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Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis

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SUMMARY

The aryl-hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates the biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In mouse embryo fibroblasts, TCDD activates expression of multiple genes, including CYP1B1, the predominant cytochrome P450 expressed in these cells. Here, we analyze constitutive functions of the AhR in primary mouse embryo fibroblasts (MEFs) and spontaneously immortalized MEF cell lines derived from wild-type (WT) C57BL/6 mice and also from congenic mice with a targeted disruption of the AhR gene ($AhR^{-/-}$). After multiple passages, primary MEFs exhibit spontaneous differentiation, growth cessation and senescence. Eventually, colonies of immortalized MEFs arise to provide clonal lines. The senescent phase occurs much earlier for $AhR^{-/-}$ MEFs, while immortalization is substantially delayed. Comparison of $AhR^{-/-}$ and WT MEFs also indicates that constitutive AhR activity is required for basal expression of CYP1B1 and suppresses lipogenesis in subconfluent cultures. Primary WT and $AhR^{-/-}$ MEFs and the corresponding lines undergo adipogenesis when treated at confluence with the appropriate hormonal inducers. Addition of TCDD before or concurrent with hormonal induction suppressed PPARγ mRNA and adipogenesis, as measured by lipid accumulation, glycerol phosphate dehydrogenase activity and stearoyl CoA desaturase type 1 mRNA expression. This effect of TCDD treatment was absent in $AhR^{-/-}$ MEFs, establishing the role of AhR in hormone-induced adipogenesis. Such hormonal activation of confluent MEFs and preadipocytes results in a limited proliferative expansion followed by irreversible growth arrest. TCDD-treated MEFs undergo the mitotic expansion but fail to exit the cell cycle. In $AhR^{-/-}$ MEFs, there is no such effect of TCDD. These findings implicate the AhR as a constitutive inhibitor of triglyceride synthesis, and as an early regulator of adipocyte differentiation. AhR interference with cell-cycle arrest in differentiation may be linked to the increased rate of senescence.

Key words: CYP1B1; Adipogenesis; Aryl-hydrocarbon Receptor (AhR)

INTRODUCTION

Primary mouse embryo fibroblasts (MEF) are cultured from trypsin digests of late-stage embryos. Multiple passaging of these cells leads to a cessation of growth (senescence), and eventual outgrowth of immortalized MEFs. The C3H10T1/2 cell line (10T1/2) is typical of such cells. 10T1/2 and other MEFs are multipotential cells that differentiate not only to adipocytes but also to myocytes and chondrocytes (Konieczny and Emerson, 1984; Taylor and Jones, 1979). Each of these fates can be potentiated by azocytosine, an agent that induces hypomethylation of DNA.

The process of adipogenesis has been extensively studied in vitro by many laboratories using 3T3-L1 cells (Green and Meuth, 1974; MacDougald and Lane, 1995a,b; Mandrup and Lane, 1997; Spiegelman, 1997). The combined hormonal activities (IDM induction) of insulin, the glucocorticoid dexamethasone (DEX) and the phosphodiesterase inhibitor isobutylmethylxanthine (MIX), produce additional cell proliferation in previously quiescent, confluent cells (Shugart and Umek, 1997). This is followed by an irreversible growth arrest, and ultimately by activation of the same genes seen in adipocytes in vivo (MacDougald and Lane, 1995b; Spiegelman and Flier, 1996). The changes in gene expression include the transitory appearance of c-myc and c-jun, which is followed by sequential elevations of C/EBPβ, C/EBPα and PPARγ (Brun et al., 1996; Lemberger et al., 1996). Drugs that activate PPARγ, such as the thiazolidinedione BRL-49653 (BRL), enhance the effects of the hormonal mix IDM. These drugs are essential for stimulation of adipogenesis in MEF

cells such as 10T1/2 (Forman et al., 1996; Kliewer et al., 1995).

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) strongly inhibits adipogenesis in 3T3-L1 preadipocytes and in primary cultures of preadipocytes (Brodie et al., 1997; Phillips et al., 1995). In mammals, TCDD causes a redistribution of triglycerides from adipose tissue to liver, and diabetic characteristics including reduced glucose uptake. In adipose tissue, TCDD lowers the activity of a series of adipogenic genes (Brodie et al., 1996; Potter et al., 1986). Inhibition of adipogenesis in 3T3-L1 preadipocytes is paralleled by inhibition of expression of late-stage nuclear regulatory proteins C/EBP$_\alpha$ and PPARγ, but expression of early-stage regulators associated with proliferation such as c-myc, c-jun, C/EBP$_\delta$ and C/EBP$_\beta$ are unaffected (Chen et al., 1997). Early addition of TCDD is required for the inhibition (Chen et al., 1997; Phillips et al., 1995).

We have previously shown that primary and immortalized MEFs express a single cytochrome P450, CYP1B1 (Alexander et al., 1997). The expression of this gene is regulated by the aryl-hydrocarbon receptor (AhR), a member of the basic-helix-loop-helix-PAS family of transcription factors, which also mediates the effects of TCDD (Rowlands and Gustafsson, 1997; Schmidt and Bradfield, 1996). High basal expression of CYP1B1, and absence of the closely related CYP1A1, even with strong AhR activators, are characteristic of MEFs (Alexander et al., 1997; Christou et al., 1993). CYP1B1 deficiency is a major cause of congenital glaucoma, thus implicating a physiological substrate of this cytochrome P450 in developmental processes (Stoilov et al., 1997). Understanding the regulation of CYP1B1 expression in MEFs may lend insight into the mechanisms controlling differentiation of mesenchymal cell lineages represented by MEFs.

The AhR mediates gene transcription by forming a nuclear heterodimer with the related helix-loop-helix protein ARNT (Probst et al., 1993). Agonists such as TCDD stimulate this complex formation at specific DNA recognition elements containing the core sequence TNGCGTG (Swanson et al., 1995) that is present in multiple copies in responsive genes like CYP1B1 (Zhang et al., 1998). Hepatoma cells deficient for the AhR show distinct morphology, do not form uniform confluent monolayers, and exhibit a prolonged G_1 stage in the cell cycle (Ma and Whitlock, 1996; Sadek and Allen-Hoffmann, 1994). In AhR-null mice, the predominant phenotype is delayed hepatic development and bile duct fibrosis (Fernandez-Salguero et al., 1995; Schmidt et al., 1996).

In this article, we demonstrate that primary and immortalized MEFs both require peroxisome proliferator activated receptor (PPAR)γ agonists to induce adipogenesis and that this response is suppressed by TCDD. We also characterize MEFs from congenic C57BL/6 mice with a targeted disruption of the AhR gene (AhR$^{-/-}$), and describe the generation of immortal MEF cell lines from these mice. We have used primary and immortalized WT or AhR$^{-/-}$ MEFs to explore the role of AhR in constitutive regulation of triglyceride synthesis, and to establish unambiguously the involvement of AhR in the TCDD-induced inhibition of adipogenesis. A direct effect of AhR on constitutive CYP1B1 gene expression is demonstrated. Finally, we also show that the AhR deficiency impacts the rate of spontaneous differentiation and senescence in sequential passaging of primary MEFs.

MATERIALS AND METHODS

Chemicals

7,12-Dimethylbenz(a)anthracene (DMBA), benz[a]anthracene and dimethylsulfoxide (DMSO) were obtained from Aldrich Chemical Co. (Milwaukee, WI). TCDD in toluene was purchased from Chemsyn Science Laboratories (Lenexa, KS). HPLC-grade methanol, ethylacetate and acetonitrile were purchased from Fisher Chemical Company (Itasca, IL), as were tissue culture plates. Oligo(dT) cellulose was purchased from Collaborative Research Inc. (Bedford, MA). Materials for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories (Richmond, CA). BCA-Protein reagents were purchased from Pierce (Rockford, IL). Nitrocellulose and Nytran membranes were purchased from Schleicher and Schuell (Keene, NH). Enhanced chemiluminescence (ECL) detection kit and hyperfilm-ECL luminescence detection film were obtained from Amersham Corporation (Arlington Heights, IL). Goat anti-rabbit horseradish peroxidase conjugate was purchased from Promega (Madison, WI). All other stock chemicals were purchased through Fisher Scientific and the University of Wisconsin Stores. Rabbit antibodies to recombinant mouse CYP1B1 and purified rat CYP1A1, and the chicken antibody to purified mouse CYP1B1 (originally P450EF), were raised in our laboratory (Savas et al., 1997). The rabbit anti-AhR antibodies were provided by Dr Richard Pollenz, University of South Carolina.

