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Altered Cell Cycle Control at the G_2/M Phases in Aryl Hydrocarbon Receptor-Null Embryo Fibroblast

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is known to mediate the toxic and carcinogenic effects of polycyclic aromatic hydrocarbons and dioxins. High-affinity AHR ligands, such as 2,3,7,8-tetrachlorodibenzeno-*p*-dioxin, have been shown to modify cell proliferation and differentiation. However, the mechanisms by which AHR affects cell proliferation and differentiation are not fully understood. To investigate the role of AHR in cell proliferation, mouse embryonic fibroblasts (MEFs) derived from AHR-null mice were obtained and characterized. Compared with wild-type MEFs, AHR-null cells exhibited a lower proliferation rate with an accumulation of 4N DNA content and increased apoptosis. The expression levels of Cdc2 and Plk, two kinases important for G_2/M phase of cell cycle, were down-regulated in AHR-null MEFs. In contrast, transforming growth factor-β (TGF-β), a proliferation inhibitor in several cell lines, was present at high levels in conditioned medium from AHR-null MEFs. Concomitant with G_2/M cell accumulation, treatment of wild-type MEFs with TGF-β3 also resulted in down-regulation of both Cdc2 and Plk. Thus, overproduction of TGF-β in AHR-deficient cells appears to be the primary factor that causes low proliferation rates and increased apoptosis. Taken together, these results suggest that AHR influences TGF-β production, leading to an alteration in cell cycle control.

The aryl hydrocarbon receptor (AHR) is a basic helix-loop-helix transcription factor that is activated by ligand binding and dimerization with the AHR nuclear translocator (Hoffman et al., 1991). AHR mediates the transcriptional activation of genes encoding xenobiotic-metabolizing enzymes such as cytochromes P450 (CYP1A1, CYP1A2, and CYP1B1), NAD(P)H:quinone oxidoreductase, and UDP-glucuronosyltransferase 6 (Rowlands and Gustafsson, 1997). It also mediates most of the toxicological effects of the halogenated aromatic hydrocarbons (HAHs) such as polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and polychlorinated biphenyls, all of which are widely disseminated in the environment (Safe et al., 1989; Fernandez-Salguero et al., 1996).

Mechanistic studies indicate that ligand binding is a limiting factor in AHR activation and function in numerous cell types (Gonzalez and Fernandez-Salguero, 1998). One of the most toxic and well studied HAHs is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a high-affinity AHR ligand. TCDD exposure produces a wide variety of toxic effects, including a wasting syndrome, immunotoxicity, hepatotoxicity, teratogenicity, and, in cases of chronic low-level exposure, tumor development and cancer (Huff et al., 1994).

TCDD also has a profound effect on the homeostasis of immune system, particularly in thymus, where it inhibits proliferation and induces changes in the differentiation pattern of thymocytes (Kremer et al., 1994). It was reported that thymic atrophy requires a functional AHR (Fernandez-Salguero et al., 1996) and that the targets for TCDD toxicity in the thymus are located in the hematopoietic compartment (Staples et al., 1998). TCDD has also been reported to induce c-*fos* and c-*jun* in Hepa-1 cell cultures (Puga et al., 1992) and to up-regulate the expression of several cytokines [interleukin (IL)-1β, IL-2, transforming growth factor (TGF)-β3, and tumor necrosis factor-α] and to down-regulate others (IL-4, IL-6, and plasminogen activator inhibitor-2; Lai et al., 1997). These data strongly suggest that AHR could play a role in the regulation of these genes, many of which are involved in cell proliferation and differentiation.

Further evidence implicating the AHR in cell cycle control was provided by studies with AHR-defective Hepa 1c1c7 cells that have lower levels of AHR expression, delayed cell

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; HAH, halogenated aromatic hydrocarbon; MEF, mouse embryonic fibroblast; DMEM, Dulbecco's modified Eagle's medium; DAPI, 4′-diamino-2-phenylindole; TCDD, 2,3,7,8-tetrachlorodibenzeno-*p*-dioxin; TGF-β, transforming growth factor-β.

growth, and longer doubling time than wild-type cells (Ma and Whitlock, 1996). A direct interaction between AHR and phosphorylated retinoblastoma protein has also been described (Ge and Elferink, 1998). Although ligand binding appears to be a prerequisite step for AHR activation, no endogenous ligand has been identified as yet. Alternative pathways for AHR activation have been proposed that involve protein kinase C-dependent phosphorylation in the absence of ligand binding (Chen and Tukey, 1996).

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transition and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. Several cell cycle transitions are dependent on the activity of cyclin-dependent kinases, and inhibition of these kinases is a mechanism by which some checkpoint pathways cause cell cycle arrest. The initiation of mitosis in eukaryotic cells is governed by a phosphorylation cascade, which culminates in the activation of the Cdc2/cyclin B complex (Basi and Draetta, 1995). Polo kinase also appears to play an important role in various steps during M phase progression such as activation of Cdc2 through Cdc25, exit from mitosis, and in cytokinesis (Glover et al., 1998).

The AHR-null mouse constitutes a valuable model to understand the role of AHR in cell biology (Fernandez-Salguero et al., 1995; Gonzalez and Fernandez-Salguero, 1998). Using AHR-null mouse embryonic fibroblasts (MEFs), the role of AHR in cell cycle progression was studied. In this communication, evidence is provided indicating that AHR plays a role in controlling cell division by influencing the expression level of the mitotic kinases, Cdc2 and Plk, as well as TGF-β. Such changes may result in lower proliferation rates, G_2/M cell accumulation, and apoptosis. Furthermore, data are provided showing that TGF-β3 is able to down-regulate the level of Cdc2 and Plk expression and induce the accumulation of MEFs at the G_2/M phase. These studies suggest that in the absent of exogenous ligand stimulation, AHR may participate in the regulation of the cell cycle through pathways likely involving TGF-β, Cdc2, and Plk.

Materials and Methods

Mice. AHR-null and wild-type mice were housed in a pathogen-free facility (SPF) using air-filtered controlled-environmental racks and fed with autoclaved Purina rodent chow with water available ad libitum. All animal manipulations were done under sterile conditions and in accordance with National Institutes of Health guidelines recommended and enforced by the National Cancer Institute Animal Care and Use Committee. Mice and MEFs were genotyped by restriction fragment length polymorphism analysis of genomic DNA as described previously (Fernandez-Salguero et al., 1995).

Cell Culture. MEFs were isolated from 14.5-day-old embryos generated by heterozygote crossbreeding. Briefly, the head and internal viscera were removed, and the remaining embryonic tissues were finely minced and digested by incubation in 0.25% trypsin-EDTA solution (Life Technologies, Grand Island, NY). After incubation for 45 min at 37°C with gentle agitation, trypsin was inactivated by adding 3 volumes of complete medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin]. Isolated cells were harvested and cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2. MEFs from the second to fourth passages were used in all experiments. To obtain mitotic cells, unsynchronized MEF cultures were treated with 0.6 μg/ml nocodazole (Sigma Chemical Co., St. Louis, MO) for 24 h, and rounded-up mitotic cells were mechanically released.

