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Monique Diry[1], Céline Tomkiewicz[1], Christoph Koehle[4], Xavier Coumoul[1], K. Walter Bock[4], Robert Barouki[1] and Catherine Transy[123]

[1]UMR-S 490 INSERM, UFR Biomédicale des Saints Pères, 75270 Paris Cedex 06, France

[2]Present address: Institut Cochin, Inserm U567, CNRS UMR8104, Département de Maladies Infectieuses, 27 rue du Faubourg Saint-Jacques, 75014 Paris, France

[3]Corresponding author: transy@cochin.inserm.fr

[4]Institute of Toxicology, University of Tübingen, Wilhelmstrasse 56, 72074 Tübingen, Germany

Running title: Promigratory action of Ahr transcription factor

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Abstract

Environmental chemicals such as dioxin adversely affect immune, neurological and reproductive functions and have been implicated in cancer development. However, the mechanisms responsible for dioxin toxicity are still poorly understood. Here, we show that dioxin and related pollutants trigger a marked morphological change in epithelial cells that remodel their cytoskeleton to increase interaction with extra cellular matrix while loosening cell-cell contacts. Furthermore, dioxin-treated cells show increased motility. These dioxin-mediated effects are mimicked by constitutive expression and activation of the intracellular dioxin receptor (AhR). They correlate with activation of the Jun NH2-terminal kinase (JNK) and are reverted by treatment with a JNK inhibitor. Dioxin-induced effects occur 48 hours post-treatment initiation, a time scale, which argues for a genomic effect of the AhR, linked to induction of target genes. This novel Ahr action on cell plasticity points to a role in cancer progression.

Introduction

In humans, acute poisoning by high doses of dioxin almost systematically triggers a dramatic skin disease termed chlorine, which may persist for years (Yamamoto & Tour, 2003). In addition, epidemiological studies have revealed a positive correlation between dioxin exposure and defects in immune, neurological and reproductive functions as well as cancer incidence (Baccarelli et al., 2002; Pelclova et al., 2001; Pesatori et al., 2003). However, the underlying mechanisms of these adverse effects of dioxin are still poorly understood.

Upon binding to dioxin and related compounds, the cytoplasmic dioxin receptor Ahr undergoes a conformational change that promotes its nuclear translocation followed by its dimerization with its nuclear partner Arnt (Denison & Nagy, 2003; Poellinger, 2000). The resulting heterodimeric transcription factor then binds to specific DNA motifs termed xenobiotic responsive elements, which up-regulates the expression of target genes. Through this mechanism, Ahr controls a cellular adaptive response to a wide range of harmful chemicals. However, dioxin toxicity is lost in Ahr null mice, showing that AhR mediates as yet poorly characterized cellular responses, which are relevant to the toxicity of its ligands.

When linked to cancer disease, pollutants have been widely investigated for their impact on cell cycle, apoptosis and genome integrity. In contrast, little is known on their possible effects on tissue plasticity, which has emerged as a key parameter in cancer invasion and metastasis (for a review, see (Gotzmann et al., 2004). For example, it is believed that carcinoma cells may partially or fully recapitulate a process known as epithelial-mesenchymal transition (EMT), which physiologically occurs during development and healing. EMT is regarded as the extreme manifestation of cell plasticity since it operates through major changes in cell shape, cytoskeleton organization, and junctions with their neighboring cells and extra cellular matrix (ECM). The resulting highly motile cells invade ECM to migrate into other locations. Various factors from the microenvironment such as TGFß and ECM

components as well as cell autonomous factors contribute to EMT (Zavadil & Bottinger, 2005).

In the present study, we show that Ahr activation is functionally connected to a signaling cascade leading to dramatic alterations of cell plasticity and increase in cell motility. This so far non-described pro-migratory action of Ahr likely represents an evolutionary conserved physiological function in development and differentiation. Its inappropriate long-term reactivation may underlie the wide array of toxic effects shown by dioxins and related compounds and contribute to cancer progression.

Results and Discussion

AhR ligands elicit morphological changes in MCF7 epithelial cells associated with cell scattering.