MEF isolation

Primary MEFs were isolated from 14-day post coitus (d.p.c.) mouse embryos (Reznikoff et al., 1973). Embryos were surgically removed, and separated from maternal tissues and the yolk sack. The bodies were minced finely and then incubated in a solution of trypsin:EDTA (0.05% trypsin; Sigma; 1 mM EDTA, glucose, 1× PBS) with shaking at 37°C for 15-30 minutes. The solution was allowed to settle for 2 minutes, and the supernatant was centrifuged for 3 minutes at 1000 ***g***. The resulting pellet was resuspended in culture medium and cells were plated at 10^4 cells/cm^2. Attached cells then constituted passage 1.

Cell culture and treatments

10T1/2 cells, purchased from ATCC (Bethesda, MD), and all primary MEFs were maintained in Dulbecco's Modified Eagle Medium:F-12 nutrient mix (DMEM:F12, Gibco BRL), supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 units of penicillin and 100 μg/ml streptomycin. Cultured cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. A standard protocol for adipocyte differentiation of 3T3-L1 cells was followed (Phillips et al., 1995; Shugart et al., 1995). Briefly, cell cultures were maintained at confluence for 1 day, then switched to base differentiation medium (DMEM supplemented with 10% FBS and 10 μg/ml insulin). Differentation medium was supplemented with 5 μM dexamethasone and 0.2 mM isobutylmethylxanthine for the first 3 days. Subsequently, cultured cells were maintained in the basal differentiation medium only. 1 μM BRL-49653 (Forman et al., 1996; Lemberger et al., 1996) was added as part of the base differentiation medium where indicated. For experiments examining inhibition of differentiation by TCDD, cell cultures were treated with 10 nM TCDD for 1 hour prior to initiating hormonal treatment.

DMBA metabolism assay

DMBA metabolism was measured in microsomes and in intact cells as previously described (Brake and Jefcoate, 1995; Christou et al.,

1993). Reactions were carried out with 0.5 mg of microsomal protein derived from 10T1/2 and WT MEF cells, and with 1 mg of microsomal protein from AhR$^{-/-}$ MEFs. For intact cells, DMBA (10 mM) was added to the medium and incubated with the cells for 1 hour in the dark. The medium was then removed and mixed 1:1 with a solution of β-glucuronidase (2000 units/ml), 0.5 M sodium acetate, pH 5.0, 100 mg/ml ascorbic acid. The medium was then extracted with ethylacetate:acetone as described for microsomes.

Microsome preparation and immunoblotting

Microsomes were prepared according to procedures previously optimized for 10T1/2 cells (Christou et al., 1993) and protein concentrations were determined following the Pierce BCA kit manufacturer's protocol. Microsomal proteins were separated by SDS-PAGE and visualized by western immunoblotting (Harlow and Lane, 1988), using the ECL detection method as per the manufacturer's protocol. Total cell lysates for detection of the AhR were prepared by lysis of cells from a 60 mm dish. 100 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1% NP-40, 0.25% deoxycholate 0.1% SDS, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) were added to cells after two PBS washes. Cells were scraped, harvested and homogenized with a 25-gauge needle. Lysates were centrifuged (12,000 ***g*** for 10 minutes at 4°C) and the soluble fraction retained for western immunoblotting.

RNA isolation and northern hybridization

Poly(A)+ RNA was isolated and separated by gel electrophoresis on 1.3% agarose gels containing formaldehyde and then transferred to Nytran Plus membranes. The membrane was hybridized using Quickhyb (Stratagene) in the presence of ^{32}P-labeled cDNA probes. Labeling was accomplished with the Stratagene random primer kit according to the manufacturer's protocol. Probes used were the full-length glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, rat β-actin cDNA, a cDNA for stearoyl-CoA desaturase gene 1 (SCD1) (Ntambi and Takova, 1996), the cDNA for aldehyde dehydrogenase-3 (ADH3) and a 1028-bp mouse CYP1B1 cDNA (Savas et al., 1994). The full-length PPARγ cDNA, obtained from Dr James Ntambi, University of Wisconsin, Madison, does not distinguish between PPARγ1 and γ2 transcripts. However, PPARγ2 is the predominant form in 3T3-L1 and 10T1/2 cells and is known to mediate the adipogenic response in these cells (Tontonoz et al., 1994). mRNA bands were visualized and quantitated using a Molecular Dynamic Phosphorimager and ImageQuant software (Molecular Dynamics).

Senescence-associated β-galatosidase staining

Cells were tested for senescence-associated β-galatosidase (SAβG) activity by a method first described for human skin fibroblasts (Hara et al., 1996), and later adapted to rodent embryo fibroblasts (Serrano et al., 1997). Briefly, cells were washed with PBS, fixed with 1% glutaraldehyde for 5 minutes at 37°C, washed with PBS, and then incubated overnight at 37°C in a fresh 1 mg/ml solution of 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside.

Transient transfections

Transfections were carried out as described elswhere (Zhang et al., 1998).

Cell proliferation following induction of adipogenesis

After the initial 60 hours of hormonal IDM induction cells were washed with PBS, detached and dispersed with a trypsin/EDTA solution (0.05% trypsin, Sigma; 1 mM EDTA, glucose, 1× PBS), and replated in 60 mm, 6-well plates at 10^4 cells/well in DMEM:10% FBS. After attaching overnight, cells were washed with PBS, detached with trypsin/EDTA, and the number of cells per well was determined by counting with a hemocytometer. Three 60 mm wells were counted for each treatment and for each cell type.

Oil-Red O staining

When differentiation was complete, as judged by visual inspection, cultures were fixed with 10% formalin in PBS for 10 minutes, stained with a 0.5% Oil-Red O solution in propylene glycol for 15 minutes, and counter-stained with Hemotoxylin for 1 minute. After rinsing, cells were mounted in glycerine and photographed.

Glycerol-3-phosphate dehydrogenase assays

Cells grown in 60 mm six-well plates were washed with PBS, harvested into 0.2 ml homogenization buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 5 mM Tris, pH 7.4), sonicated, and centrifuged at 13,000 ***g*** for 10 minutes at 4°C. Supernatants were removed and assayed immediately. Glycerol-3-phosphate dehydrogenase (GPDH) activity was determined spectrophotometrically as described elsewhere (Brodie et al., 1996). Activity was normalized to protein content of the supernatant fractions as determined by the Pierce BCA kit.

RESULTS

Early onset of proliferative crisis in AhR$^{-/-}$ MEFs

Primary fibroblasts eventually enter a non-growing senescent phase when placed in culture. Maintenance of primary AhR$^{-/-}$ MEFs in culture was limited to only 2 or 3 passages before the cultures entered proliferative crisis (Fig. 1). Equivalent MEFs generated from congenic WT C57BL/6 mice entered proliferative crisis at passage 7-8. The decreased replicative potential observed in cultures of both these MEFs occured in parallel, with the appearance of multinucleated cells with a spindle-like morphology, rounded cells containing lipid droplets and small pyramid-shaped cells. These morphologies are consistent with, respectively, myoblasts, adipocytes and osteoblasts. Differentiated cells are not retained in immortalized MEF cultures, which were composed of large flattened cells with numerous processes.

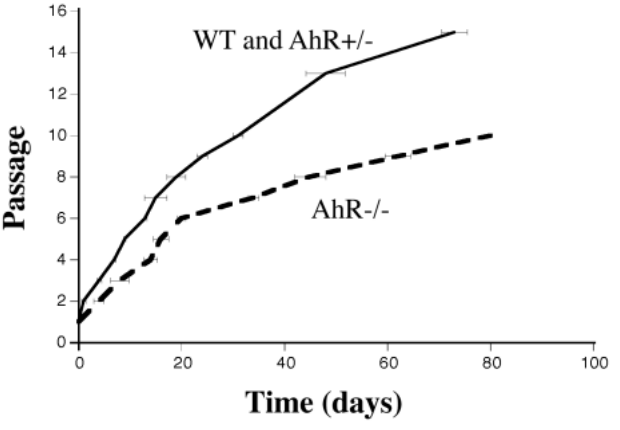


Fig. 1. Early onset of proliferative crisis in AhR$^{-/-}$ MEFs. Passage number is plotted against the days in culture to describe a line showing the frequency of passage through continuous culture. MEFs were maintained in 10% FBS-containing DMEM:F12 medium and replated to 30% of confluent cell density by trypsin/EDTA dispersion when 95% confluence was reached. Passage 1 denotes the initial isolation of MEFs. Data represents the mean ± range for three isolations of AhR$^{-/-}$ MEFs and three WT. The two AhR$^{+/-}$ preparations done could not be distinguished from WT.

