Dioxin, a potent tumor promoter, activates the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor belonging to the basic helix-loop-helix-PAS family, to enhance tumorigenesis via unknown mechanisms. We undertook this study to determine the mechanisms underlying the impact of dioxin on cell fate, in particular senescence that occurs in normal human cells and is considered to play important tumor suppressive function. We have previously shown that in primary human keratinocytes, dioxin attenuates senescence while retaining the proliferative capacity and represses expression of the tumor suppressors, p16\(^{INK4a}\) and p53. Here, we show that repression of p16\(^{INK4a}\) and p53 transcriptional activity by dioxin absolutely requires the AHR and is accompanied by promoter methylation. Furthermore, dioxin alone is sufficient to immortalize normal human keratinocytes. Our data introduce a previously unrecognized regulatory pathway, that of the AHR, that impacts senescence. More importantly, this is the first report of a tumor promoter capable of inhibiting senescence in a receptor mediated manner and introduces a novel mechanism by which this carcinogen may contribute to human malignancies.

Dioxin or TCDD\(^1\) (2,3,7,8-tetrachlorodibenzo-p-dioxin) is an ubiquitous environmental contaminant and a highly potent tumor promoter. It has been classified as a "class I" human carcinogen by the EPA (1, 2), albeit amid considerable controversy that is primarily due to the unknown mechanisms that underlie its carcinogenic actions and a lack of sufficient epidemiological data (3). Most effects of TCDD are mediated by the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor belonging to the bHLH-PAS family. Human exposures to AHR agonists with varying potencies occur frequently as a result of combustion processes such as those most recently associated with September 11, 2001 (5).

Upon agonist activation, the AHR translocates into the nucleus and dimerizes with ARNT (HIF\(\delta\)) to modify gene regulation by binding to dioxin response elements (DREs) (6). In this manner, the AHR transcriptionally up-regulates a battery of genes involved in xenobiotic metabolism and consequently aids in the elimination of these xenobiotics by decreasing their biological half-lives. Currently, the endogenous role of the AHR is controversial. On one hand, it may be a component of an adaptive response that through evolutionary pressures has evolved to aid in the elimination of xenobiotics. On the other hand, it may play an important physiological role that is independent of its actions in xenobiotic metabolism. Support for the latter idea is found in the accumulating evidence that the AHR is a regulator of the cell cycle. For example, absence of the AHR alters cell cycle progression by allowing the cells to accumulate in the G\(\_\)S/M phase (7). In addition to the role that it plays as a DNA binding partner with ARNT, the AHR has been shown to impinge on the functions of other transcription factors, such as the retinoblastoma protein (Rb) and NF-kB, through mechanisms that presumably involve direct protein-protein interactions (8).

Cellular senescence is characterized by a state of irreversible growth arrest in response to many stimuli. Senescence was initially associated with the phenomenon of finite proliferative capacity mediated by telomere attrition/dysfunction (replicative senescence). However, it is now clearly recognized that a number of extrinsic/intrinsic stimuli (stress-induced or premature senescence) can initiate the senescent phenotype (9–11) and often involves the activation of both the p53 and the p16\(^{INK4a}/\)Rb pathway, which results in the irreversible, growth-arrested state. These stimuli/stressors (i.e. telomere dysfunction, DNA damage, activated oncogenes) have in common the potential to initiate or promote neoplastic transformation, and this has led to the hypotheses that the senescent phenotype represents an inherent tumor suppressor mechanism akin to that of apoptosis. Accordingly, for a precancerous cell to achieve immortalization, a necessary first step in its journey to malignant transformation, it must be able to bypass the senescence barrier. The idea that inactivation of the senescent pathway is an important first stage in the development of a cancerous cell is also supported by the observations that many human cancers are characterized by a lack of functional cyclin-dependent kinase inhibitor, p16\(^{INK4a}\) and p53 expression (12, 13), the two major regulators of the senescent program (14–17).

