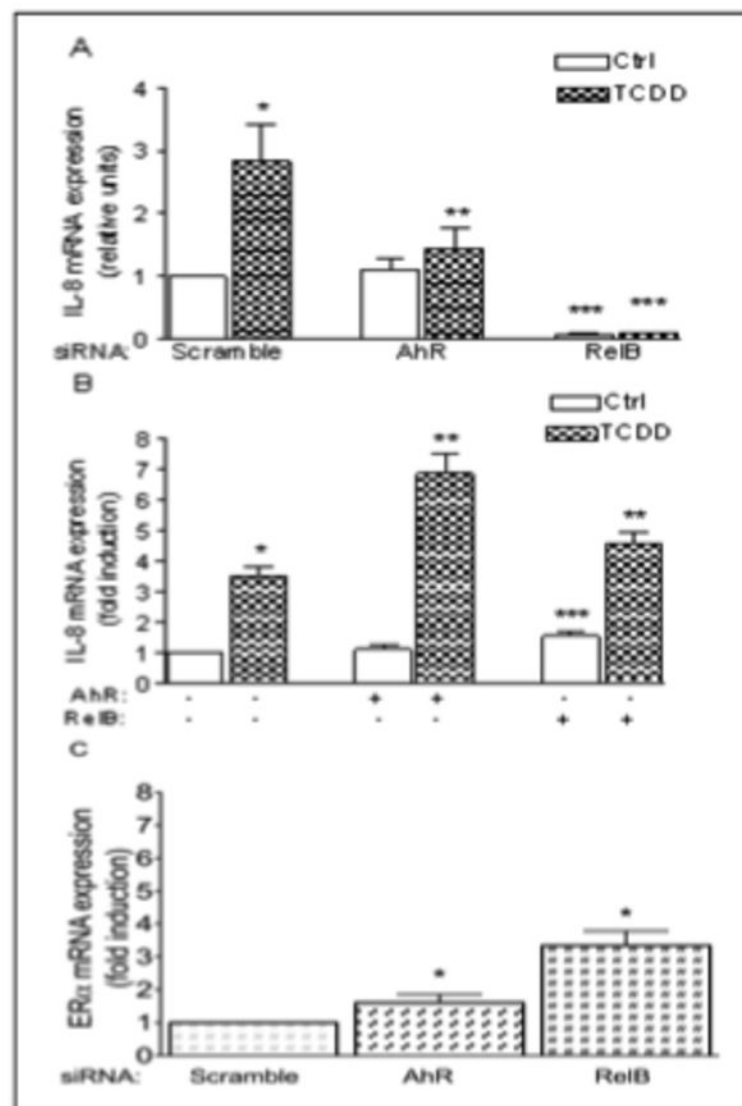


Fig. 7.