DNA Synthesis. DNA synthesis was determined by [^{3}H]thymidine incorporation. Cells (5×10^4) were seeded into 24-well dishes (final volume, 0.5 ml), and 0.5 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added to each well 2 h before harvest. Cells were then fixed with methanol/acetic acid (3:1 v/v) and washed with 80% methanol. Then, 0.5 ml of 0.25% trypsin-EDTA was added, and the plates were incubated for 30 min at 37°C before harvesting into a 24-well filter plate. Incorporated radioactivity was counted in a Beckman LS 5000TD scintillation counter.

Flow Cytometry. MEFs were harvested and washed in PBS. Cells were resuspended in 0.5 ml of cold PBS and fixed by slowly adding 4.5 ml of cold 100% ethanol while vortexing gently. Samples were incubated at −20°C overnight. After fixation, cells were pelleted out of ethanol, washed once with PBS, and resuspended in PBS containing 20 μg of propidium iodide (Sigma Chemical Co.) and 200 μg/ml DNase-free RNase (5 Prime-3 Prime, Boulder, CO). Cells were incubated at 37°C for 30 min and then allowed to stain for at least 8 h at 4°C. Samples were analyzed for DNA content on a Becton Dickinson (Mountain View, CA) FACScan as previously described (Lanni and Jacks, 1998).

Western Blot Analysis. Affinity-purified Plk antibody (Lee et al., 1995) was used at 0.5 μg/ml. Goat polyclonal ERK-1/2 and rabbit polyclonal Cdc2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used at 0.5 μg/ml. Western blot analysis was carried out as reported previously (Lee et al., 1995), and proteins that interact with antibodies were detected by an enhanced chemiluminescence Western detection system (Amersham).

Northern Blot Analysis. Total RNA from wild-type and AHR-null MEFs was isolated by homogenizing cells in guanidine-phenol solution (Biotex Laboratories, Houston, TX). Total RNA (20 μg) was subjected to electrophoresis in a 1% agarose/2.2 M formaldehyde gel and transferred to Gene Screen Plus membranes in 20× SSC (3 M NaCl, 30 mM sodium citrate, pH 7.0). The RNA was fixed to the membranes by baking at 80°C for 2 h, prehybridized in SSC/formamide solution, and hybridized at 42°C with the ^{32}P-labeled probes. cDNAs were labeled by random priming with DNA polymerase I Klenow fragment using [α-^{32}P]dCTP (Pharmacia, Piscataway, NJ). Labeled probes were added to the membranes at 2.0×10^6 cpm/ml. Filters were washed in 0.1× SSC and 0.5% SDS, and the membranes were exposed to autoradiographic film overnight at −80°C.

Immunoprecipitation and Kinase Assays. MEFs ($\sim 5 \times 10^6$ cells) were lysed in TBSN buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 20 mM *p*-nitrophenyl phosphate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin]. For measuring Cdc2 kinase activity, the lysate was incubated with 10 μl of p13^{suc1} agarose conjugate (Calbiochem, San Diego, CA) for 2 h. The precipitates were washed and subjected to kinase reaction in a kinase cocktail containing 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 5 μM cold ATP, 10 μCi of [γ-^{32}P]ATP, and 3 μg of histone H1 (Calbiochem, San Diego, CA) as a substrate. All reactions were carried out at 30°C for 15 min, and the reactions were terminated by the addition of 5× Laemmli's sample buffer (Laemmli, 1970) and boiled for 5 min. Proteins were separated on an SDS-10% polyacrylamide gel, and ^{32}P incorporation was detected by autoradiography.

For Plk immunoprecipitation, MEF lysates were incubated with affinity-purified Plk antibody for 2 h. Protein A-Sepharose 4B (Pharmacia) was added and incubated for an additional 1 h to precipitate the antibodies. To determine Plk specific kinase activity, Plk antibody preincubated with its epitope-peptide was used as a control. The immunoprecipitates were washed and subjected to kinase reactions as described earlier, with 3 μg of casein (Sigma Chemical Co.) as a substrate.

TGF-β Assay. To prepare conditioned medium, MEF cultures were grown in fresh medium without serum (OPTI-MEM I; Life Technologies). After incubation for 48 or 72 h, the supernatants were

centrifuged at 2000 rpm for 15 min at 4°C. The resulting supernatants were supplemented with BSA (Sigma Chemical Co.) and phenylmethylsulfonyl fluoride (Sigma Chemical Co.) at final concentrations of 100 μg/ml. Conditioned medium was aliquoted and stored at −80°C until the measurement of TGF-β activity. To prepare the conditioned media used to study its effects on DNA distribution, MEF cultures were grow in fresh OPTI-MEM I medium supplemented with 2% of fetal bovine serum (Life Technologies). After incubation for 72 h, the conditioned medium was obtained as described earlier.

Bioassays of TGF-β activity were performed using the mink lung epithelial cell line CCL64. Cells were plated onto 24-well plates at a density of 2.5×10^4 cells/well in 1 ml of DMEM with 10% FBS and allowed to adhere overnight. The next day, cells were washed with fresh medium containing 1% FBS. Conditioned medium prepared from wild-type or AHR-null MEFs were diluted in DMEM with 1% serum and added to the plates. TGF-β antibody 1D11 (25 μg/ml; Genzyme, Cambridge, MA) or control IgG was added to the conditioned medium. To quantify TGF-β secretion, additional wells were treated with medium plus TGF-β at various concentrations as standard. CCL64 cells were grown for 24 h, and 0.5 μCi of [^{3}H]thymidine was added for the final 2 h of incubation. Cells were harvested, transferred to a 24-well filter plate, and counted with a Beckman LS 5000TD scintillation counter.

Analysis for Apoptosis. MEFs were harvested at 24, 48, and 72 h. Cells were washed in PBS, fixed in ice-cold absolute methanol for 5 min, rehydrated in PBS, and incubated with 4′-diamino-2-phenylindole (DAPI) solution for 30 min in the dark. Cells were then washed and analyzed using an Olympus fluorescence microscope at 420 nm. Apoptotic cells exhibiting crescent-shaped areas of condensed chromatin located near the periphery of the nucleus and/or fragmented nuclei were scored as positive. Apoptotic nuclei were counted in five to seven randomly selected fields using a 40× neofluar objective. At least 500 to 1000 nuclei were counted for each genotype at different time points.