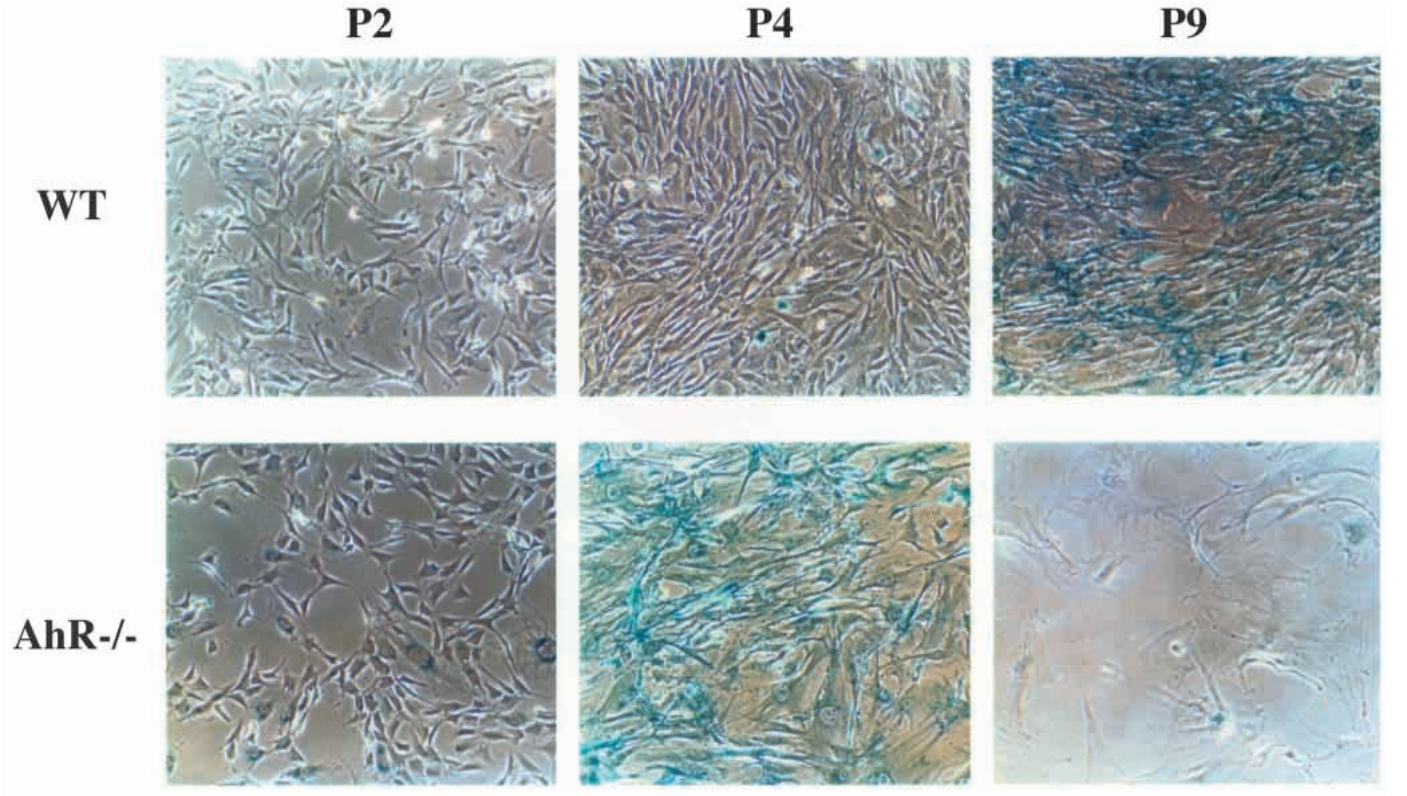


Fig. 2. Senescence associated β-galactosidase activity marks the onset of proliferative crisis in primary MEF cell cultures. Micrographs of primary WT and AhR$^{-/-}$ MEFs at passages 2, 4 and 9 were fixed and stained for β-galactosidase activity as described in Materials and methods. Micrographs are all 40×.

We next determined whether the early decline in replicative potential of the AhR$^{-/-}$ MEF cultures was due to senescence. Lysosomal SAβG is observed at increased passage numbers when fibroblasts exhibit decreased replication in parallel with a loss of telomeres (Belair et al., 1996; Campisi, 1997). The early onset of proliferative crisis observed in AhR$^{-/-}$ MEFs is paralleled by expression of SAβG (Fig. 2). At passage 4 nearly all AhR$^{-/-}$ MEFs are positive for SAβG activity. WT MEFs do not show the same proportion of SAβG-positive cells until passage 7-8, when they too enter proliferative crisis.

Expression of CYP1B1 in primary AhR$^{-/-}$ MEF cells

Primary MEFs isolated from 14-15 d.p.c mouse embryos express constitutive CYP1B1 that can be induced through activation of the AhR (Alexander et al., 1997). Primary AhR$^{-/-}$ MEF cells have greatly decreased constitutive and TCDD-induced CYP1B1 protein (Fig. 3), mRNA (Fig. 4) and the associated DMBA metabolic activity (not shown) compared to WT MEFs and 10T1/2 cells. By contrast, aldehyde dehydrogenase-3 (ADH3), another AhR-responsive gene, shows no change in constitutive expression, but is similarly non-responsive to TCDD treatment in primary AhR$^{-/-}$ MEFs (Fig. 4).

Isolation of immortal WT and AhR$^{-/-}$ MEFs

When late passage MEF cultures are maintained at near 50% confluency for at least 7 weeks, colonies of immortalized proliferating cells appear. We have isolated and expanded three such colonies from both the AhR$^{-/-}$ and congenic WT MEF

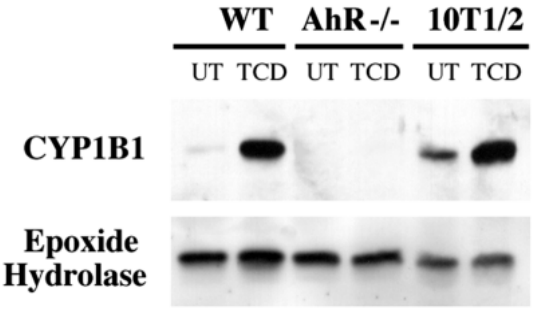


Fig. 3. Expression of CYP1B1 immunoreactive protein in primary MEFs is AhR-dependent. Targeted disruption of the AhR results in elimination of detectable immunoreactive CYP1B1 in MEFs. AhR$^{-/-}$ and WT MEFs were grown to 90% confluence and then treated with 10 nM TCDD (TCD) or with DMSO alone (UT) for 20 hours. Cells were then harvested and microsomal fractions isolated as described in Materials and methods. Each lane contains 10 μg of microsomal protein. Epoxide hydrolase was immunoblotted as an example of a non-AhR responsive gene and as a loading control. The data are representative of three or more independent observations from each cell type.

cultures. In each of five preparations, AhR$^{-/-}$ MEF cells remained in crisis without immortalization for longer than their WT counterparts (WT, 80±32 days; AhR$^{-/-}$, 140±25 days). The WT and AhR$^{-/-}$ lines have been carried through continuous culture for more than 1 year (>60 passages) without changes in morphology or growth rate.

