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# Repression of Ah Receptor and Induction of Transforming Growth Factor-β Genes in DEN-Induced Mouse Liver Tumors

**Li Peng**[1], **Christopher N. Mayhew**[2], **Michael Schnekenburger**[1], **Erik S. Knudsen**[2], and **Alvaro Puga**[1*]

1*Center for Environmental Genetics, Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, Ohio, 45267-0056*

2*Department of Cell and Cancer Biology, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, Ohio, 45267-0056*

## Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the biologic and toxic effects of its xenobiotic ligands. In recent years it has become evident that in the absence of ligand the AHR promotes cell cycle progression and that its activation by high-affinity ligands results in interactions with the retinoblastoma protein (RB) that lead to perturbation of the cell cycle, $G_0/G_1$ arrest, diminished capacity for DNA replication and inhibition of cell proliferation. Hence, the AHR has diametrically opposed pro-proliferative and anti-proliferative functions that have yet to be reconciled at the molecular level. Work from our own and from other laboratories suggests that the AHR may function as a tumor suppressor gene that becomes silenced in the process of tumor formation. To develop preliminary support for a more thorough examination of this hypothesis we characterized the expression levels of various tumor suppressor genes, transforming growth factor-β (*Tgfb*) genes and the *Ahr* gene in liver tumor samples from mice with a liver-specific RB ablation and their wild-type littermates. In tumors arising in RB-positive livers, *Cdkn2d* and *Tgfb1* were repressed and *Cdkn2c*, *Tgfb2*, *Tgfb3* and *Pai1* were induced, whereas in RB-negative tumors, only *Cdkn2c* and *Tgfb3* were induced. *Ahr* was significantly repressed in tumors from both sets of mice, supporting the concept that *Ahr* silencing may be associated with cancer progression.

### Keywords

Ah receptor; tumor suppressor genes; TGFβ; hepatocellular carcinoma

## INTRODUCTION

The AHR is a ligand-activated member of the bHLH/PAS family of transcription factors that plays an important role in controlling a variety of developmental and physiological events, including induction of drug metabolizing enzymes, xenobiotic detoxification, neurogenesis, tracheal and salivary duct formation, circadian rhythms, response to hypoxia, and hormone receptor function (Schmidt and Bradfield, 1996; Crews and Fan, 1999; Pocar et al., 2005). So far, more than 400 environmental toxicants and naturally occurring compounds have been

*Corresponding author: Alvaro Puga, Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056. Cincinnati, OH 45267-00567, (Messenger Mail, use: 123 E. Shields St. Cincinnati, OH 45220), Phone: (513) 558-0916; FAX: (513) 558-0925, E-mail: Alvaro.Puga@UC.EDU.

reported to be AHR ligands (Denison et al., 2002). The cytosolic unliganded AHR is one component of a multi-protein complex containing the immunophilin-like protein XAP2/AIP/ARA9, the 23-kD co-chaperone protein p23 and two molecules of a 80-kD Hsp90 protein (Ma and Whitlock, Jr., 1997; Petrulis et al., 2003). Upon ligand binding, the complex translocates to the nucleus where AHR dissociates from the multi-protein complex and dimerizes with ARNT, a second bHLH/PAS protein, to form a heterodimeric transcription factor. The AHR/ARNT heterodimer binds to DNA recognition sequences, termed AHREs/XREs/DREs, found in the enhancer region of genes coding for many phase I drug metabolizing enzymes, such as the cytochromes P450 CYP1A1, CYP1A2, CYP1B1, and several phase II conjugating enzymes, such as ALDH3A1 and NQO1. Binding leads to chromatin and nucleosome disruption, recruitment of the basal transcriptional machinery and other transcriptional regulators, and initiation of downstream transcription (Hankinson, 1995; Whitlock, Jr., 1999; Schnekenburger, Peng and Puga, 2007; Schnekenburger, Talaska and Puga, 2007). Following nuclear export, the AHR is degraded via the 26S proteasome pathway (Pollenz, 2002).