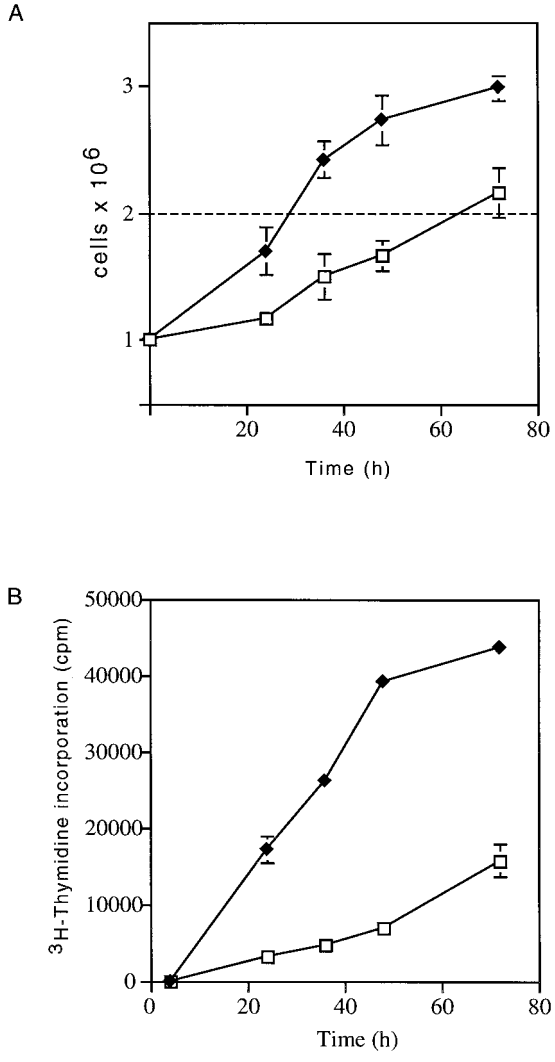

Fig. 1. Doubling time and DNA synthesis in wild-type (♦) and AHR-null (□) MEFs. A, 1×10^6 wild-type and AHR-null MEFs were cultured as described in *Materials and Methods*, and the cell number was determined at the indicated time points. B, wild-type (♦) and AHR-null (□) MEFs were cultured and pulse-labeled with [^{3}H]thymidine. Data represent mean ± S.D. values from three samples for each time point.

Results

Accumulation of AHR-Null MEFs in G_2/M Phase of Cell Cycle. To examine whether AHR plays a role in normal cell proliferation, MEFs derived from wild-type and AHR-null mice were analyzed for cell doubling time and DNA synthesis rates. The doubling time of wild-type MEFs was 24 h, whereas that for AHR-null MEFs was about 65 h, almost 3-fold slower (Fig. 1A). The DNA synthesis rate was also markedly different; by 24 h, wild-type MEF cultures had incorporated 3-fold more [^{3}H]thymidine than AHR-null MEFs (Fig. 1B). By 72 h, AHR-null MEFs incorporated only 35% of [^{3}H]thymidine compared with the control cultures. These results indicate that AHR plays an important role in cell proliferation.

To examine whether the differences in the doubling time reflect changes in the cell cycle progression between the two cell types, flow cytometry analysis was carried out. In wild-type MEFs, 53.8% of the cells were present in G_1, whereas 30.1% of the cells were present in G_2/M at 72 h (Table 1, Fig. 2). In contrast, 40.3% of AHR-null MEFs were in G_1, whereas 45.2% were in G_2/M, indicating that a large population of AHR-null MEFs were delayed in G_2/M phase. Although the difference was less pronounced, a similar tendency was found at 24 and 48 h (data not shown). These data suggest that AHR-null MEFs have an altered rate of cell division that leads to slow progression at the G_2/M phase (Table 1).

Elevated Apoptosis in AHR-Null MEFs. Apoptosis may contribute to the longer doubling time observed in AHR-null MEFs. To determine whether AHR-null MEFs have increased rates of apoptosis, the cells were stained with DAPI to detect the morphological features of apoptosis. As shown in Fig. 3, A and B, wild-type MEFs exhibited low levels of apoptotic cells, less than 5% of the total cell population, after 24, 48, and 72 h in culture. In contrast, in AHR-null MEF cells cultured for 48 and 72 h, approximately 30% of the total cell population showed peripheral accumulation of chromatin

TABLE 1
Cell cycle analysis of wild-type and AHR-null MEFs
MEFs were cultured as described in *Materials and Methods*. After 72 h, the cells were harvested and stained with propidium iodide, and the percentage of cells in each phase was determined by flow cytometry. The mean and S.D. values were determined from three independent experiments. G_0/G_1 and G_2/M are significantly different among wild-type and AHR-null MEFs ($P < .05$, Student's t test).

MEF Type	DNA Distribution of Cell Cycle Phase		
	G_0/G_1	S	G_2/M
Wild-type	53.8 ± 4.0	16.0 ± 4.8	30.1 ± 2.2
AHR-null	40.3 ± 1.2	14.6 ± 4.7	45.2 ± 4.0

in their nuclei, indicating apoptosis. In support of this observation, the increased apoptosis appears to correlate with decreased cell proliferation in AHR-null MEFs. Doubling times then were normalized to apoptotic cell rates (22.8 h for wild-type and 45.5 h for AHR-null). It should be noted that AHR-null cells exhibit a 3-fold slower DNA synthesis rate than wild-type MEFs, although they were approximately 2-fold slower in doubling time. This apparent discrepancy may be due in part to a continued high rate of apoptosis in the AHR-null cells. Combining the normalized doubling times with flow cytometric data of wild-type and AHR-null MEFs allows us to estimate the time that each cell type spends in each phase of cell cycle (Table 2). Although AHR-null MEFs possess significantly longer G_0/G_1 and S phases than wild-type cells, they spend approximately three times as much time as wild-type MEFs in G_2/M phase (20.6 versus 6.9 h, respectively). These data reveal that the increased doubling time observed in AHR-null MEFs is largely due to the prolonged G_2/M phase.

Cdc2 and Plk mRNA Levels Are Down-Regulated in AHR-Null MEFs. Cyclin-dependent kinases play key roles at multiple checkpoints during progression of the cell cycle (Grana and Reddy, 1995). The entry of the cells into mitosis is regulated by the Cdc2/cyclin B kinase complex that is required for the G_2/M phase transition (Murray and Kirschner, 1989). In addition, Cdc2/cyclin B kinase inhibition results in a G_2/M phase arrest (Nurse, 1990). Plk is a member of polo kinase subfamily that appears to play important roles at various stages in G_2/M phase of the cell cycle (DM Glover et al., 1998).