Previously, we have reported that in primary human epidermal keratinocytes (HEK), TCDD repressed both the expression of the tumor suppressor proteins, p16\(^{INK4a}\) and p53, and as well as the onset of senescence (18). From studies performed over the past decade, it is clear that the bHLH-PAS proteins play...
imported roles in the detection and adaptation to environmental changes (6). While members of the bHLH-PAS family have not previously been shown to be associated with the regulation of senescence, Id-1, a member of its sister family, the bHLH proteins, has been identified to play an important role in this cellular process (19). The results of the current study put forth a role of the AHR in regulating the senescence program in normal human cells. We propose that inappropriate alteration of the senescence program by the AHR agonist, TCDD, is an important mechanism by which TCDD exerts its tumorigenic effects in human cells. To support this idea, we report herein that TCDD immortalizes primary keratinocytes through an AHR-mediated bypass of cellular senescence that involves hypermethylation and transcriptional silencing of p16INK4a and p53.

**EXPERIMENTAL PROCEDURES**

**Materials**

TCDD and MNF (3'-methoxy-4'-nitroflavone) were gifts from Dr. Stephen H. Safe (Texas A & M University, College Station, TX) and Dr. Thomas A. Gasewicz (University of Rochester, Rochester, NY). Unless otherwise mentioned, all other chemicals were purchased from Sigma or Fischer Scientific. The 2.4-kbp fragment upstream of the p53 gene promoter (p53P1) inserted into the p220-CAT vector was obtained from Dr. David Reisman (University of Wisconsin, Madison, WI).

**Cell Culture**

Experiments were performed with neonatal normal HEKs (catalog number C-001-5C, Cascade Biologics) (18). The cells were grown in Epilife medium (Cascade Biologics, Epilife® Defined Growth Supplement, 0.06 mM Ca²⁺).

**Culturing Protocol and Treatments—**At 90% confluence, the medium was changed to Dulbecco’s modified Eagle’s medium supplemented with 1.5 mM Ca²⁺, which was changed to Dulbecco’s medium supplemented with 10% fetal bovine serum and 100 units/ml Pen-Strep (Invitrogen) and containing 1.5 mM Ca²⁺. At the same time, the cells were treated with either 0.01% Me₆SO (control) or 1 nm TCDD in presence or absence of 1 μM MNF, 1 μM 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma) or 100 μM geldanamycin (Sigma) for the indicated time points. The medium was replenished every other day with fresh addition of the chemicals, and passage number for the HEKs was limited to four.

**Population Doubling Curves—**Primary HEKs were grown in Epilife medium as described above in the continuous presence of either 0.01% Me₆SO or 1 nm TCDD in presence or absence of 1 μM MNF. The cells were passaged when ~70% confluent and were cultured until they ceased to grow. Cells were counted at each passage and the population doubling (PD) determined. PD = log₂ (number of cells at each subculture/number of cells initially plated). No corrections were made for the cells that failed to reinitiate growth at subculture. Cumulative PD was plotted against total time in culture to determine replicative life span. Experiments were done using cells from two different donors and viability of the plated cells was determined using trypan blue staining. One representative graph is shown.

**Western Blot Analysis**

The HEKs were harvested, whole cell lysates were prepared in F-buffer, and Western blot analysis was performed as described previously under “Experimental Procedures” (18). Primary antibodies used were as follows: AHR (MA1-514, ABR Affinity Bioreagents Inc. and 27188

**Table I**

| Gene   | Primer sequence               | Annealing temperature | Cycles |
|--------|-------------------------------|-----------------------|--------|
| AHR    | 5'-GGA TCA CAT GGA GAC CCT CG-3' | 55                    | 35     |
| CYP1A1 | 5'-CTT CAA CCT CAG TTG GCT TTG-3' | 55                    | 35     |
| 16S    | 5'-TCC AAG GGT CGG CTG CAG-3'  | 55                    | 35     |
| p53    | 5'-TCC AAG GGT CGG CTG CAG-3'  | 55                    | 35     |
| (hnRNA)| 5'-TCC AAG GGT CGG CTG CAG-3'  | 55                    | 35     |

**Table II**

| Target gene | siRNA sequence |
|-------------|----------------|
| AHR (siAHR) | 5'-AAU ACU UCC ACC ACC GUU GCC GGC-3' |
| Scrambled (siSCR) | 5'-AAC GUU GCG CUC AUC AAC UCU-3' |

**RNase Protection Assay**

The HEKs were harvested at the indicated time points and total cellular RNA was extracted using TRIzol Reagent (Invitrogen). The RNase protection assays were performed using the human cell cycle hCC2 multiprobe template set (Pharmingen) as described previously under “Experimental Procedures” (18).