As a model system, we used MCF7, a well-differentiated breast cancer epithelial cell line, which shows estrogen dependence and expresses AhR as a latent transcription factor (Coumoul et al., 2001). When applied on these cells for 48 hours, TCDD and 3-methyl cholanthrene (3-MC), which are typical halogenated and non-halogenated AhR ligands, led to similar changes. Cells displayed multiple membrane protrusions and markedly increased their surface (Figure 1A and 1B). This morphological change was associated with a prominent cytoskeletal remodeling including formation of actin lamellipodia and redistribution of vinculin (Figure 1A). In addition, when cells were seeded at low density to allow the formation of tight colonies before treatment, both TCDD and 3-MC induced cell scattering with loosening of cell-cell contacts, in keeping with the down-regulation of E-cadherin, a known component of homotypic cell junctions (Figure 1C). Western blots confirmed the down-regulation of E-cadherin, which was observed by immuno-histochemistry (Figure 1C).

TCDD exerts a promigratory action in a JNK dependent way.

Cytoskeletal remodeling and cell scattering are both observed in epithelial cells that transiently acquire high motility such as during wound closure and presumably carcinoma metastasis (Gotzmann et al., 2004).Cell movement is a highly coordinated process, which involves successive adhesion and de-adhesion events with the extra cellular matrix (ECM). Therefore on the whole, cell migration requires an increased ability to engage contacts with ECM components. To address the role of dioxin on these processes, MCF7 cells were pretreated with TCDD or vehicle alone and visualized at different time points after incubation on fibronectin-coated cover slips. As shown in Figure 2A, TCDD-treated cells spread far more rapidly than their vehicle-treated counterparts, indicating an enhanced capacity for

interacting with ECM substratum. Finally, TCCD-treatment led to a two-fold increase in cell velocity as measured in a classical wound-healing assay (Figure 2B).

The above data indicate that TCCD and related compounds modulate several characteristics of epithelial plasticity in a way reminiscent of EMT. The whole process, which is not fully characterized, is believed to rely on complex cascades of molecular events that usually convey cues from the environment. In this respect, the jun N-terminal kinase (JNK) has recently been recognized as a major integrator of pathways leading to increased migratory potential of epithelial cells (Xia & Karin, 2004). We therefore addressed its potential implication in the TCDD-mediated effects on MCF7 cell plasticity. As shown in Figure 3A, the phosphorylated active pool of JNK was markedly increased in cells treated for 48 hours with either TCDD or 3-MC. In contrast, under the low serum conditions used to perform these treatments, we observed a significant down-regulation of the phosphorylated pool of ERK kinases (Figure 3A and 3B), which are known to transduce mitogenic signals (Seger & Krebs, 1995). Noticeably, pharmacological inhibition of JNK activity markedly reduced the occurrence of both the membrane protrusions and the cell scattering elicited by TCDD treatment (Figure 3C). Furthermore, it also prevented TCDD-mediated down-regulation of E-Cadherin (Figure 3D and counteracted the effect of dioxin on MCF-7 migration in wound healing experiments (figure 3E, SI-Figure 1. Taken together, these data point to a prominent role of increased JNK signaling in the particular cell response to TCDD that is documented here. Of note, studies of Drosophila development indicate that JNK signaling is involved not only in epithelial sheets migration but also in the disruption of cell-cell contacts necessary for pseudo-epithelial-mesenchymal transition processes (Pastor-Pareja et al., 2004).

Constitutive expression and activation of AhR mimics TCDD effects on cell morphology and JNK activation.