Because Cdc2 and Plk are important regulatory proteins known to play pivotal roles in the G_2/M phase, we examined whether the altered cell cycle observed in AHR-null MEFs reflects changes in Cdc2 and Plk expression level. The steady-state levels of Cdc2 protein are relatively constant through the cell cycle (Draetta and Beach, 1988). In AHR-null MEFs, however, Cdc2 level was drastically reduced at 48 and 72 h, whereas only a slight decrease in expression was observed in wild-type MEFs (Fig. 4A). Similarly, Plk protein levels were reduced in AHR-null MEFs after 48 and 72 h in culture. In contrast, no changes were observed in Erk 1 and 2 expression in either wild-type or AHR-null MEFs.

To examine whether the expression of Cdc2 and Plk are down-regulated at the mRNA level, Northern analysis was

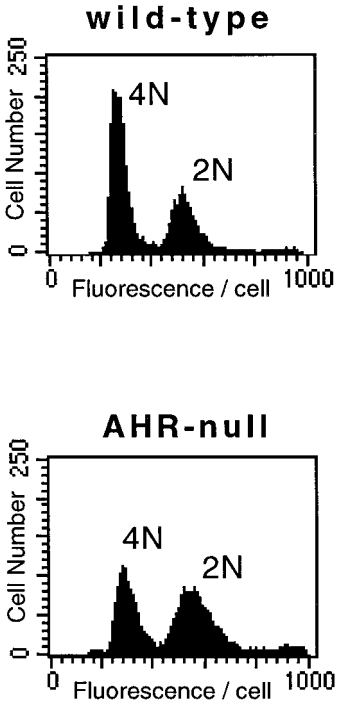

Fig. 2. DNA profile content of wild-type and AHR-null MEFs at 72 h. DNA content is represented on the *x*-axis, and the number of cells counted is given on the *y*-axis. Data shown are representative of three experiments (see Table 1).

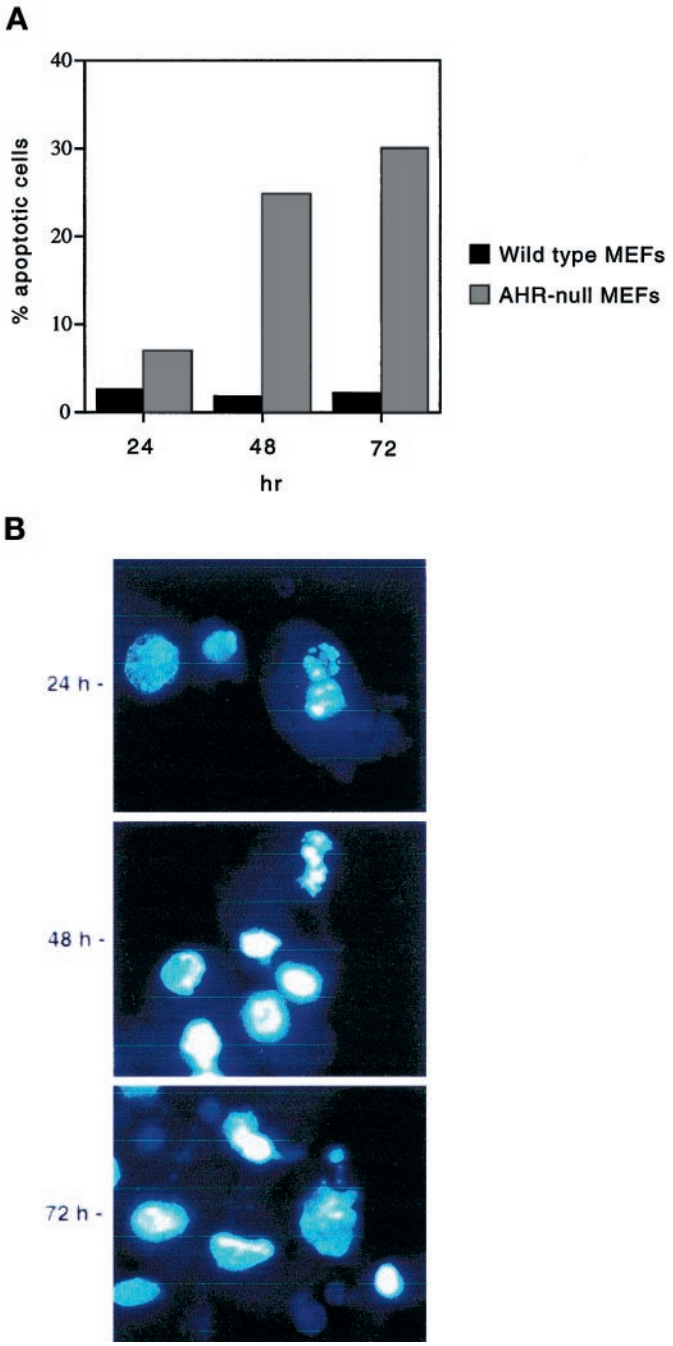

Fig. 3. Morphological analysis for apoptosis in MEFs. Fibroblasts were cultured, and DAPI immunofluorescence was carried out as indicated in *Materials and Methods*. A, percentages of apoptotic cells in wild-type and AHR-null MEFs. B, AHR-null MEFs showing chromatin condensation.

performed. A marked reduction in both Cdc2 and Plk mRNA levels was found in AHR-null MEFs compared with wild-type MEFs (Fig. 4B). Although the possibility of post-transcriptional regulation cannot be excluded, these data suggest that the lower Cdc2 and Plk protein levels in AHR-null MEFs could be the result of down-regulation at the level of transcription.

To examine whether the AHR deficiency affected the Cdc2 and Plk activities, immunocomplex kinase assays were carried out to measure the specific kinase activities. To enrich the mitotic population, MEFs were treated with nocodazole, a microtubule destabilizing agent, and the mitotic cell fractions were used. When equal amounts of Cdc2 and Plk proteins were precipitated, specific activities remained similar between wild-type and AHR-null MEFs (Fig. 5, A and B). These data indicate that the specific activities of Cdc2 and Plk are not affected. However, the absolute amounts of Cdc2 and Plk are lower in AHR-null compared with wild-type MEFs, thus accounting for the total activity of these kinases.