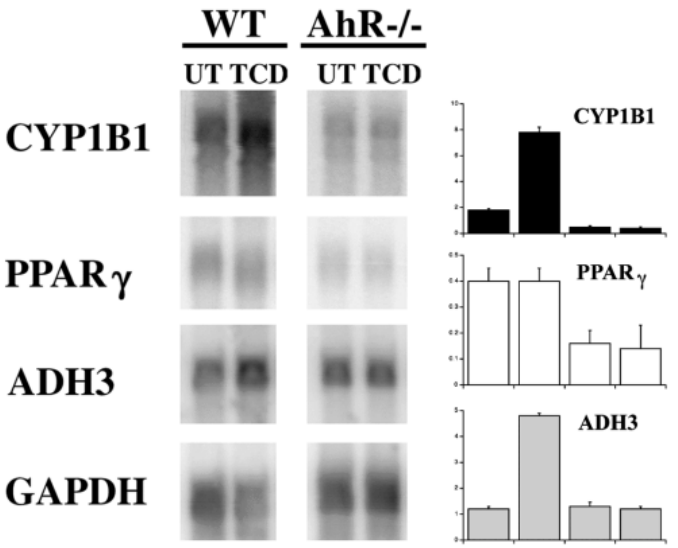


Fig. 4. Selectively decreased basal expression of CYP1B1 and PPARγ in AhR$^{-/-}$ MEFs. Northern hybridization of 4 μg poly(A)+ mRNA from primary WT and AhR$^{-/-}$ MEFs with CYP1B1, ADH3 and PPARγ cDNAs. Subconfluent cells were exposed to 10 nM TCDD in culture medium for 8 hours prior to mRNA isolation. Densitometric analyses of two separate mRNA preparations and hybridizations normalized for GAPDH mRNA expression are shown adjacent to a representative hybridization. Data are presented as the mean ± range.

The expression of CYP1B1 in these lines is comparable to that in primary MEFs. WT MEF lines all express similar levels of AhR together with basal and TCDD-inducible levels of CYP1B1, but not CYP1A1 (Alexander et al., 1997). The AhR$^{-/-}$ MEF lines exhibit no detectable AhR protein (not shown) and the same selective decrease in basal CYP1B1 mRNA expression relative to WT cells that is seen in primary cells (Figs 4 and 7).

Two AhR$^{-/-}$ lines were similar in morphology and growth characteristics to WT lines and the 10T1/2 cell line. These two lines, designated B6AhR$^{-/-}$(B) and B6AhR$^{-/-}$(D), and the WT lines were diploid, and had crescent or fibroblastic shape with a doubling time of 16-18 hours like the prototypical 10T1/2 cell line. A third line, B6AhR$^{-/-}$(F), which was tetraploid, exhibited a more irregular morphology with an elongated spindle shape and multiple projections, and proliferated more slowly. Only B6AhR$^{-/-}$(B) and B6AhR$^{-/-}$(D) were used for subsequent transfection and differentiation experiments.

Restoration of CYP1B1 expression in AhR$^{-/-}$ MEF lines

We transiently transfected AhR$^{-/-}$ lines with either pSV.Sport1, which contains an insert encoding the full AhR open reading frame, or the vector alone (Zhang et al., 1998). Fig. 5 shows that transfection restores 80% of constitutive CYP1B1-dependent DMBA-metabolism activity compared to the congenic WT line B6WT(A) and 10T1/2 cells. Similar results were obtained from transfection of a second pair of

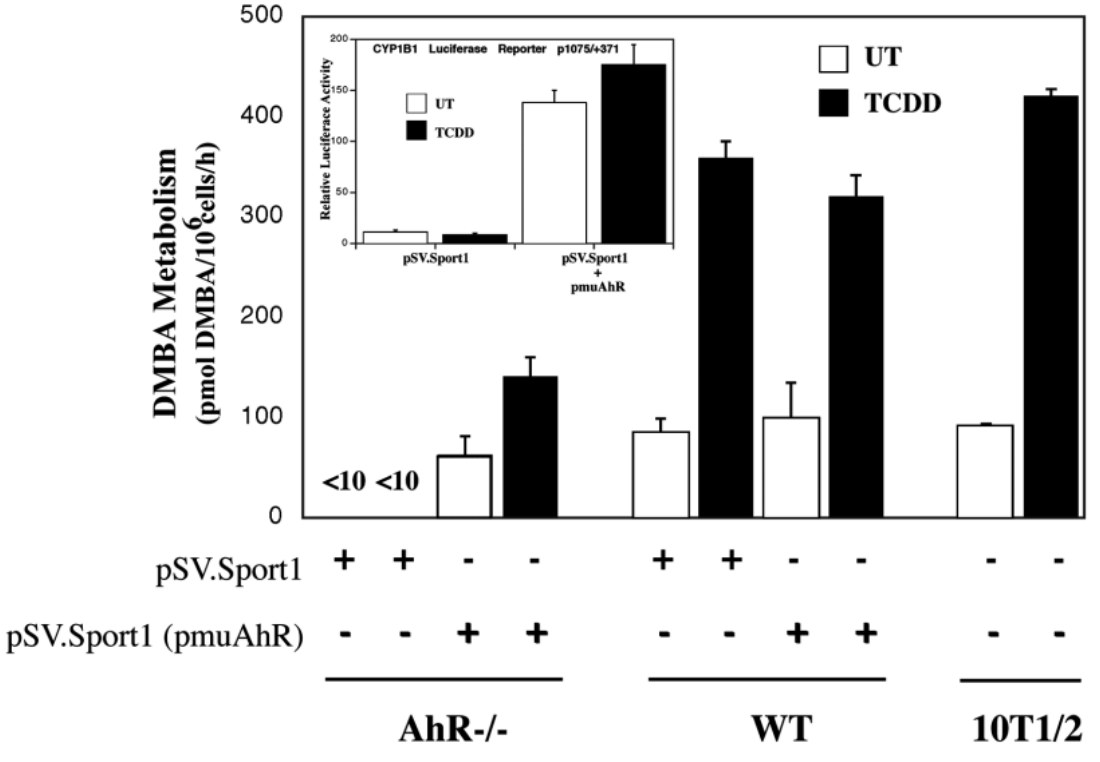
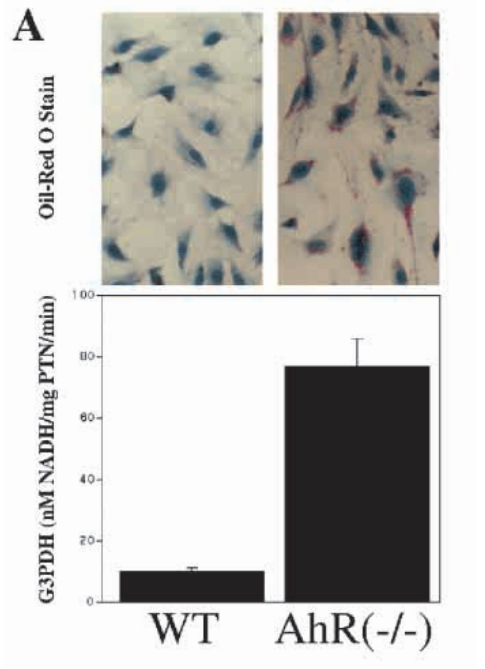
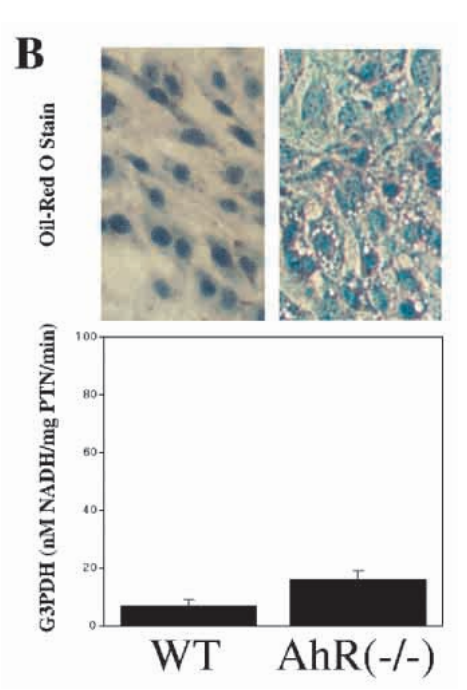


Fig. 5. Restoration of basal and TCDD-induced CYP1B1 DMBA metabolism in AhR$^{-/-}$ MEFs transiently transfected with the AhR. AhR$^{-/-}$ and WT MEFs were transfected with either the plasmid pSV.Sport1 or pSV.Sport1 containing the AhR-expressing cDNA (pmuAhR). Following transfection, cells were treated with 10 nM TCDD for 24 hours (filled columns) or left untreated (open columns) and subsequently assayed for whole cell DMBA metabolism. The data represent the mean and range of duplicate measurements. Similar results were obtained from AhR transfection of the separately derived B6AhR$^{-/-}$(D) cell line (not shown). (Inset) Previously published data of parallel experiments using a CYP1B1 luciferase reporter construct co-transfected into B6AhR$^{-/-}$(B) with the AhR expression plasmid. Values presented are normalized for transfection efficiency and described in Zhang et al. (1998).