Tan et al. have previously reported that AhR ligands may induce JNK activation (Tan et al., 2002). In their study, JNK activation occurred independently of AhR expression and represented an early and transient response to the drug treatment. This is in contrast with our present finding of a late and persistent JNK activation induced by TCDD and 3-MC treatment. This raised the possibility that the drugs impact on MAPK activities in a dual way, one by the rapid and non genomic action described by Tan et al. (Tan et al., 2002) and the other by the slower process described here, which would most likely rely on AhR activation. In this view, the latter pathway should be triggered when activation of AhR is obtained independently of drug treatment. To address this hypothesis, we used a MCF7-derived clone engineered for conditional expression of a constitutively active AhR recombinant protein (CA-AhR) referred to here as MCF7 [CA-AhR] (Kohle et al., 2002). When doxycyclin (DOX) was withdrawn from the growth medium, which relieved repression of CA-AhR transgene, cells underwent a cytoskeleton remodeling that was similar to that observed in parental MCF7 cells treated with TCDD (compare Figure 1A with Figure 4A) and that became visible after three days following DOX removal. Importantly, constitutive AhR expression and AhR activity also resulted in JNK activation (Figure 4B) and in increased cell motility (Figure 4C, SI-Figure 3). Furthermore, treatment with a JNK inhibitor counteracted the effect of CA-AhR activation (figure 4C, SI-Figure 2?). We conclude that AhR activation *per se* is sufficient to trigger JNK activation and changes in MCF7 cell shape. Weiss et al. recently reported a rapid AhR-dependent p38 MAPK activation that occurred in a highly restricted cell type manner and did not require AhR transcriptional activity (Weiss et al., 2005). Our data do not exclude that a similar mechanism operates in AhR-induced cytoskeleton remodeling and JNK activation. However, since these events occur in a time course > 48 hours in MCF7 [CA-AhR], they more likely result from activities of proteins up regulated by AhR than from a direct effect of AhR protein.

AhR ligands-mediated changes in cell plasticity do not depend on estrogen receptor and might be related to the activation of several AhR target genes.

There have been numerous reports of interference between AhR and estrogen receptor (ER) activities (Safe & Wormke, 2003). Taken together with the recent finding that ER activation contributes to inhibit cell movement in MCF7 cells (Sisci et al., 2004), this raised questions as to the generality of the AhR-mediated effects documented here. However, the well-differentiated HepG2 hepatocarcinoma cells, which do not respond to estrogens, responded to AhR ligand treatment in a way similar to MCF7 cells (SI-Figure 3). This observation has several implications. First, it excludes that AhR agonists modulate cell plasticity only in the context of ER-dependent cells. Second, when taken together with our other data and with the recent report that AhR null mouse mammary fibroblasts show impaired motility (Mulero-Navarro et al., 2005), it argues for a general role of AhR in tissue plasticity that was previously overlooked. Third, since large-scale analyzes of TCDD-induced genes were almost exclusively performed in HepG2 cells, it offered the opportunity to re-analyze the generated data in light of our present findings. Several of the genes that were found up-regulated in at least two of the three compared data sets (Frueh et al., 2001; Marchand et al., 2005; Puga et al., 2000) are highly suggestive of a role in cell migration and /or tumor progression. Thus the GTP exchange factor (GEF) Sos-1 initially discovered as a GEF for Ras has been shown to also activate Rac (Nimnual et al., 1998), which positively acts on cell motility (Evers et al., 2000). Second HEF-1 also termed CASL, which is involved in integrin-signaling, controls migration of lymphocytes and its artificial over-expression in MCF7 cells induced a migratory phenotype (O'Neill et al., 2000). Finally, the protease inhibitor Serpin 1 also termed PAI-1, a previously reported TCDD-induced gene and its protease target Urokinase (uPA), which was also induced by TCDD in our own transcriptome

analysis, are now considered strong predictive factors of breast cancer invasive phenotype (Foekens et al., 2000). We therefore used a real time PCR method (Marchand et al., 2005) to investigate the response of these genes to TCDD and 3-MC in MCF7 as well as in T47D, another cell model of estrogen-dependent breast cancer (Coumoul et al., 2001). As shown in SI-Table 1, the AhR agonist 3-MC significantly induced this whole subset of genes, both in MCF7 and T47D. Thus, 3-MC behaved like TCDD in HepG2 cells. In contrast, TCDD and CA-AhR expression induced a more restricted response where HEF-1 represented the most consistently induced target. As expected, all conditions of AhR activation resulted in strong induction of CYP1A1, the prototype target of AhR transcription factor. Taken together, these data suggest that depending on the cell context and on the AhR agonist, diverse pathways may contribute to AhR-mediated effects on tissue plasticity with the possible common involvement of HEF-1 induction. In this respect, we note that in one study, induced HEF-1 overexpression increased cell motility (Fashena et al., 2002) whereas in another it activated JNK with apoptosis as an outcome (Law et al., 2000). Additional studies are therefore required to delineate how AhR activation impinges signaling cascades that control cell motility and invasion.