AHR-Null MEFs Present Higher Levels of Active TGF-β. The TGF-β family consists of three polypeptides (TGF-β1, TGF-β2, and TGF-β3) that regulate morphogenesis, differentiation, proliferation, and adhesion (Massague, 1990). In chondrocytes and vascular smooth muscle cells, TGF-β2 and TGF-β3 decrease cell proliferation rates by extending the G_2/M phase (Grainger et al., 1994; Boumediene et al., 1995). It has also been observed that TGF-β inhibits Cdc2 synthesis (Eblen et al., 1994) and can elicit programmed cell death in endometrial cells (Rotello et al., 1991), normal and transformed hepatocytes (Fukuda et al., 1993), and a number of other cell types. Recently, increased TGF-β activity has been reported in AHR-null primary hepatocytes (Zaher et al., 1998). Thus, the observed G_2/M phase cell accumulation, together with the down-regulation of Cdc2 and Plk, could be due to an alteration in TGF-β activity. To examine whether AHR-null MEFs secreted active TGF-β into the medium, TGF-β bioassays were performed (Fig. 6). No inhibitory effect was observed when conditioned medium from wild-type MEF cultures was used (data not shown). In contrast, a 5-fold dilution of conditioned medium from the AHR-null MEFs inhibited CCL64 cell growth by more than 50% (equivalent to 50 pmol of TGF-β). This inhibition was partially blocked by the addition of the anti-TGF-β blocking antibody 1D11 but not by control IgG. In addition, the activation of latent TGF-β through heating of the conditioned medium at 80°C for 8 min results in cell growth inhibition by around 90% (equivalent to 450 pmol of TGF-β), which was also partially blocked by the 1D11 antibody but not by the control IgG (Fig. 6). These data suggest that the inhibition of cell growth observed in AHR-null MEFs is due to an increased level of TGF-β secreted into the medium and that it may contribute to the higher apoptosis rate observed in AHR-null MEFs.

AHR-Null Conditioned Medium Induces Accumulation of Cells in G_2/M. To determine whether AHR-null conditioned medium contains factors that could account for the accumulation of cells in G_2/M phase, conditioned medium collected from AHR-null MEF cultures was tested for its ability to influence the cell cycle. No effects were found when conditioned medium prepared from wild-type MEF was used (Fig. 7, A and B). However, enrichment of G_2/M phase cells was evident when wild-type MEFs were treated with conditioned medium collected from AHR-null MEFs (Fig. 7C). This effect was also partially blocked by the addition of 1D11 antibody (Fig. 7D). This result is in agreement with data showing an accumulation of AHR-null MEFs in G_2/M phase (Fig. 2).

TGF-β3 Treatment Decreases Cdc2 and Plk mRNA Levels with a G_2/M Phase Cell Accumulation. To establish a link between the high TGF-β levels and the G_2/M phase cell accumulation observed in AHR-null MEFs, wild-type cells were treated with TGF-β3, which is abundant in livers of AHR-null mice (Zaher et al., 1998), and Cdc2 and Plk mRNA expression was analyzed by Northern blotting. After treatment, a marked reduction was found in the expression of Cdc2 and Plk mRNAs (Fig. 8C). Treatment with TGF-β3 resulted in an accumulation of cells in G_2/M phase (Fig. 8B) but to a lesser extent than that produced by conditioned medium collected from AHR-null MEFs. These results suggest that in addition to TGF-β, other factors may contribute to the observed enrichment of G_2/M phase cells in AHR-null MEFs.

TABLE 2

Analysis of wild-type and AHR-null MEF cell cycle time

MEFs were harvested at 72 h and stained with propidium iodide, and the percentage of cells in each phase was determined by flow cytometry. The mean and S.D. values were determined from three independent experiments and were multiplied by the normalized doubling times of each cell type (22.8 h for wild-type and 45.5 h for AHR-null) to calculate the length of each cell cycle phase. G_2/M time is approximately three times longer in AHR-null MEFs than in wild-type cells ($P < .05$, Student's t test).

MEF Type	Length of Cell Cycle Phase		
	G_0/G_1	S	G_2/M
		h	
Wild-type	12.3 ± 0.9	3.6 ± 1.1	6.9 ± 0.5
AHR-null	18.3 ± 0.6	6.6 ± 2.1	20.6 ± 1.8

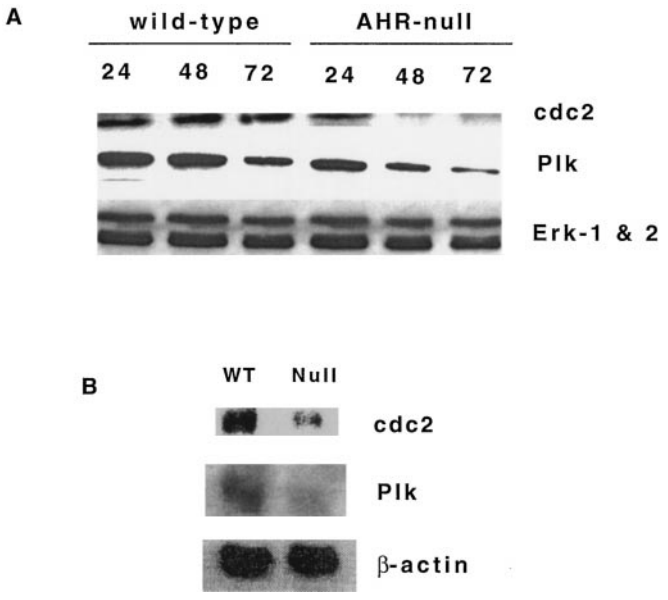

Fig. 4. Expression of Cdc2 and Plk transcripts and their products in wild-type and AHR-null MEFs. A, total cellular extracts were prepared at the indicated time points and subjected to Western blot analysis. The same membrane was used for blotting with mitogen-activated protein kinase Erk 1/2 antibody to provide an internal control for equal loading. B, 20 μg of total RNA from MEFs cultured for 48 h was subjected to Northern blot analysis. An *Sac*I 1-kb fragment within the PLK coding sequence and a Cdc2 cDNA were used as probes. β-Actin was used as a control for equal loading and RNA integrity.

Discussion

Several studies have suggested that the AHR plays an important role in the regulation of cell growth and differentiation (Gaido et al., 1992; Ma and Whitlock, 1996; Ge and Elferink, 1998). As shown here, the AHR indirectly regulates the expression of proteins, such as Cdc2 and Plk, that are involved in cell cycle control. MEFs derived from AHR-null mice present a prolonged doubling time and lower DNA synthesis compared with wild-type MEFs. In both cases, the difference between wild-type and AHR-null MEFs was about 3-fold. The lower rate of DNA synthesis observed in AHR-null MEFs was reversed with reintroduction of AHR by transfection with an AHR cDNA-containing expression vector (unpublished data). When DNA distribution in the cell cycle was analyzed, an accumulation of AHR-null MEFs in G_2/M phase was noted. Others observed similar cell proliferation behavior for Hepa 1c1c7 cells, a transformed hepatoma cell line with low levels (up to 10%) of AHR expression (Ma and Whitlock, 1996). However, these authors found that the AHR-defective Hepa 1c1c7 cells are arrested in G_1. This discrepancy could be due to the following reasons: 1) Hepa 1c1c7 is a transformed hepatoma-derived cell line that has an altered cell cycle, 2) Hepa 1c1c7 cells still express variable amounts of AHR and thus do not represent a true AHR-deficient cell line, and 3) differences in cell types could reflect intracellular differences in the mechanism by which AHR affects cell cycle control.