**RT-PCR and Real Time RT-PCR**

The HEKs were harvested at the indicated time points, and total cellular RNA was extracted as described before. cDNA was synthesized according to the supplier’s protocol from 5 μg of RNA using random primers and SuperScript II RTase (Invitrogen). According to the supplier’s protocol, 2 μl of cDNA was used for the PCR reactions using primers (Integrated DNA Technologies) described in Table I for 35 cycles. The PCR products were applied to 10% polyacrylamide gels, and the bands were visualized by staining with SYBR green (Molecular Probes). For real time RT-PCR, cDNA was synthesized as described, and the PCR reactions were carried out according to supplier’s instruction using Assay-On-Demand for CYP1A1 and TaqMan® PCR Reagent for β-actin expression (Applied Biosystems). The PCR conditions were: 1) hold at 50 °C for 2 min, 2) hold at 95 °C for 10 min, and 3) denature at 95 °C for 15 s and anneal/extend at 60 °C for 1 min for 40 cycles. The results for CYP1A1 expression were normalized with respect to β-actin.

**RT-PCR for Heterogeneous Nuclear RNA (hnRNA) for p53 and p16INK4a**

The HEKs were harvested at the indicated time points, and total cellular RNA was extracted as described before. Contaminating DNA was removed by subjecting the samples to RQ1 RNase free DNase (Promega) digestion. cDNA was synthesized as described above, and 2 μl of cDNA was used for the PCR reactions for 40 cycles using [α-32P]dATP and [α-32P]dCTP (PerkinElmer Life Sciences). The primers (Integrated DNA Technologies) were specifically designed to amplify regions covering parts of both introns and exons (Table I). PCR products were applied to 10% polyacrylamide gels, and gels were dried for an hour at 80 °C. Exposed PhosphorImager screens were scanned and quantitated using Image Quant 5.0 (Amersham Biosciences).

**siRNA Transfection and Luciferase Reporter Assay**

siRNA was designed using siDESIGN (Dharmacon) and is depicted in Table II. The luciferase reporter regulated by the DRE were prepared in the following manner: (1) transiently transfecting HEK cells with DRE-regulated luciferase plasmid DNA (1 μg) and the TransIT-TKO protocol (Mirus) for siRNA (25 nm). After an overnight incubation, the cells were treated with either 0.01% Me₆SO or 1 nm TCDD for an additional 24 h. The luciferase activities were measured using the
Luciferase Reporter Assay System (Promega). For RNA analyses, the HEK cells (70% confluent) were transfected with 25 nM siRNA using TransIT-TKO and treated as described above. Following the incubation, RNA was isolated using TRIzol and was analyzed either by RNase protection assay or RT-PCR.

Methylation-specific PCR for p16\(^{INK4a}\)

After the indicated days of treatment with either Me\(_2\)SO or TCDD (as described above), the HEK cells were harvested, and genomic DNA was isolated using a DNA extraction kit (Chemicon). 1 \(\mu\)g of DNA was subjected to bisulfite modification using CpGenome\(^{TM}\) DNA Modification Kit (Chemicon). The modified DNA (100 ng) was analyzed using CpG\(^{TM}\) WIZ p16 Amplification Kit (Chemicon). The amplified products were run on 10% polyacrylamide gel and visualized using SYBR green staining.

**Gel Shift Assay**

Primary HEK cells were cultured as described above. Upon reaching 90% confluence, the cells were treated with 0.01% Me\(_2\)SO or 1 nM TCDD in the presence or absence of 1 \(\mu\)M MNF or 100 \(\mu\)M geldanamycin (GA) for 1 h. Nuclei were isolated using the NucBuster\(^{TM}\) Protein Extraction Kit (Novagen). The annealed oligonucleotides HIS 17 and HIS 18 (20) were used as the radiolabeled probe for the electrophoretic mobility shift assays. The annealed oligonucleotides were radiolabeled with \(\gamma\)-\(^{32}\)P[ATP by end labeling with T4 polynucleotide kinase (20). 9 \(\mu\)g of the nuclear extracts were incubated with non-specific competitor (salmon sperm DNA, 2 \(\mu\)g), and the KCI concentration was adjusted to 100 mM. After incubating the mixture for 10 min at room temperature, the probe was added (100,000 cpm, ~0.5 ng), and the mixture was incubated for an additional 10 min at room temperature. When indicated, 2 \(\mu\)g of either AntiAHR (Abcam) or AntiARNT (Santa Cruz Biotechnology, Inc.) were added, and the samples were incubated an additional 10 min. The samples were subjected to 4% acrylamide non-denaturing gel electrophoresis using 0.5 \(\times\) TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0) as the running buffer.