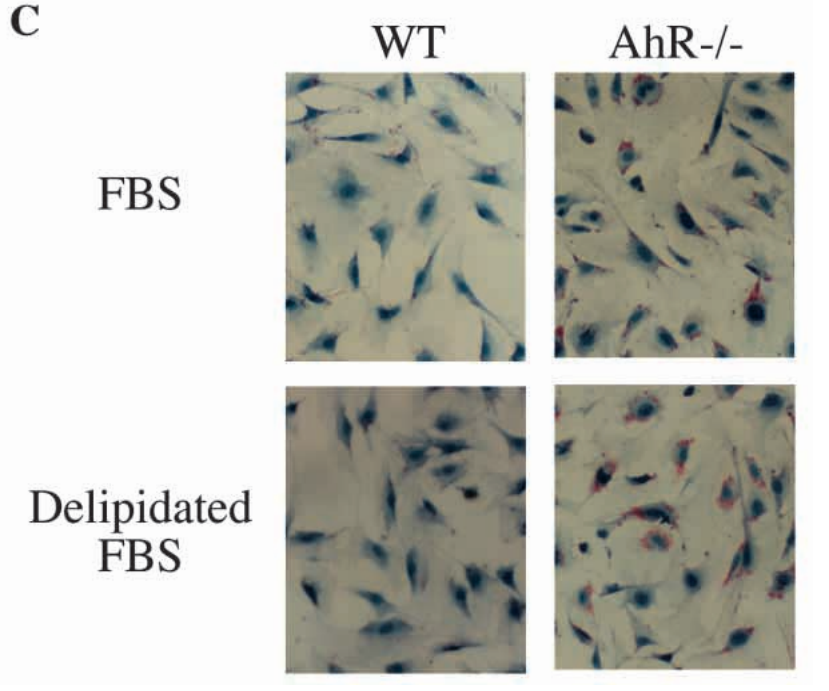


Fig. 6. AhR$^{-/-}$ MEFs exhibit marked microvesicular lipid accumulation in subconfluent culture. Upper panels, Oil-red O stain with hemotoxylin counter-stain. Lower panels, GPDH activity. (A) Wild type and AhR$^{-/-}$ MEFs in subconfluent culture. (B) Confluent culture. Cells were maintained in 10% FBS containing DMEM/F12 medium. GPDH activity is presented as the mean ± s.d. from triplicate determinations of two AhR and WT cell lines. (C) Lipid accumulation in similarly treated normal and lipid-depleted serum. All micrographs are 40×.

lines (B6AhR$^{-/-}$(D) and B6WT(B), not shown). TCDD induction was, however, much lower in transfected AhR$^{-/-}$ MEFs than in WT and 10T1/2 cells. A similar pattern of high basal activity and low TCDD induction was observed using a CYP1B1 luciferase reporter (inset in Fig. 5). Thus, two distinct assays, one a direct measure of promoter activity, and the other an enzymatic measure of CYP1B1 levels, show similar results. Transfection of WT MEFs with the AhR expression plasmid did not significantly alter constitutive expression. Efforts to measure AhR in the lysates of the transfected AhR$^{-/-}$ cells were unsuccessful, indicating an expression level that is at least 10 times lower than in WT cells. We conclude that basal CYP1B1 is highly stimulated by recombinant AhR, and that this high basal activity limits the potential for induction.

Proliferating AhR$^{-/-}$ MEFs show enhanced triglyceride synthesis

In subconfluent cultures, both primary and the AhR$^{-/-}$ MEF cell lines exhibit a microvesicular lipid accumulation not seen in WT MEFs (Fig. 6A). Lipogenesis was assayed in the AhR$^{-/-}$ lines by staining triglyceride droplets with Oil-Red O and by assaying the GPDH activity. Subconfluent B6AhR$^{-/-}$ (B) MEFs show fivefold greater GPDH activity levels than B6WT(A) MEFs (Fig. 6A). In confluent AhR$^{-/-}$MEFs, Oil-Red O-staining droplets are no longer observed and GPDH

activity decreases to levels seen in WT MEFs (Fig. 6B). Lipid droplets were also observed in an equivalent lipid-depleted serum (Fig. 6C). This establishes that de novo lipogenesis is being affected by removal of the AhR as opposed to lipid uptake from the medium.

Induction of adipocyte differentiation

The IDM differentiation medium that induced 3T3-L1 adipogenesis was not sufficient to induce differentiation in WT and AhR$^{-/-}$ primary cells or lines, or in the 10T1/2 cell line. More than 75% of 3T3-L1 cells had differentiated 7-9 days after IDM stimulation whereas less than 5% of WT, AhR$^{-/-}$ or 10T1/2 cells contained large lipid droplets. Only the 3T3-L1 preadipocytes showed IDM-induced increases in GPDH activity (Table 1) or PPARγ and SCD1 mRNA levels (Fig. 7), which are markers of adipogenesis. Where comparisons were made, similar results were seen for primary and equivalent MEF lines.

Addition of the PPARγ agonist BRL to the differentiation medium resulted in increased GPDH activity in each of the previously unresponsive cell lines (Table 1). These levels are 10-20 times greater than the levels seen for lipogenesis in subconfluent AhR$^{-/-}$ MEFs. Visual examination of the cultures showed that >90% of cells contained large lipid droplets consistent with the adipocyte morphology. AhR$^{-/-}$ MEF cells had threefold less constitutive PPARγ mRNA than WT MEFs and the 10T1/2 cell lines (Figs 4, 8). However, BRL induced adipogenesis to a comparable extent in all these cells. Adipogenesis in AhR$^{-/-}$ lines is also associated with marked induction of PPARγ expression although levels were lower than in WT cells (Fig. 8).

CYP1B1 expression in differentiation

Based on earlier results, CYP1B1 expression can provide a direct measure of AhR activity in MEFs. CYP1B1 mRNA expression did not change between undifferentiated MEFs and the hormone-stimulated adipocytes (Fig. 7). Recently, the AhR was reported as undetectable following adipogenesis in 3T3-L1 cells (Chen et al., 1997). We also observed a decrease in the AhR with differentiation but low levels were clearly detectable (Fig. 9). Interestingly, CYP1B1 was basally expressed and inducible in fully differentiated 3T3-L1 cultures. Indeed, basal CYP1B1 protein expression was elevated by differentiation in these cells (Fig. 9). AhR is typically downregulated following activation in MEFs (Alexander et al., 1997). The decrease in AhR and increase in CYP1B1 following differentiation is consistent with increased turnover and activity of the AhR.

Table 1. GPDH activity in WT MEFs, AhR$^{-/-}$ MEFs, 10T1/2 and 3T3-L1 cells: effect of different hormonal treatments

	Treatment			
Cells	Untreated	IDM	IDM/BRL	IDM/BRL +TCDD
WT	27.2 (0.9)	36 (6.5)	2099 (127)	452 (200)
AhR$^{-/-}$	15 (2.0)	18 (2.0)	840 (150)	950 (220)
10T1/2	34 (8.7)	31 (5.1)	800 (90)	180 (90)
3T3-L1	168 (28)	822 (84)	1215 (147)	60 (18)

Values are activity in moles NADPH produced/mg protein/hour (s.d.).