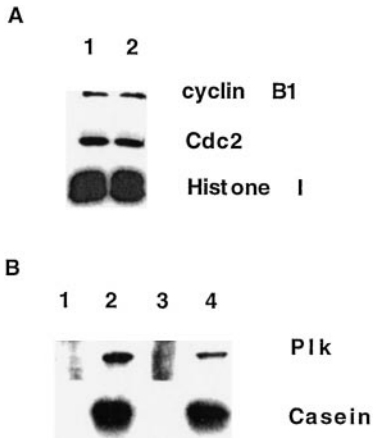

Fig. 5. Protein kinase activity of Cdc2 and Plk. Cells were synchronized by nocodazole, and the mitotic fraction was analyzed by flow cytometry. Mitotic lysates were used to examine the kinase activity of Cdc2 and Plk using histone H1 and casein as substrates, respectively. The same samples were used to quantify the amount of cyclin B, Cdc2, and Plk present in the lysate by Western blot analysis as controls. A, protein kinase activity of Cdc2. Lane 1, wild-type mitotic cells. Lane 2, AHR-null mitotic cells. B, protein kinase activity of Plk. Lane 1, wild-type mitotic cells plus Plk peptide. Lane 2, wild-type mitotic cells. Lane 3, AHR-null mitotic cells plus Plk peptide. Lane 4, AHR-null mitotic cells.

Because AHR-null MEFs are enriched in G_2/M phase of the cell cycle, the expression level and kinase activities of two mitotic kinases, Cdc2 and Plk, were examined. AHR-null MEFs exhibited lower Cdc2 and Plk protein content due to decreased levels of their respective mRNAs compared with wild-type MEFs. These lower protein contents result in decreased total Cdc2 and Plk activity (data not shown). However, no differences were observed for the specific activities of both kinases, suggesting that the activation steps of these enzymes have not been influenced by the absence of AHR. Although no changes were observed for the specific Cdc2 and Plk activities, AHR-null MEF total activities have to be decreased due to the lower expression level. Moreover, up to 3 times more protein from AHR-null mitotic lysates compared with wild-type mitotic lysates was required to immunoprecipitate equal amounts of Cdc2 and Plk proteins. These data suggest that the regulation of Cdc2 and Plk may occur at the transcriptional level. However, TCDD, which is known to activate gene expression through the AHR, failed to induce Cdc2 and Plk mRNA levels in wild-type MEFs

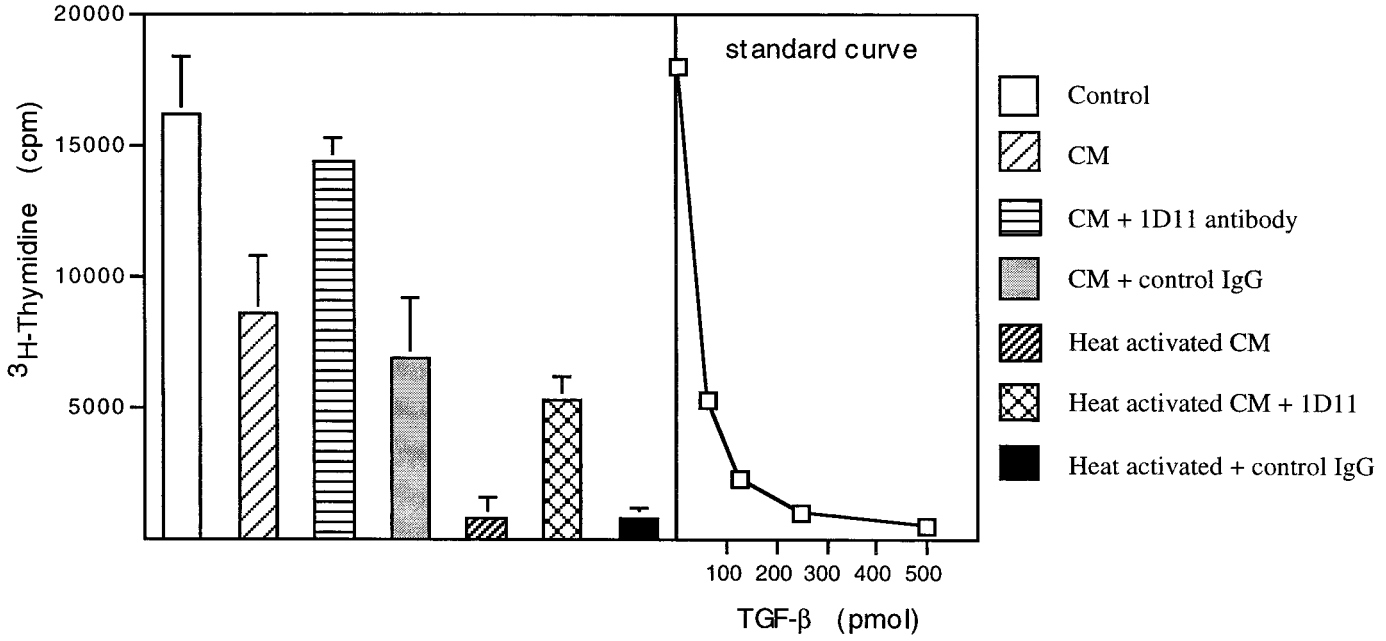

Fig. 6. Increased secretion of TGF-β in medium conditioned (CM) by AHR-null MEFs. MEFs were cultured in OPTI-MEM I reduced serum medium. After 72 h, the medium was collected, and TGF-β was quantified as described in *Materials and Methods*. Data are shown as mean ± S.D. Three samples were analyzed for each condition.

(unpublished data), suggesting that these genes are not regulated by a direct AHR mechanism.

Recently, it was reported that livers from AHR-null mice expressed higher levels of TGF-β1 and TGF-β3 compared with wild-type animals (Zaher et al., 1998). TGF-β has been associated with diminished cell proliferation and elevated apoptosis (Jurgensmeier et al., 1994). MEFs from AHR-null mice secreted higher levels of active and latent TGF-β into conditioned medium as determined by its ability to inhibit proliferation of mink lung epithelial cells. However, hepatic TGF-β expression was not regulated by the AHR because AHR-null mice presented TGF-β1 and TGF-β3 mRNA levels similar to those found in control mice (Zaher et al., 1998). Transglutaminase activates TGF-β by cleavage of a latent form (Glick et al., 1989). The induction of type II transglutaminase activity, resulting from retinoic acid accumulation, was found in the livers of AHR-null mice (Andreola et al., 1997). Therefore, an interaction between retinoic acid and TGF-β signaling pathways could be involved in maintaining higher levels of active TGF-β in AHR-null MEFs. Further investigations will be necessary to understand the mechanisms by which the TGF-β latent form is elevated in AHR-null MEFs.