**Statistical Analysis**

The data generated from different experiments were analyzed using GraphPad Prism software using either one-way or two-way analysis of variance.

**RESULTS AND DISCUSSION**

**TCDD-induced Decrease in the Expression of p16\(^{INK4a}\) and p53 Requires the AHR**—In our previous study, we reported that in normal human keratinocytes, TCDD attenuated the senescence program while inhibiting the expression levels of the key regulators of senescence p16\(^{INK4a}\) and p53 (18). To elucidate the mechanism by which TCDD exerts its effects on these tumor suppressors, we first determined whether TCDD down-regulates the expression levels of p16\(^{INK4a}\) and p53 during senescence in a manner that requires the AHR. Toward this end, we treated preconfluent HEK cells with the AHR agonist TCDD, the AHR antagonist MNF, a flavonoid ligand that competes with TCDD for AHR binding (21), or vehicle control Me\(_2\)SO and harvested them at various time points after confluence had been reached. This experimental paradigm has been shown to induce the characteristic phenotype of “stress-induced premature senescence,” i.e. high expression levels of p16\(^{INK4a}\) and p53 and positive staining for senescence-associated \(\beta\)-galactosidase (18, 22).

To determine that MNF appropriately antagonizes the actions of TCDD in this cultured system, we monitored its ability to block the induction of TCDD of cytochrome P450 1A1 (CYP1A1), the best characterized target gene of the TCDD/AHR signaling pathway (6). As expected, increasing concentrations of TCDD induced a corresponding rise in the CYP1A1 mRNA in a manner that was antagonized by MNF (Fig. 1a). Given these results, we used the optimal concentrations of MNF and TCDD (1 \(\mu\)M and 1 nM, respectively) to ascertain whether MNF would similarly antagonize the ability of TCDD to repress the expression levels of p16\(^{INK4a}\) and p53. As shown, MNF blocks the ability of TCDD to repress the mRNA (see supplemental Fig. 1) and protein levels of both p16\(^{INK4a}\) and p53 (Fig. 1b). Additionally, as determined by senescence-associated \(\beta\)-galactosidase staining (18), MNF also blocked the ability of TCDD to inhibit senescence in HEKs (data not shown).

We validated our pharmacological approach by using siRNA targeted to block the expression of the AHR. The efficacy of the siAHR was first determined by using a luciferase reporter that is regulated by two copies of the DNA recognition site of the AHR/ARNT heterodimer (24). As shown (Fig. 2, a and b), the presence of siAHR completely blocked the TCDD-induced luciferase activity, significantly inhibited the mRNA levels of the AHR, and compromised the ability of TCDD to induce the CYP1A1 mRNA. Experiments using siRNA were restricted to a 24-h time point, since longer incubations yielded a loss in the effectiveness of the siAHR. As expected, in the presence of siSCR, treatment with TCDD represses the mRNA levels of p16\(^{INK4a}\) and p53; however, treatment with siAHR prevents the TCDD-induced decrease of these tumor suppressors (Fig. 2c).