Concluding remarks

Based on our finding that AhR activation results in strong alteration of the plastic characteristics of epithelial cells, we may envision a new interplay between the pathological and the physiological roles of AhR.

In contrast to other AhR agonists, dioxin is stored in the organism for very long periods owing to its intrinsic chemical stability and to its resistance to AhR-induced metabolic machinery. On the other hand, AhR ligands present in tobacco smoke are efficiently metabolized but may be recurrently delivered in heavy smokers. Therefore, dioxin exposure

and tobacco smoking represent two settings of sustained AhR activation. Our data indicate that prolonged AhR activation triggers a marked cytoskeleton remodeling associated with JNK activation and a pro-migratory action. We hypothesize that this AhR-initiated cascade is physiologically significant in other settings. Supporting the view that AhR is not a mere xenobiotic sensor controlling a metabolic cascade, invertebrate species do possess AhR orthologs with no known endogenous or exogenous ligand. In *C.* elegans development, the corresponding gene has recently been shown to play a role in proper migration and differentiation of specific neurons (Huang et al., 2004; Qin & Powell-Coffman, 2004). In addition, AhR null mice exhibit a number of developmental defects (Fernandez-Salguero et al., 1995). Thus the tissues that are the most sensitive to the pathological effects of dioxin may well represent those where AhR plays an important role during development and differentiation.

Dioxin has long been suggested to act in the initiation or in the promotion of human cancer, in agreement with observations made in laboratory animals. Our data suggest another possible action of dioxin, namely on tumor progression. In support of this hypothesis, Nesaretnam et al showed that DMBA-induced breast tumors in rats were strikingly more invasive when the animals were co-treated with the dioxin-like compound TCB (Nesaretnam et al., 1998). In addition, Mulero-Navarro et al recently reported that immortalized fibroblasts from AhR null mice show impaired ability to induce sub-cutaneous tumors, in keeping with reduced motility and response to angiogenic factors (Mulero-Navarro et al., 2005). Therefore, the relation between dioxin exposure or tobacco smoking and human cancer might deserve further investigation from the viewpoint of severity rather than incidence of the disease.

Finally, AhR-mediated action on cell plasticity might provide an explanation for the remarkable diversity of toxic effects shown by dioxins and related pollutants. It would also

place AhR in the growing family of transcription factors whose physiological functions in development are hijacked during pathological processes.

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Figure legends

Figure 1: The AhR ligands TCDD and 3-MC induce cytoskeleton remodeling and cell scattering in MCF7 breast cancer cells. Cells were obtained from the American Tissue Culture Collection (ATCC) and were routinely grown in DMEM medium supplemented with 10% fetal calf serum (FCS). Treatments with TCDD (25 nM), 3-MC (5 μM) or vehicle (DMSO < 0.1 %) were performed for 48 hours in medium lacking phenol red and supplemented with 3 % charcoal-stripped FCS. For fluorescent staining, cells were seeded on glass coverslips before treatment initiation, fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton. Vinculin and E-cadherin were detected by green fluorescence using murine monoclonal antibodies from Sigma and Zymed laboratories followed by incubation with FITC-labelled anti-mouse Ig (Vector laboratories). Actin was visualized in red using TRITC-labelled phalloidin (Sigma), and cell nuclei were stained in blue using Dapi. In panel B, quantification of cell areas was performed using the MetaMorph software (Universal imaging Downingtown, PA). Ten random fields, i.e. more than 100 cells, were analyzed for each condition of treatment. In panel C inset, equal amounts of protein lysates prepared from treated cells in M-PER extraction buffer (Pierce) were subjected to western blot analysis using anti-E-cadherin antibody and a chemiluminescence procedure (Tropix).