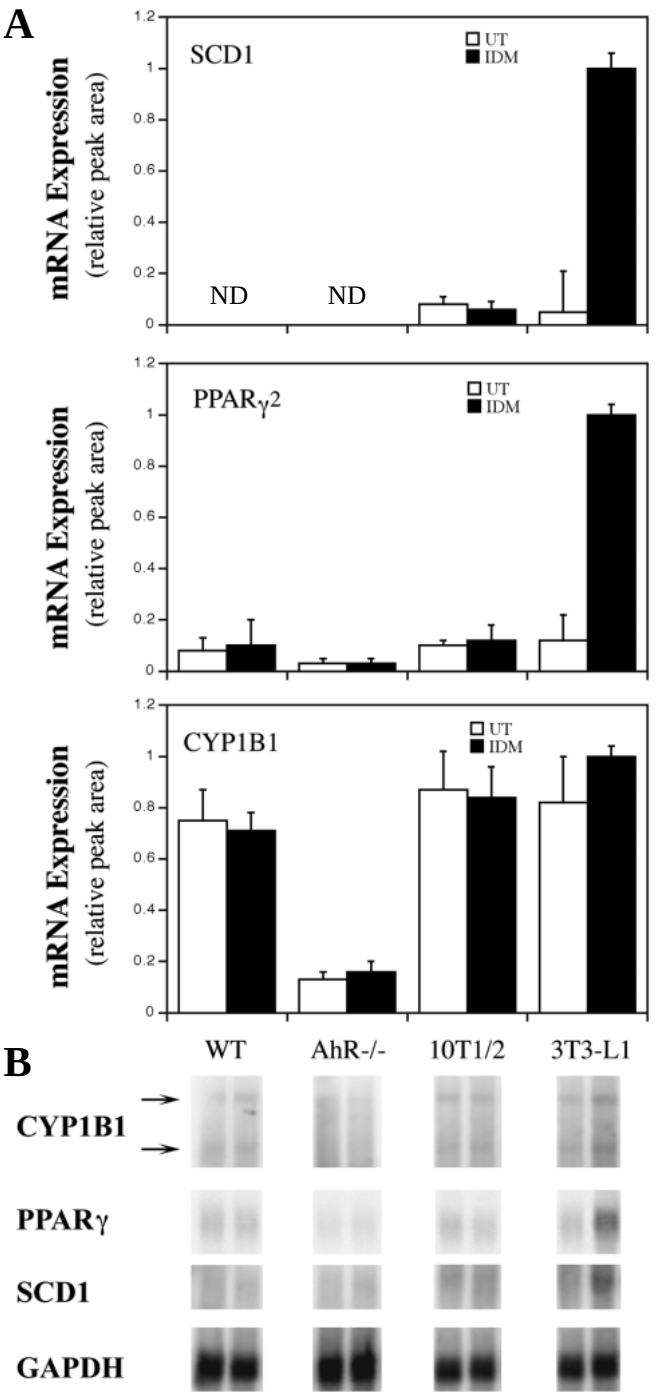


Fig. 7. IDM hormonal stimulation is insufficient to induce adipogenesis in MEFs. PPARγ and SCD1 mRNA expression are shown as markers for terminal adipocyte differentiation. CYP1B1 is used as a marker for AhR activity. 10T1/2, 3T3-L1, and immortal MEF lines B6WT(A) (WT) and B6AhR$^{-/-}$(B) (AhR$^{-/-}$) received hormonal treatment (IDM) or base differentation medium (UT). 7 days after initiating treatment cells were lysed for mRNA isolation. 4 μg of poly(A)+ mRNA were hybridized with cDNA probes for the indicated genes. (A) Data are presented normalized for β-actin expression, and expressed as peak-area relative to the most intense signal for each specific cDNA hybridization. The values are the mean of two independent experiments and error bars represent the range. (B) Representative hybridizations. Hybridizations where no signal above background was detected are designated as not detected (ND).

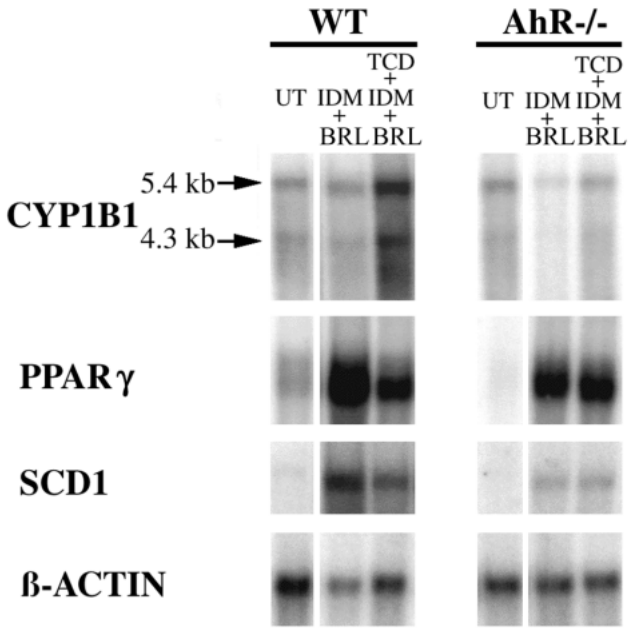


Fig. 8. AhR$^{-/-}$ MEFs are resistant to TCDD inhibition of adipogenesis. Northern hybridization of 4 μg poly(A)+ mRNA from wild type and AhR$^{-/-}$ MEFs with CYP1B1-, PPARγ2-, SCD1- and β-actin-specific cDNAs. MEF cultures were harvested for mRNA isolation on day 8 after induction of adipogenesis. The WT MEF cell line B6WT(A) and the AhR$^{-/-}$ MEF line B6AhR$^{-/-}$(B) were stimulated to induce adipocyte differentiation with IDM in addition to 1.0 μM BRL-49563 (IDM+BRL); pretreated with 10 nM TCDD for 1 hour prior to adipocyte induction (TCD+IDM+BRL); or maintained without hormonal stimulation (UT). The northern hybridization shown is representative of three independent experiments.

TCDD inhibition of adipogenesis is mediated by the AhR

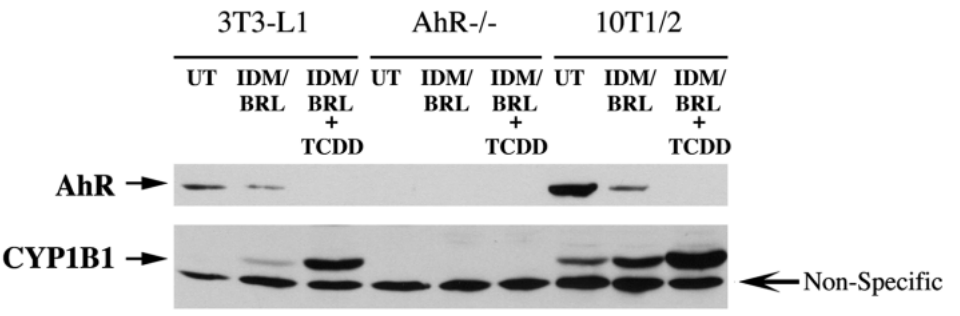
The inhibition of adipogenesis by TCDD and other AhR ligands in 3T3-L1 and 10T1/2 cells has been described previously (Phillips et al., 1995). Based on Oil-Red O and GPDH activity, pretreatment with 10 nM TCDD blocked adipogenesis in 10T1/2, 3T3-L1 and WT MEFs. By contrast, there was absolutely no effect on differentiation in AhR$^{-/-}$ MEF lines. TCDD decreased GPDH activity 4- to 5-fold in each cell type except in AhR$^{-/-}$ MEF cell lines. Fig. 8 shows that TCDD treatment also decreased expression of PPARγ following IDM/BRL treatment in WT but not B6AhR$^{-/-}$(B) MEFs. Following TCDD treatment, PPARγ levels in WT cells were comparable to the initially lower levels in AhR$^{-/-}$ cells. Only the latter differentiated, indicating that TCDD does more than lower PPARγ expression. TCDD-treated cultures showed only very limited differentiation seen as scattered clusters of bright, lipid-droplet containing cells against a confluent field of lipid-free cells (Fig. 10).