Thus, using both pharmacological and molecular approaches, we have demonstrated that TCDD represses the expression of p16\(^{INK4a}\) and p53 in an AHR-dependent manner. To probe more fully the relationship between the AHR signaling pathway and the senescence program in primary HEKs, we examined the impact of TCDD and MNF on the protein expression of the AHR. As shown in Fig. 3a, the protein levels of the AHR increase in a time-dependent manner. In addition, TCDD decreases the protein levels of the AHR presumably via agonist-induced degradation (25), and MNF blocks the impact of TCDD on AHR levels. Further characterization revealed that MNF blocks the ability of TCDD to induce formation of the AHR/ARNT DNA binding complex (Fig. 3b, lanes 2 and 8). The specificity of the AHR/ARNT complex was determined by per-
treated as described for mRNA levels of AHR and CYP1A1 induction by TCDD. HEK cells were harvested, and assayed for luciferase activity. b) In the presence or absence of 100 μM GA, nuclear extracts were prepared, and aliquots (9 μg) were subjected to gel shift analysis. The annealed oligonucleotides that were used as the radiolabeled probe contained the DRE (GCCGTG). Specificity of the AHR/ARNT complex was determined using antibodies to the AHR and ARNT (lanes 3–6). Relative band intensities (within brackets) were calculated with respect to vehicle control Me 2SO in lane 1. c) GA inhibits the ability of TCDD to induce DNA binding of the AHR/ARNT heterodimer. Primary HEKs were treated for 1 h with either 0.01% Me 2SO or 1 nM TCDD in the presence or absence of 1 μM GA. Nuclear extracts were prepared, and aliquots were subjected to gel shift analysis using the same protocol as above. d) GA inhibits the TCDD-induced down-regulation of p53. Primary HEKs were cultured as described in the legend to Fig. 1a and were treated with either 0.01% Me 2SO or 1 nM TCDD in the presence or absence of 100 μM GA for 24 h. The cells were then harvested, and p53 levels were analyzed by Western blotting. Similar experiments performed to analyze p16\(^{INK4a}\) levels were unsuccessful due to low expression of p16\(^{INK4a}\) protein at this time point. Here, D = Me 2SO-treated and T = TCDD-treated cells.

Fig. 3. Impact of MNF and GA on AHR expression levels and DNA binding of the AHR/ARNT heterodimer. a) MNF blocks the ligand-induced degradation of AHR. Primary HEK cells, treated as described in the legend to Fig. 1a, were harvested at the indicated time points, and the protein expression of AHR was determined using Western blot analysis. b) MNF inhibits the ability of TCDD to induce DNA binding of the AHR/ARNT heterodimer. Primary HEKs were treated for 1 h with either 0.01% Me 2SO or 1 nM TCDD in the presence or absence of 1 μM MNF. Nuclear extracts were prepared, and aliquots (9 μg) were subjected to gel shift analysis. The annealed oligonucleotides that were used as the radiolabeled probe contained the DRE (GCCGTG). Specificity of the AHR/ARNT complex was determined using antibodies to the AHR and ARNT (lanes 3–6). Relative band intensities (within brackets) were calculated with respect to vehicle control Me 2SO in lane 1. c) GA inhibits the ability of TCDD to induce DNA binding of the AHR/ARNT heterodimer. Primary HEKs were treated for 1 h as described above in the presence or absence of 100 μM GA. Nuclear extracts were prepared, and aliquots were subjected to gel shift analysis using the same protocol as above. d) GA inhibits the TCDD-induced down-regulation of p53. Primary HEKs were cultured as described in the legend to Fig. 1a and were treated with either 0.01% Me 2SO or 1 nM TCDD in the presence or absence of 100 μM GA for 24 h. The cells were then harvested, and p53 levels were analyzed by Western blotting. Similar experiments performed to analyze p16\(^{INK4a}\) levels were unsuccessful due to low expression of p16\(^{INK4a}\) protein at this time point. Here, D = Me 2SO-treated and T = TCDD-treated cells.