Figure 2: TCDD increases spreading capacity and motility of MCF7 cells. Cells were treated for 48 hours as described in Figure 1 legend. (A) Cells were harvested and incubated for the indicated times on fibronectin-coated glass coverslips to assess spreading capacity. Fixed cells were visualized after staining by fluorescent phalloidin (red) and dapi (blue). (B) Confluent cell monolayers were subjected to a wound-healing assay to monitor cell motility. TCDD

treatments were performed 48h before wounding. Triplicate wells of vehicle- or TCDD-pretreated cells were grown 24h without serum before wounding to avoid cell proliferation and wounded, still in serum free medium, by removing a 300-500 µm strip of cells using a standard 200 µl pipet tip. The wounded areas were marked and cells were photographed at different time points. The upper part of the panel shows a typical result of wound closure ability of vehicle- *versus* TCDD-treated cells. The lower part of the panel shows quantification of cell motility expressed as the average linear speed of cells (µm/hour), which was calculated from the distance decrease between the wound edges over time.

Figure 3: AhR ligands mediate cytoskeleton remodeling and E-cadherin down-regulation through increased JNK signaling. Cells were treated for 48 hours with TCDD or vehicle as described in Figure 1 legend. When indicated, cells were co-treated with 1.7 µM JNK inhibitor 1(JNK-I) from Calbiochem. After lysis, equal amounts of protein extracts were subjected to western blot analysis as described in Figure 1 legend. Phosphorylated JNK (P-JNK) was revealed by a mouse monoclonal antibody from BD Biosciences. Total JNK (JNK), phosphorylated ERK (P-ERK), and total ERK (ERK) were revealed by rabbit polyclonal antibodies from Cell Signaling (A). Imagequant software was used to quantify the bands and the bar graphs show the relative levels of P-JNK and P-ERK (B). In panel C, cells grown on glass coverslips were fixed and permeabilized for subsequent fluorescence staining of actin, as described in Figure 1 legend. In Panel D, a western blot shows the effect of JNK inhibitor 1 on TCDD-induced? E-Cadherin down regulation. In panel E, cells were subjected to a wound-healing assay as described in Figure 2B legend in presence or absence of JNK-I.

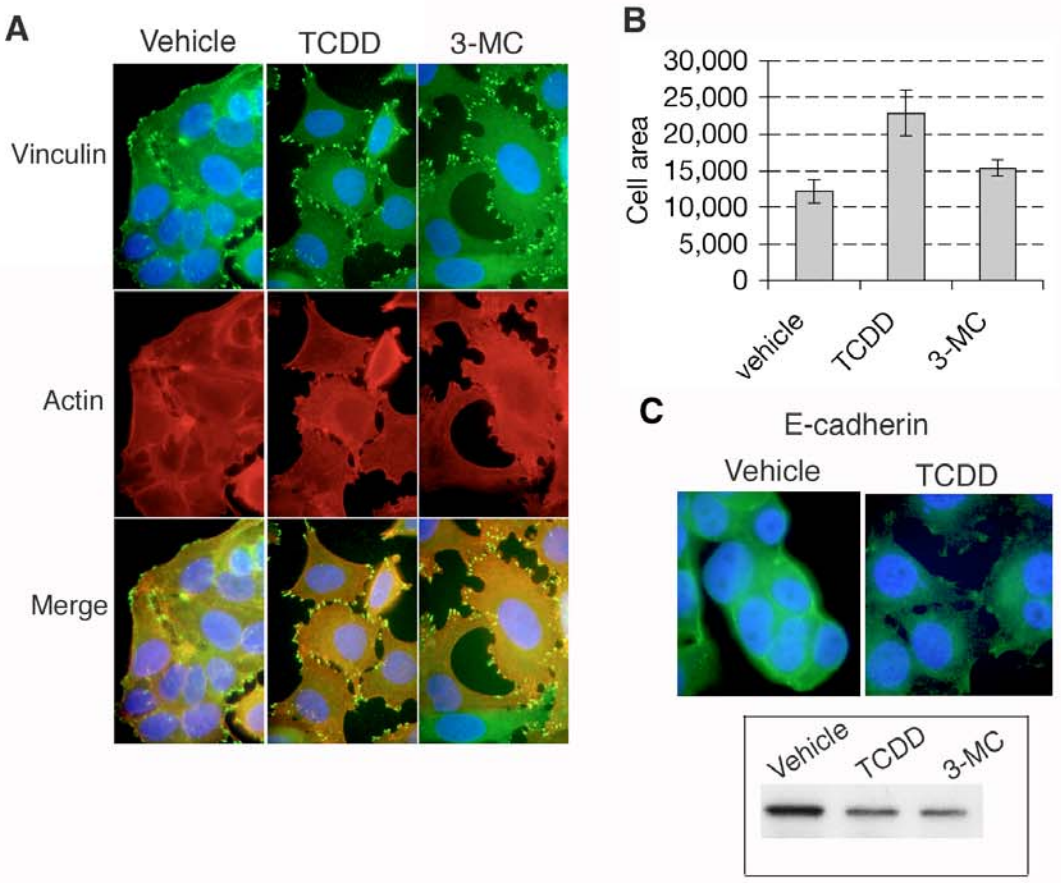
Figure 4: Constitutive expression and activation of AhR mimics TCDD and 3-MC effects. MCF7 [CA-AhR] were routinely grown in the presence of 1 µg/ml of doxycyclin (DOX)