We next determined whether TCDD pretreatement altered the post-mitotic cell-cycle arrest indicative of commitment to differentiation. Cells were replated in DMEM:F12 medium at subconfluent densities (10^4 cells/60 mm plate) following the 2-day hormonal induction. Replated, subconfluent 3T3-L1 and 10T1/2 cells exhibited a lag of 60 hours before cell numbers began to increase. Only 3T3-L1 cells exhibited this lag with IDM treatment alone (no BRL). Parallel treatments with TCDD completely restored cell proliferation following replating. AhR$^{-/-}$ MEFs, like 10T1/2 cells, showed a marked lag in proliferation when BRL was added to the differentiation medium that was, however, retained in the presence of TCDD (Fig. 11). The difference in growth arrest exactly parallels the treatment requirements for a differentiation response.

DISCUSSION

In this work, we have shown that the absence of AhR accelerates entry into senescence. Extensive research with primary cultures of rodent and human fibroblasts and epithelial cells has shown that suppression of p53 or Rb proteins delays senescence (Chen et al., 1996; Harvey et al., 1993, 1995; Shay et al., 1993). Entry into senescence and differentiation of these multipotential MEFs are clearly parallel processes since, as these cells enter senescence, we also observe increasing numbers of myoblasts and adipocytes in the culture. These and chondrocytes are derived from MEF cultures treated with azocytosine, although in different proportions to what is seen here spontaneously (Konieczny and Emerson, 1984). Rb clearly plays a major role in the commitment to adipogenesis since Rb$^{-/-}$ MEFs fail to undergo adipogenesis (Chen et al., 1996). E2F/Rb complex formation is key to the regulation of cell cycle progression. Recent work suggests that PPARγ activation leads to changes in the phosphorylation of the alternative E2F/DP1 complex (Altiok et al., 1997). AhR may alter processes that involve exit from the cell cycle, possibly

Fig. 9. AhR decreases during MEF adipogenesis, but remains TCDD responsive. Immunoreactive AhR and CYP1B1 proteins were detected in 3T3-L1, B6AhR$^{-/-}$(B) and 10T1/2 cells induced to undergo adipocyte differentiation (IDM/BRL), or were maintained in differentiation medium without hormonal stimulation (UT). After 7 days, when >90% of cells contained lipid droplets, differentiated adipocytes were treated with 10 nM TCDD for 18 hours (IDM/BRL+TCDD). The blot shown is representative of duplicate experiments.



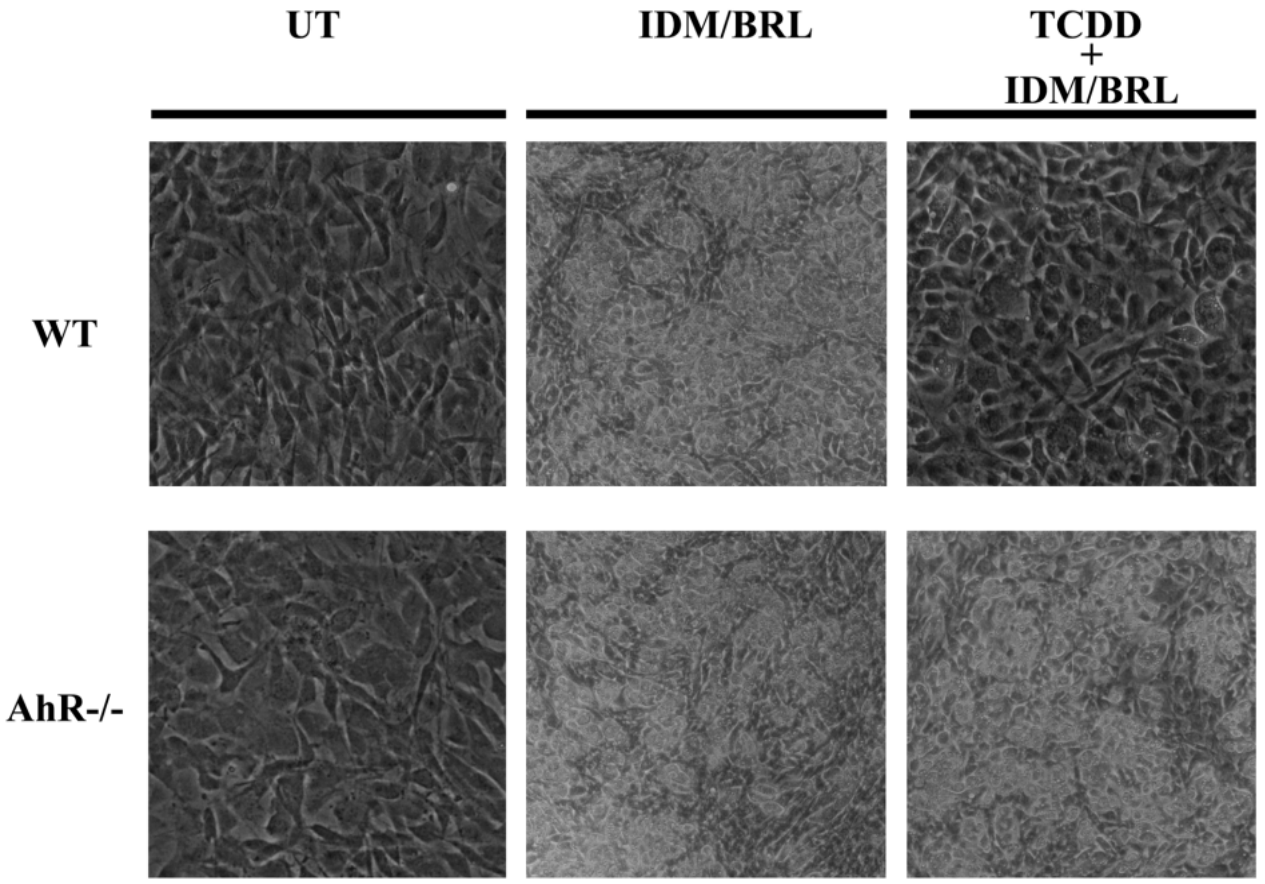


Fig. 10. TCDD suppression of adipogenesis in MEFs is AhR dependent. The WT MEF cell line B6WT(A) and the AhR$^{-/-}$ MEF line B6AhR$^{-/-}$(B) were stimulated to induce adipocyte differentiation with IDM and and 1.0 μM BRL-49563 (IDM/BRL); pretreated with 10 nM TCDD for 1 hour prior to adipocyte induction (TCDD+IDM/BRL); or maintained without hormonal stimulation (UT). Undifferentiated cells retain their fibroblastic morphology whereas differentiated adipocytes appear as bright, compact, rounded cells. Micrographs were taken 8 days after initiating treatments. UT panels, 40×; IDM/BRL, 20×; TCDD+IDM/BRL, 40× for WT and 20× for AhR$^{-/-}$ MEFs. The micrographs show representative fields of each cell type/treatment for multiple experiments.

by interaction with Rb family members (Ma and Whitlock, 1996).

We also report here two distinct types of effects of the AhR on triglyceride (TG) accumulation in MEFs and preadipocytes. First, deletion of the AhR results in elevated TG synthesis in parallel with increased cytosolic GPDH activity in both primary MEFs and derived cell lines. This indicates that one of the constitutive functions of the AhR is to suppress TG synthesis. This effect was, however, highly dependent on cell density. AhR loss only elevated GPDH activity and TG synthesis in subconfluent cells. The loss of lipogenesis in confluent cells suggests that the AhR inhibits expression of certain lipogenic genes in proliferating cells or at low cell density, and that these genes are suppressed by other mechanisms in confluent cultures. This transient lipogenic response is reminiscent of the liver fatty metamorphosis observed in AhRΔ2-null mice (Schmidt et al., 1996).

The second effect of AhR on TG accumulation is the suppression of hormone-induced adipogenesis when the receptor is activated by TCDD during the early stages of this differentiation process. Adipogenesis results in 60-fold increases in GPDH activity and parallel increases in TG accumulation in 10T1/2, 3T3-L1, WT and AhR$^{-/-}$ MEFs. TCDD treatment during the 2-day period of IDM/BRL stimulation suppresses GPDH activity and TG accumulation except in AhR$^{-/-}$ MEFs. Matsumura and coworkers have previously shown that TCDD acts during the proliferative phase to block the morphological and biochemical transition to adipocytes (Phillips et al., 1995). We have thus confirmed these findings, extended them to primary MEFs and have shown that the selection processes inherent in spontaneous immortalization do not affect either the adipogenic response or the sensitivity to TCDD. Most important, we have rigorously established the involvement of the AhR in this response by showing that adipocyte differentiation of AhR$^{-/-}$ MEFs is completely unaffected by TCDD.