Fig. 2. Use of siRNA designed to specifically down-regulate AHR (siAHR) validates the absolute requirement of AHR for the TCDD-induced down-regulation of p53 and p16\(^{INK4a}\). a) siAHR inhibits the ability of TCDD to induce the DRE-regulated luciferase activity. At 70% confluence, HEK cells were cotransfected with either 25 nM siAHR (siRNA for AHR) or siSCR (scrambled siRNA) and a luciferase reporter gene regulated by two copies of the DRE using TransIT-TKO and TransIT-Keratinocyte reagents. After an overnight incubation, the cells were treated with 0.01% Me 2SO (IT-TKO and TransIT-Keratinocyte reagents. After an overnight incubation, the cells were treated with 0.01% Me 2SO (DMSO) or 1 nM TCDD in medium containing 1.5 mM Ca\(^{2+}\), incubated for an additional 24 h, harvested, and assayed for luciferase activity. b) siAHR decreases the mRNA levels of AHR and CYP1A1 induction by TCDD. HEK cells were treated as described for a except that only the siAHR or siSCR was transfected. mRNA levels of AHR and CYP1A1 were analyzed using RT-PCR and 16S as a loading control (*, p < 0.05; **, p < 0.01, comparing AHR mRNA expression between siSCR and siAHR in the Me 2SO (DMSO)- and TCDD-treated groups respectively; #, p < 0.05, comparing CYP1A1 mRNA expression between siSCR and siAHR in the TCDD-treated group). c) siAHR suppresses the ability of TCDD to repress p53 and p16\(^{INK4a}\) expression. The RNA samples obtained from b were analyzed for p53 and p16\(^{INK4a}\) mRNA levels using RNase protection assay. Here, D = Me 2SO-treated and T = TCDD-treated cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Forming super shifts using antibodies that recognize AHR and ARNT (lanes 4 and 6). Thus, in agreement with previous studies performed in mouse hepatoma cells (21), MNF blocks the actions of TCDD in human keratinocytes by inhibiting formation of the AHR/ARNT DNA binding complex presumably by inhibiting nuclear translocation of the AHR. Further evidence supporting the idea that the AHR is required to mediate the effect of TCDD in these primary human keratinocytes is provided by experiments performed with GA. GA is a benzoqui...
that the transcriptional regulation of p16 INK4a and p53 are of both tumor suppressor genes. From studies performed in other laboratories, it is evident that the transcriptional regulation of p16INK4a and p53 are distinct. In many cases, the regulation of p16INK4a expression is mediated either via homozygous deletion or by epigenetic alterations; however, in the case of p53 transcriptional regulation is primarily mediated by the two regulatory promoter regions, P1 and P2 (31). To address the possibility TCDD may repress transcription of p53 via direct repression of its promoter, we performed transient transfections using a time course similar to that described in the legend to Fig. 1 and a reporter plasmid that is regulated by a 2.4-kbp fragment of the human p53 promoter. Within this promoter fragment is the P1 promoter, which is thought to allow the cells to escape senescence and that is blocked by TCDD. Additional studies performed using actinomycin D indicate that TCDD does not alter the mRNA stability or degradation of the p16INK4a and p53 mRNAs (30) and support our conclusion here that TCDD represses the transcription of both tumor suppressor genes.

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cells continued to divide for at least 50 PD (>80 PD total) beyond their normal limit/threshold. Immortalization has previously been defined as an event that extends the life span of the primary cells by a minimum of 50 PD (36). As expected, MNF alone did not significantly alter the life span of these cells but blocked the TCDD induced bypass of senescence, again indicating a role of the AHR in these TCDD-induced events.

We then determined whether immortalization of the normal human keratinocytes cultured in these conditions involved a down-regulation of p16\textsuperscript{INK4a} and p53 similar to that observed during our previously described protocol (i.e. during senescence induced by high calcium and high cell density, Figs. 1–5). As shown in Fig. 6\textit{b}, treatment with TCDD using this experimental paradigm decreased the expression levels of both p16\textsuperscript{INK4a} and p53. However, comparison of the mRNA expression patterns revealed that the impact of TCDD on these two gene products is distinct. With respect to p16\textsuperscript{INK4a}, TCDD induced a progressive decrease in its expression levels during entire time course. In contrast, the TCDD-induced expression patterns of p53 were similar to that of the Rb proteins (i.e. Rb, p107, and p130) and p27\textsuperscript{KIP1}. Here, the expression levels were indistinguishable from that of the Me\textsubscript{S}O control until passage 7 (P7) but plummeted at some time point after the loss in viability of Me\textsubscript{S}O-treated cells (i.e. between P7 and P16). As shown in Fig. 6\textit{b}, p14\textsuperscript{ARF}, which regulates the p53 protein levels via regulation of the MDM2 pathway, was also found to be down-regulated by TCDD under the current experimental paradigm. Our preliminary examinations have thus far failed to detect a TCDD-induced increase in hTERT expression.

To determine whether the expression of AHR or its transcriptional activity was altered during TCDD-induced immortalization of the primary human keratinocytes, we performed RT-PCR to measure the mRNA levels of AHR and its target gene, CYP1A1. As shown in Fig. 6\textit{c}, although the AHR mRNA expression was not significantly altered during this process, there was a steady and time-dependent increase in the CYP1A1 mRNA levels. These results indicate that the transcriptional activity of the AHR may be necessary for the bypass of senescence observed in these normal HEKs.