(Sigma) for repression of the constitutively activated AhR (CA-AhR) transgene. The effect of CA-AhR expression on cell morphology and cytoskeleton (panel A) was examined after 72-hour growth and on JNK induction (panel B) after 48-hour growth in presence or in absence of DOX in medium lacking phenol red supplemented with 3 % charcoal-stripped FCS. Panel C shows an experiment similar to that performed in Figure 3E. JNK-I treatment and/or DOX removal were performed 48h before wounding.

SI-Figure 1: (A) representative wounded areas used to perform quantification in figure 3E. (B) Magnified areas showing the morphology of the cells in the wounding zone.

SI-Figure 2: representative wounded areas used to perform quantification in figure 4C

SI-Figure 3: AhR ligand-mediated changes in cell plasticity are not confined to estrogen-dependent cells. Human HepG2 hepatoblastoma cells were obtained from ATCC. Cells were seeded on glass cover slips and grown in the indicated conditions for 48 hours in serum-free medium. Cells were then processed for fluorescence staining as described in Figure 1 legend.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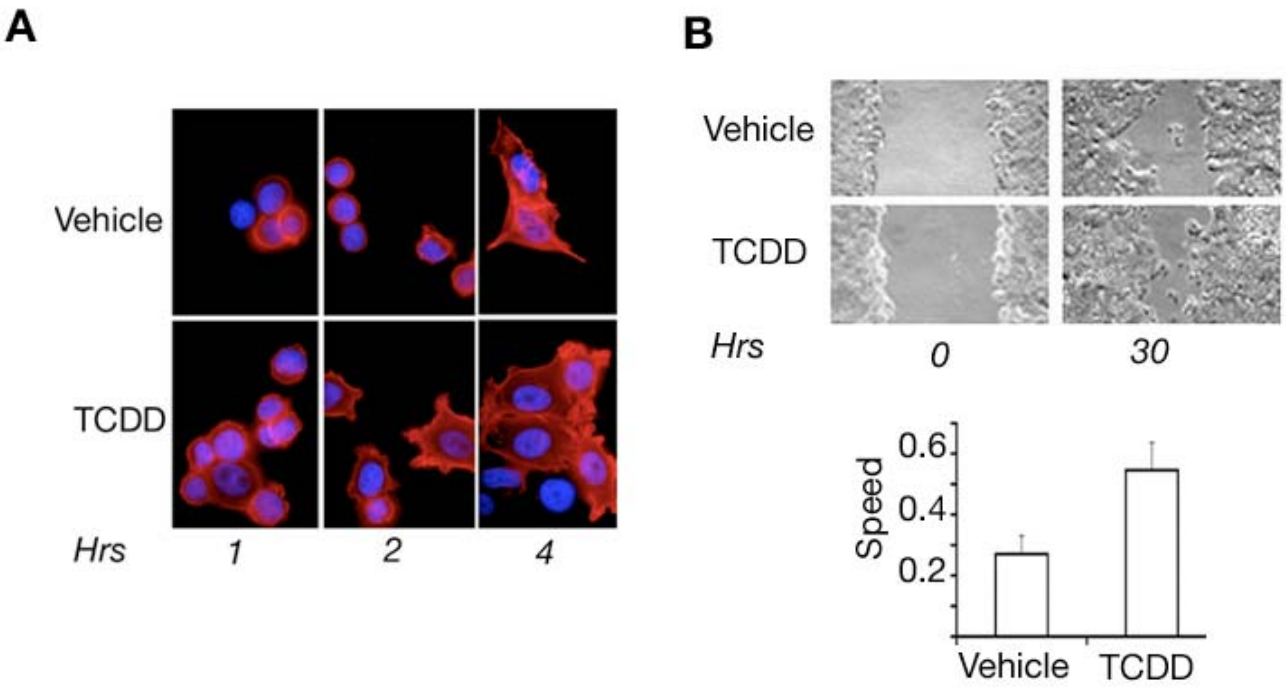
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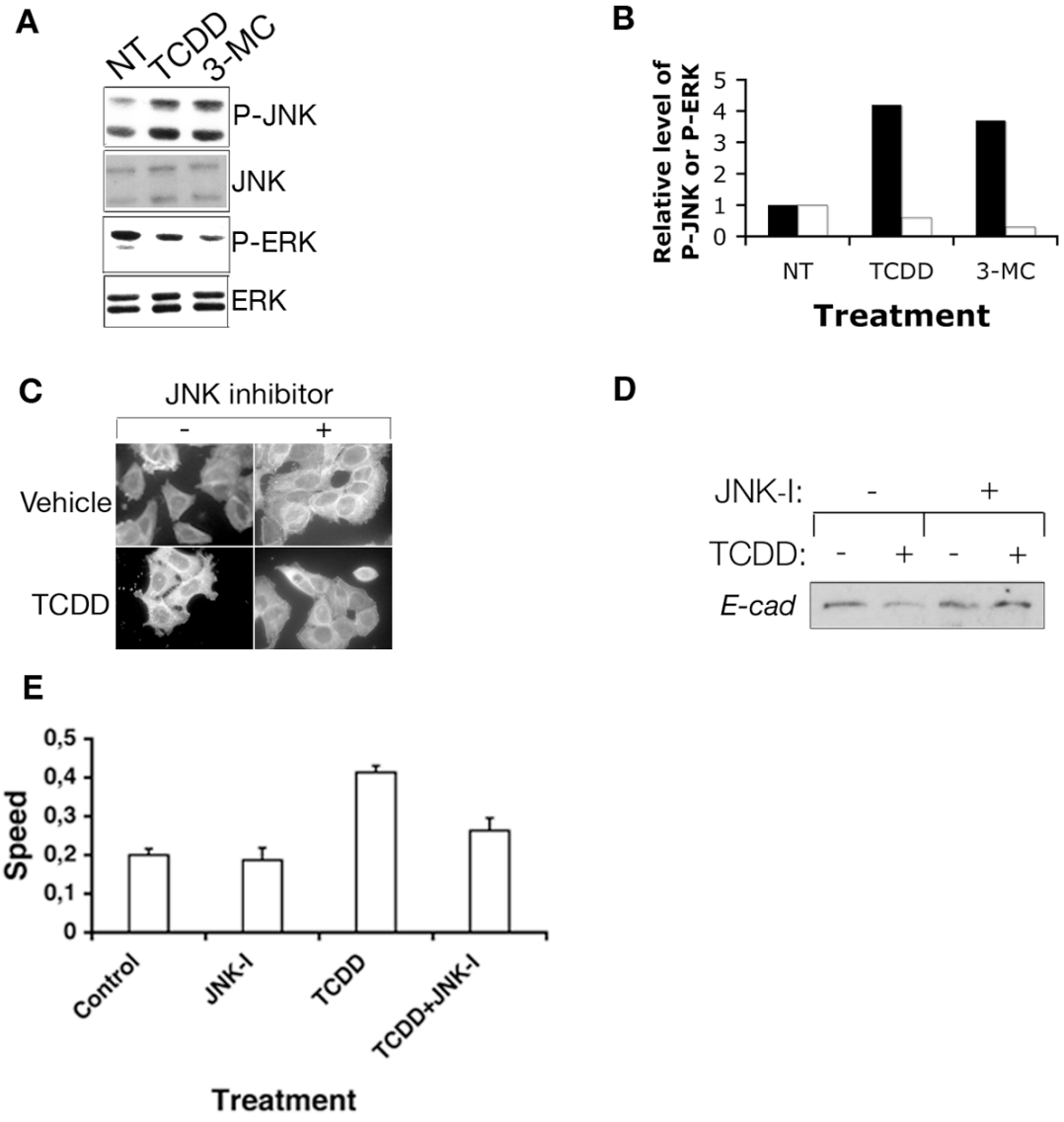
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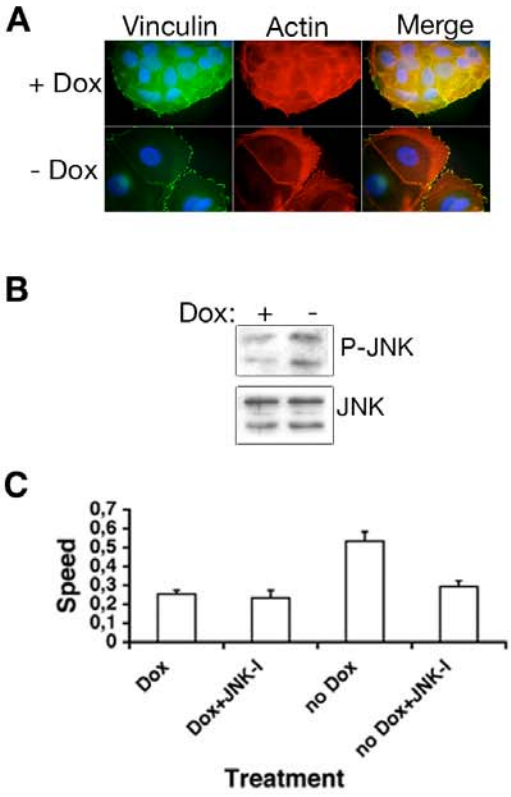
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Dox+JNK-I
no Dox
no Dox+JNK-I
Treatment