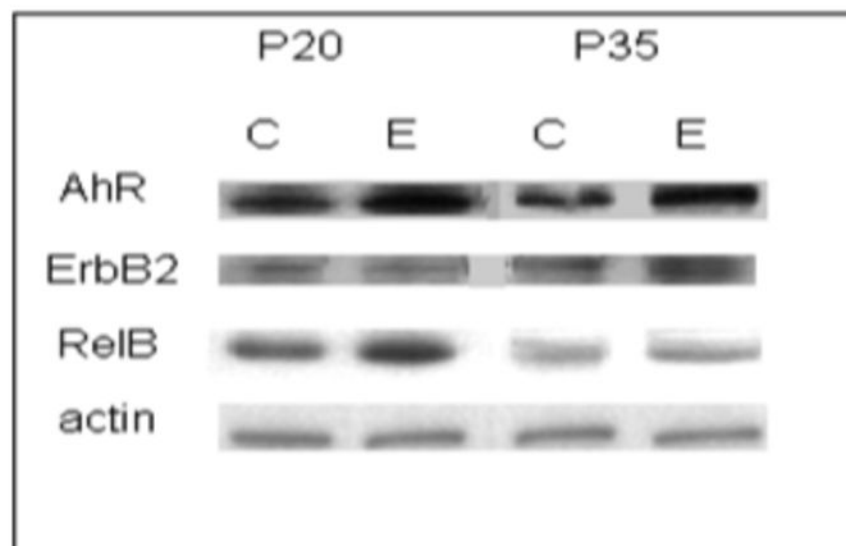


Fig. 8

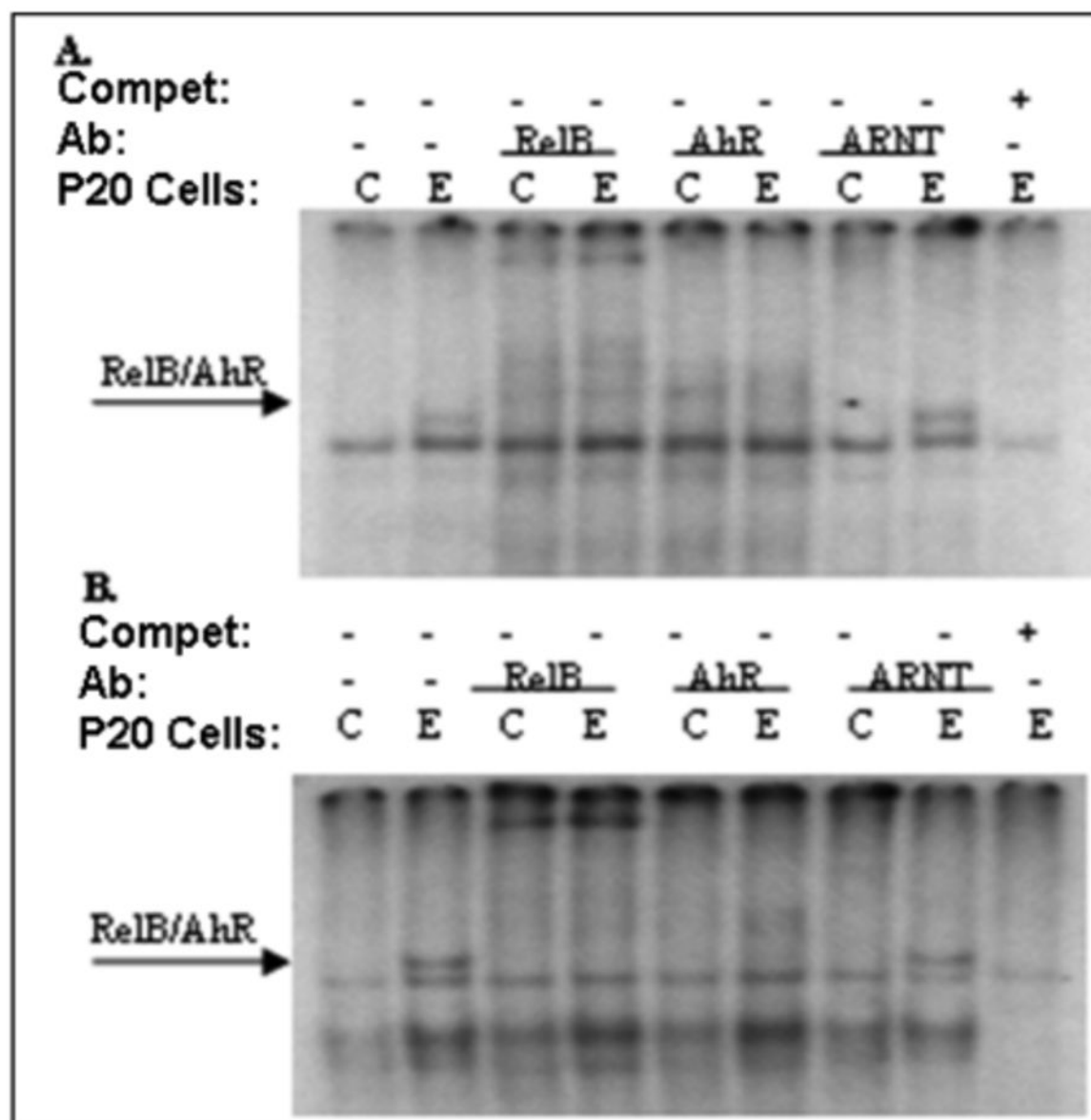


Fig. 9

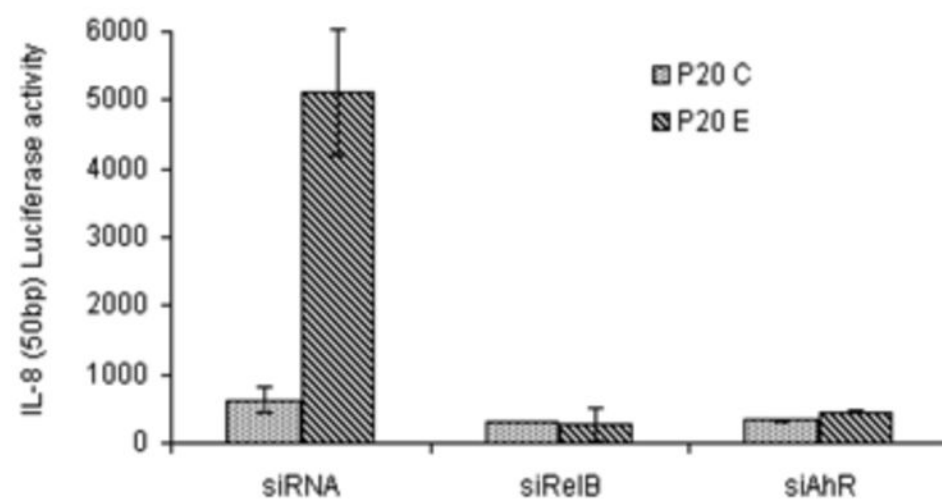


Fig. 10