CONCLUSIONS

To summarize, we report that normal human keratinocytes exposed to TCDD alone become immortalized in an AHR-dependent manner, presumably through the suppression of the key initiators of senescence, p16\textsuperscript{INK4a} and p53. Additionally, we have shown that this suppression is accompanied by promoter methylation. Although from the slope of the graph (36) in Fig. 6\textit{c} it can be predicted that treatment with TCDD alone is sufficient for immortalization, the requirement for additional mutations cannot be ruled out. While senescence has been shown to be induced by a number of chemical agents, i.e. those involved in chemotherapies, to our knowledge, this is the first report of a known tumor promoter inhibiting this important cancer preventive pathway.

A central question that arises from the work presented in the current study is whether the AHR pathway is a critical player in the senescence program or whether it only impinges on its regulation. Evidence that supports the former idea is the observation that as the keratinocytes undergo senescence, the protein levels of the AHR increase by ~17-fold (Fig. 3 and Ref. 18) while remaining essentially unchanged in the TCDD-treated cells that bypass senescence and undergo immortalization (Fig. 6\textit{c}). Given that in similar experiments performed in an immortalized human keratinocyte cell line, the expression of the AHR is not altered during differentiation, it is our view that the increase in AHR expression levels observed in the current study is a consequence of senescence rather than dif-

![Fig. 6. TCDD extends the life span of primary human keratinocytes in culture.](http://www.jbc.org/)

**Fig. 6.** TCDD extends the life span of primary human keratinocytes in culture. \textit{a,} impact of TCDD and MNF on the replicative life span of proliferating normal human keratinocytes. Primary human keratinocytes were cultured in low Ca\textsuperscript{2+} medium (0.06 mM, Epilife medium) with either 0.01% Me\textsubscript{S}O (DMSO) or 1 mM TCDD in the presence or absence of 1 mM MNF. The cells were passaged when ~70% confluent until they ceased to grow, and cumulative population doublings were plotted against total time in culture. \textit{b,} serial culturing of proliferating HEK cells with TCDD represses the mRNA expression of p53, p16\textsuperscript{INK4a}, p14\textsuperscript{ARF}, and Rb. The HEKs were cultured as described for \textit{a}. At the indicated passage numbers, the cells were harvested, and the mRNA expression was analyzed by RNase protection assay using the hCC2 probe (Pharmingen). The expression patterns of the Rb family members (p107 and p130) and p27\textsuperscript{KIP1} were similar to that of p53 (*, p < 0.01, comparing TCDD (P16) to all other treatment groups). \textit{c,} impact of TCDD-treatment during immortalization of normal human keratinocytes on AHR and CYP1A1 mRNA. The RNA samples obtained from \textit{b} were analyzed for AHR and CYP1A1 mRNA levels using RT-PCR and 16S as loading control.
ferentiation. However, if the canonical AHR signaling pathway is critical for the maintenance of the senescence program, then it would have been expected that inhibiting transcriptional activity of the AHR with the use of the antagonist alone would have had an impact on the ability of the cells to undergo senescence. The lack of an MNF-mediated effect (Fig. 6a) in addition to the observed increase in CYPIA1 levels (Fig. 6c) during immortalization suggests either that it is only activation of the AHR pathway by TCDD that alters regulation of the senescence program or that the AHR plays an endogenous role in senescence in a manner that cannot be altered by MNF (e.g., a ligand-independent role). This issue will be addressed by our future experiments.

These results not only offer a plausible mechanism by which TCDD may exert its tumor promoting effects but also implicate the importance of epigenetic misregulation in mediating the effects of a nongenotoxic compound. It not only puts forth a role of the AHR in replicative senescence, which is supported by the observations that mice deficient in the AHR exhibit hyerplasia in the skin and gastrointestinal tract epithelia (37), but also elucidates mechanism underlying the events involved in the hyperproliferative responses that occur during chloracne, a hallmark of TCDD exposure in humans (38). Thus, the AHR may be an important player in determining epithelial cell fate via its ability to regulate p16INK4a, p53, and senescence and may in fact serve as a link in understanding the existing enigma between carcinogenesis and aging (23).

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