Similar to the results reported in primary hepatocyte cultures, the elevated levels of TGF-β found in AHR-null MEFs could be responsible for the higher number of apotic cells present in these cells. These results are in agreement with several studies showing that overexpression of TGF-β can result in increased levels of apoptosis (Jurgensmeier et al., 1994). Moreover, because TGF-β can effectively inhibit cell proliferation (Massague, 1990; Nielsen-Hamilton, 1990; Roberts and Sporn, 1990), its higher level in AHR-null MEFs may constitute an important factor in the low proliferation rate observed in these cells.

The treatment of MEFs with TGF-β3 resulted in down-regulation of Cdc2 and Plk mRNA with a 4N DNA cell accumulation. These data are in agreement with previous reports showing a decreased cell proliferation rate by extending the G_2/M phase in chondrocytes and vascular smooth muscle cells after treatment with TGF-β3 (Grainger et al., 1994; Boumediene et al., 1995). Furthermore, G_2/M phase arrest and decreased Cdc2 activity have been associated with apoptosis (Wood and Earnshaw, 1990; Ucker, 1991; Norbury et al., 1994), and microinjection of anti-Plk antibody into cultured mammalian cells leads to a temporary mitotic arrest (Lane and Nigg, 1996).

Wild-type MEFs treated with conditional medium from AHR-null MEFs showed an accumulation of cells in G_2/M similar to that observed in AHR-null MEFs. Nevertheless,

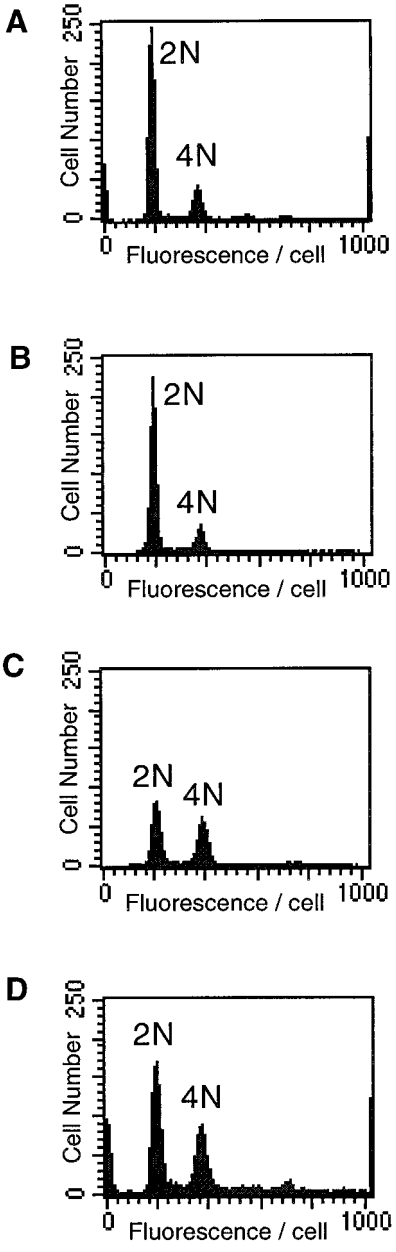

Fig. 7. Effect of conditioned medium (CM) on DNA distribution along the cell cycle. MEFs were cultured in OPTI-MEM-I reduced serum medium supplemented with 2% fetal bovine serum. After 72 h, CM was collected and a 5-fold dilution was prepared. A, wild-type MEFs. B, wild-type MEFs plus wild-type CM. C, wild-type MEFs plus AHR-null CM. D, wild-type MEFs plus AHR-null CM plus 50 μg of 1D11. DNA content is represented on the x-axis, and the number of cell counted is represented on the y-axis.

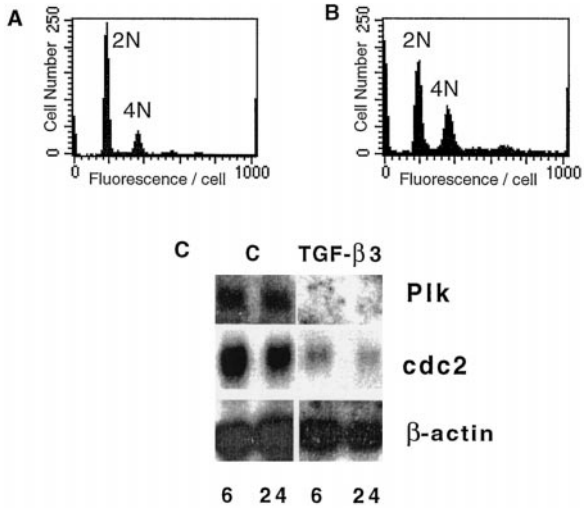

Fig. 8. Effect of TGF-β3 on DNA distribution and Cdc2 and Plk mRNA level. A, DNA profile content of wild-type MEFs at 72 h. B, wild-type MEFs plus TGF-β3 (10 ng/ml). DNA content is represented on the x-axis, and the number of cells counted is represented on the y-axis. C, total RNA (20 μg) isolated from wild-type MEFs treated with TGF-β3 (10 ng/ml, 6 and 24 h) was subjected to Northern blot analysis using an *Sac*I 1-kb fragment within the PLK coding sequence and Cdc2 cDNA as a probes. β-Actin was determined as a control for equal loading and RNA integrity.

this effect was only partially blocked by 1D11 antibody, indicating that in the 4N DNA cell accumulation process, factors others than elevated TGF-β activity may contribute to the observed altered cell cycle control in AHR-null MEFs.

In conclusion, AHR-null MEFs presented increased levels of latent and active TGF-β that appear to be responsible for down-regulation of Cdc2 and Plk mRNAs. The production of TGF-β appears to be the primary factor leading to a delay in AHR-null MEFs in G_2/M and lower proliferation rates with increased apoptosis. These data suggest that AHR plays a role in the cell cycle control through a mechanism involving TGF-β. The role of the AHR in maintaining appropriate levels of TGF-β in MEF and primary hepatocyte cultures awaits further investigation.

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References

Andreola F, Fernandez-Salguero PM, Chiantore MV, Petkovich MP, Gonzalez FJ and De Luca LM (1997) Aryl hydrocarbon receptor knockout mice (AHR−/−) exhibit liver retinoid accumulation and reduced retinoic acid metabolism. *Cancer Res* **57:**2835–2838.

Basi G and Draetta G (1995) The cdc2 kinase:structure, activation and its role at mitosis in vertebrate cells, in *Cell Cycle Control* (Hutchison C and Glover DM eds) pp 106–143, Oxford University Press, New York.

Boumediene K, Vivien D, Macro M, Bogdanowicz P, Lebrun E and Pujol JP (1995) Modulation of rabbit articular chondrocyte (RAC) proliferation by TGF-beta isoforms. *Cell Prolif* **28:**221–234.