IDM/BRL stimulation of confluent WT and AhR$^{-/-}$ MEF lines results in additional proliferation and subsequent increase in PPARγ expression in a parallel manner to IDM-treated 3T3-L1 preadipocytes (Chawla et al., 1994). Previous work has established PPARγ2 as a key regulator of the commitment to differentiation in these cells (Spiegelman and Flier, 1996; Tontonoz et al., 1994). Interestingly, PPARγ expression levels are decreased in both primary and immortal AhR$^{-/-}$ MEFs, but show a similar pattern of increase following IDM/BRL stimulation of adipogenesis.

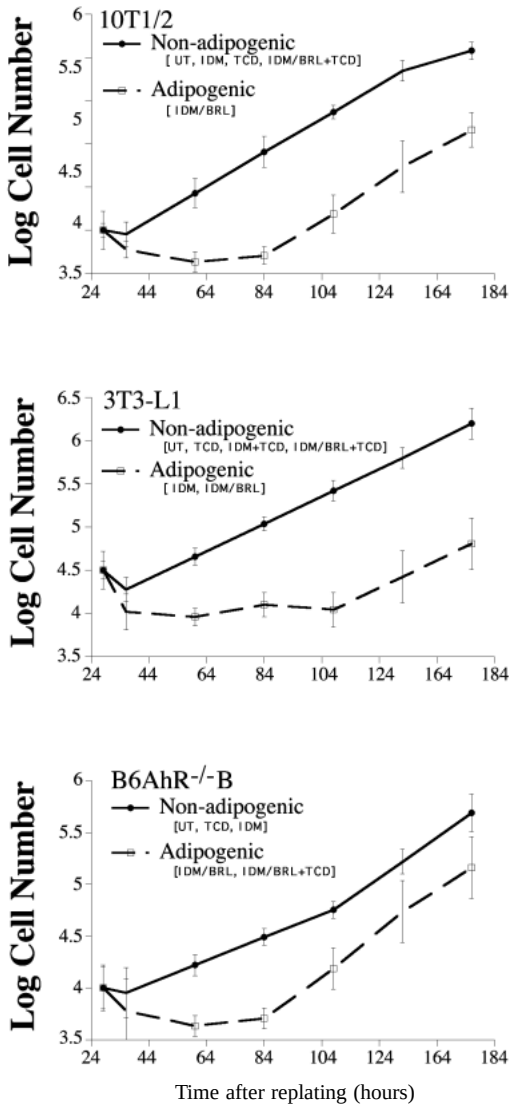


Fig. 11. TCDD treatment blocks commitment to adipogenesis and permanent withdrawal from cell cycle. The data are presented in a simplified form as trend lines: one for nonadipogenic and one for adipogenic treatments as indicated for each cell type. 3T3-L1 preadipocytes, 10T1/2 fibroblasts and AhR$^{-/-}$ MEFs were treated according to the adipogenesis induction protocol with and without TCDD (10 nM) pre-treatment, or left untreated. 60 hours after initiating treatment, a period sufficient to cause commitment to adipogenesis, cells were trypsin/EDTA dispersed and replated at 10^4 cells per 60 mm dish. Cell numbers were determined every 24 hours thereafter for 6 days. The mean cell number and range from two experiments was determined for each treatment. Two narrow response patterns were apparent. Thus, for clarity, the data for each cell type were combined into two lines representing the mean of all treatments giving an adipogenic response, and the mean of all treatments failing to induce adipocyte differentiation.

Paradoxically, both WT and AhR$^{-/-}$ MEFs require a PPARγ agonist (BRL) for the transition to adipogenesis, as previously seen for 10T1/2 cells (Kliewer et al., 1995). The decreased PPARγ expression in AhR$^{-/-}$ MEFs does not appear to adversely affect the response, indicating that this is not a limiting factor. SCD1 message levels are much lower in AhR$^{-/-}$ MEFs, consistent with the direct sensitivity of this gene to a diminished PPARγ2 stimulation (Miller and Ntambi, 1996). However, activation of the AhR blocks adipogenesis, suggesting that AhR may decrease the effectiveness of activated PPARγ to stimulate differentiation. Our work suggests that the major difference between MEFs and the preadipocyte line 3T3-L1 is that the latter exhibits heightened responsiveness to PPARγ stimulation, possibly due to increased levels of an endogenous activator. Interestingly, heptane extraction of FBS removes a lipophilic stimulant of adipogenesis in 3T3-L1 cells that can be replaced by PPARγ activation (Chawla and Lazar, 1994; Tontonoz et al., 1994). CYP1B1 may possibly metabolize an endogenous PPARγ activator that is either in the medium or that is produced in the cells.

We have further defined the mechanism of action of TCDD to the stage of growth arrest. Following the IDM/BRL-induced proliferation and arrest, replated cells remain growth-inhibited at low densities. When TCDD is included with IDM/BRL, MEFs exhibit normal stimulation and arrest, but grow continuously when the inhibitory cell-cell contacts are removed. This response exactly parallels changes seen previously when DEX is removed from the hormonal stimulation (Shugart and Umek, 1997). However, TCDD does not affect C/EBPβ expression, which is stimulated by glucocorticoids (Phillips et al., 1995; Rowlands and Gustafsson, 1997). Previous work with 3T3-L1 cells shows that TCDD lowers expression of PPARγ, C/EBPα and all lipogenic genes that are transcriptionally activated by these regulators (Phillips et al., 1995). We also see a strong suppression of PPARγ after treatment with TCDD. Previous work indicates that C/EBPβ is critical for this differentiation but this is not affected by TCDD (Wu et al., 1995; Yeh et al., 1995). Together, these findings suggest a site of action for TCDD between C/EBPβ and PPARγ, which is consistent with the presence of the responsive CCAAT elements in the PPARγ promoter (Zhu et al., 1995).

The mechanism of action of the TCDD-activated AhR parallels the activity of several other receptor-mediated processes. PGF2α acts through the plasma membrane PGF2α receptor to inhibit adipogenesis at the same stage, possibly by activation of Ca/calmodulin kinases (Miller et al., 1996). Retinoids also inhibit differentiation of these cells via RAR receptors (Schwarz et al., 1997). Interestingly, RXR receptors heterodimerize with PPARγ as an essential part of the stimulation of adipogenesis (Chawla and Lazar, 1994). Mouse hepatoma cells with impaired AhR activity exhibit a more rounded morphology with fewer cell-cell and cell-substrate contacts. Cell-cell contact appears to be an essential part of adipogenesis. The cell-surface proteins Notch-1 and Pref-1, which function through cell-cell interaction, have been implicated in adipogenesis (Garces et al., 1997; Xing et al., 1997).

CYP1B1 expression provides a direct assay for constitutive AhR activity in MEFs as well as for the stimulation by TCDD. Constitutive and TCDD-induced CYP1B1 are seen in MEFs that contain the AhR both before and after adipogenesis. The absence of CYP1B1 in AhR$^{-/-}$ cells implicates the receptor in constitutive expression at both stages. Interestingly, CYP1B1 expression increases following differentiation, suggesting increased AhR activity. The inducibility of CYP1B1 in differentiated adipocyte cultures of MEFs indicates that the

decrease in AhR described here and elsewhere (Chen et al., 1997) does not explain the impotence of TCDD to inhibit adipogenesis when added after hormonal induction.

We have shown that CYP1B1 expression under regulation of the AhR is a regular feature of the phenotype of MEFs and human fibroblasts (Eltom et al., 1998). Since CYP1B1 has been implicated in developmental regulation (Stoilov et al., 1997), our current studies are directed towards identifying the mechanism of this participation. Recent studies of CYP1B1$^{-/-}$ MEFs in this laboratory show a greatly enhanced predisposition to adipogenesis, suggesting that CYP1B1 imposes additional limitations on differentiation. We have described both constitutive activities of the AhR and made a functional charaterization of the regulation of CYP1B1 by the AhR in MEF cells (Alexander et al., 1997; Zhang et al., 1998). Linking ligand metabolism and cell-cycle regulation in the interplay between AhR and CYP1B1 may be crucial to understanding the mechanisms modulating repression of adipogenesis.

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