SI-Table 1: AhR ligands differentially induce genes involved in cell motility and breast cancer progression.

	MCF-7		T47D		MCF7 [CA-AhR]
	TCDD[1]	3-MC[2]	TCDD[1]	3-MC[2]	-DOX[3]
SOS1	1.1	2.1	1.8	5.7	1.4
HEF-1	2.7	12.4	10.6	19.1	2.5
PAI-1	0.7	30.1	1	2.3	0.4
u-PA	0.7	10	4.2	13.5	0.8
CYP1A1	1590	5350	6160	2670	177

MCF7 and T47D were treated for 48 hours with either vehicle alone or the indicated drugs: [1]10 nM TCDD, [2]5 μM 3-MC. [3] MCF7[CA-AhR] were treated for 72 hours in the absence or the presence of 1μg/ml DOX. Total RNAs were reverse-transcribed and quantitatively analyzed for their contents in the indicated gene transcripts using real time PCR as previously described. Results are expressed as fold-induction relative to [1,2] cells treated with vehicle only or [3] cells grown in DOX-containing medium. The following forward (F) and reverse (R) primers were used:
CYP1A1-F, 5´-GACCACAACCACCAAGAAC-3´,
CYP1A1-R, 5´-AGCGAAGAATAGGGATGAAG-3´;
HEF-1-F, 5'-TGAGAGGCAACAGAAAGAGC-3';
HEF-1-R, 5'-GAGAAGGGAAATGAAATGGG-3';
SOS1-F, 5'-GACCTTCATTCCATTGCTG-3',
SOS1-R, 5'-GTGTGTGTGCTCCCTTTTG-3';
PAI-1-F, 5'-AGAGAACCTGGGAATGACC-3',
PAI-1-R, 5'-GCGGGCTGAGACTATGAC-3';
uPA-F, 5´-TCACACCAAGGAAGAGAATG-3,
uPA-R, 5´-TGACAACCAGCAAGAAAGC-3´.