Chen YH and Tukey RH (1996) Protein kinase C modulates regulation of the CYP1A1 gene by the aryl hydrocarbon receptor. *J Biol Chem* **271:**26261–26266.

Draetta G and Beach D (1988) Activation of cdc2 protein kinase during mitosis in human cells: Cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* **54:**17–28.

Eblen ST, Fautsch MP, Burnette RJ, Joshi P and Leof EB (1994) Cell cycle-dependent inhibition of p34cdc2 synthesis by transforming growth factor beta 1 in cycling epithelial cells. *Cell Growth Differ* **5:**109–116.

Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM and Gonzalez FJ (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah (see comments). *Science (Wash DC)* **268:**722–726.

Fernandez-Salguero PM, Hilbert DM, Rudikoff S, Ward JM and Gonzalez FJ (1996) Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8- tetrachlorodibenzeno-p-dioxin-induced toxicity. *Toxicol Appl Pharmacol* **140:**173–179.

Fukuda K, Kojiro M and Chiu JF (1993) Induction of apoptosis by transforming growth factor-beta 1 in the rat hepatoma cell line McA-RH7777: A possible association with tissue transglutaminase expression. *Hepatology* **18:**945–953.

Gaido KW, Maness SC, Leonard LS and Greenlee WF (1992) 2,3,7,8-Tetrachlorodibenzo-p-dioxin-dependent regulation of transforming growth factors-alpha and -beta 2 expression in a human keratinocyte cell line involves both transcriptional and post-transcriptional control. *J Biol Chem* **267:**24591–24595.

Ge NL and Elferink CJ (1998) A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein: Linking dioxin signaling to the cell cycle. *J Biol Chem* **273:**22708–22713.

Glick AB, Flanders KC, Danielpour D, Yuspa SH and Sporn MB (1989) Retinoic acid induces transforming growth factor beta-2 in cultured keratinocytes and mouse epidermis. *Cell Regul* **1:**87–97.

Glover DM, Hagan IM and Tavares AA (1998) Polo-like kinases: A team that plays throughout mitosis. *Genes Dev* **12:**3777–3787.

Gonzalez FJ and Fernandez-Salguero P (1998) The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metab Dispos* **26:**1194–1198.

Grainger DJ, Kemp PR, Witchell CM, Weissberg PL and Metcalfe JC (1994) Transforming growth factor beta decreases the rate of proliferation of rat vascular smooth muscle cells by extending the G_2 phase of the cell cycle and delays the rise in cyclic AMP before entry into M phase. *Biochem J* **299:**227–235.

Grana X and Reddy EP (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* **11:**211–219.

Hoffman EC, Reyes H, Chu FF, Sander F, Conley LH, Brooks BA and Hankinson O (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science (Wash DC)* **252:**954–958.

Huff J, Lucier G and Tritscher A (1994) Carcinogenicity of TCDD: Experimental, mechanistic, and epidemiologic evidence. *Annu Rev Pharmacol Toxicol* **34:**343–372.

Jurgensmeier JM, Schmitt CP, Viesel E, Hofler P and Bauer G (1994) Transforming growth factor beta-treated normal fibroblasts eliminate transformed fibroblasts by induction of apoptosis. *Cancer Res* **54:**393–398.

Kremer J, Gleichmann E and Esser C (1994) Thymic stroma exposed to arylhydrocarbon receptor-binding xenobiotics fails to support proliferation of early thymocytes but induces differentiation. *J Immunol* **153:**2778–2786.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227:**680–685.

Lai ZW, Hundeiker C, Gleichmann E and Esser C (1997) Cytokine gene expression during ontogeny in murine thymus on activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Mol Pharmacol* **52:**30–37.

Lane HA and Nigg EA (1996) Antibody microinjection reveals an essential role for human polo-kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J Cell Biol* **135:**1701–1713.

Lanni JS and Jacks T (1998) Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol Cell Biol* **18:**1055–1064.

Lee KS, Yuan YL, Kuriyama R and Erikson RL (1995) Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. *Mol Cell Biol* **15:**7143–7151.

Ma Q and Whitlock JP Jr (1996) The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. *Mol Cell Biol* **16:**2144–2150.

Massague J (1990) The transforming growth factor-beta family. *Annu Rev Cell Biol* **6:**597–641.

Murray AW and Kirschner MW (1989) Dominoes and clocks: The union of two views of the cell cycle. *Science (Wash DC)* **246:**614–621.

Nielsen-Hamilton M (1990) Transforming growth factor β and its action on cellular growth and differentiation. *Curr Top Dev Biol* **24:**95–136.

Norbury C, MacFarlane M, Fearnhead H and Cohen GM (1994) Cdc2 activation is not required for thymocyte apoptosis. *Biochem Biophys Res Commun* **202:**1400–1406.

Nurse P (1990) Universal control mechanism regulating onset of M-phase. *Nature (Lond)* **344:**503–508.

Puga A, Nebert DW and Carrier F (1992) Dioxin induces expression of c-fos and c-jun proto-oncogenes and a large increase in transcription factor AP-1. *DNA Cell Biol* **11:**269–281.

Roberts A and Sporn M (1990) Peptide growth factors and their receptors, in *The Transforming Growth Factors βs* (Sporn M and Roberts A eds) pp 419–472, Springer-Verlag, Berlin.

Rotello RJ, Lieberman RC, Purchio AF and Gerschenson LE (1991) Coordinated regulation of apoptosis and cell proliferation by transforming growth factor beta 1 in cultured uterine epithelial cells. *Proc Natl Acad Sci U S A* **88:**3412–3415.

Rowlands JG and Gustafsson J (1997) Aryl hydrocarbon receptor-mediated signal transduction. *Crit Rev Toxicol* **27:**109–134.

Safe S, Mason G, Sawyer T, Zacharewski T, Harris M, Yao C, Keys B, Farrell K, Holcomb M, Davis D and et al (1989) Development and validation of in vitro induction assays for toxic halogenated aromatic mixtures: A review. *Toxicol Ind Health* **5:**757–775.

Staples JE, Fiore NC, Frazier DE Jr, Gasiewicz TA and Silverstone AE (1998) Overexpression of the anti-apoptotic oncogene, bcl-2, in the thymus does not prevent thymic atrophy induced by estradiol or 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Appl Pharmacol* **151:**200–210.

Ucker DS (1991) Death by suicide: One way to go in mammalian cellular development? *New Biol* **3:**103–109.

Wood ER and Earnshaw WC (1990) Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J Cell Biol* **111:**2839–2850.

Zaher H, Fernandez-Salguero PM, Letterio J, Sheikh MS, Fornace AJ Jr, Roberts AB and Gonzalez FJ (1998) The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis. *Mol Pharmacol* **54:**313–321.

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