The AHR has also been recognized as a cell cycle regulator (Ma and Whitlock, 1996; Weiss et al., 1996; Ge and Elferink, 1998; Kolluri et al., 1999; Puga et al., 2000; Strobeck et al., 2000; Marlowe et al., 2004) although the precise molecular mechanisms responsible for this role have not been fully elucidated. In the absence of an exogenous ligand, or after deletion of the ligand-binding PAS-B domain, the AHR has been shown to promote cell cycle progression (Ma and Whitlock, 1996; Elizondo et al., 2000; Chang et al., 2007), whereas exposure to its prototypical ligand, TCDD, inhibits cell cycle proliferation in an AHR-dependent manner (Bauman et al., 1995;Hushka and Greenlee, 1995;Levine-Fridman, Chen, and Elferink, 2004; Marlowe et al., 2004). Mechanistically, at least two separate signaling pathways contribute to the AHR role in cell cycle regulation. First, AHR can repress expression of TGF-β1 (Elizondo et al., 2000) by accelerating TGF-β1 mRNA degradation (Chang et al., 2007) and blocking TGF-β1-dependent inhibition of cell proliferation and promotion of apoptosis. Conversely, overproduction of TGF-β in fibroblasts from AHR knockout mice causes low proliferation rates and increased apoptosis (Elizondo et al., 2000; Chang et al., 2007). Consistent with these observations, livers from AHR knockout mice showed increased levels of TGF-β1 and TGF-β3 proteins and elevated numbers of hepatocytes undergoing apoptosis compared to wild-type mice (Zaher et al., 1998). Second, protein interactions between AHR and RB further repress the RB-dependent repression of the transcription factor E2F and prevent entry of the cells into S-phase (Ge and Elferink, 1998; Puga et al., 2000; Strobeck et al., 2000; Marlowe et al., 2004). Hence, under certain circumstances the AHR could be considered as a pro-proliferative gene with the properties of an oncogene, and under others as a tumor suppressor gene. Consistent with this view, AHR has been found to be silenced by promoter hypermethylation in a significant number of human acute lymphoblastic leukemia cases (Mulero-Navarro et al. 2006).

Epidemiologic studies have shown that the retinoblastoma tumor suppressor protein RB is functionally inactivated in most human neoplasms (Liu et al., 2004) with loss of heterozygosity being frequently observed in hepatocellular carcinomas (Ashida et al., 1997; Kondoh et al., 2001). In good agreement with the epidemiologic observations, mice with a liver-specific ablation of the *Rb* gene showed aberrant ploidy and increased multiplicity of liver tumors when exposed to DEN (Mayhew et al. 2005; Mayhew et al. 2007; Srinivasan et al. 2007). To explore the role of AHR/RB/TGF-β interactions in liver tumorigenesis, we have examined the expression status of *Tgfb* genes, tumor suppressor genes, *Ahr* and several of its downstream targets in DEN-induced liver tumors and normal livers of mice with a liver-specific ablation of the *Rb* gene and in their wild-type littermates. The central question in these experiments was to determine whether AHR expression would be repressed, favoring the hypothesis that it functioned as a tumor suppressor gene, or would be over-expressed or unaffected, favoring the hypothesis that it functioned as an oncogene. We find distinct patterns of gene expression that

for the most part show a significant increase of expression of *Tgfb* genes and repression of *Ahr* in RB-positive and RB-negative tumors.

## MATERIALS AND METHODS

### Mice

Liver-specific *Rb* conditional knockouts and their normal littermates were derived from $Rb^{fl/fl}$ mice, harboring *Rb* alleles in which exon 19 is flanked by loxP sites, crossed with *Alb-Cre* mice to obtain mice homozygous for the floxed *Rb* locus and hemizygous for *Alb-Cre*. These mice were then interbred with $Rb^{fl/fl}$ mice to produce $Rb^{fl/fl}$ (wild type) and $Rb^{fl/fl}$ *Alb-*$Cre^{+/0}$ (RB-knockout) littermates at a 1:1 ratio (Mayhew et al., 2005). Mice were housed in a pathogen-free animal facility under standard 12-hour light/12-hour dark cycle with *ad libitum* water and chow. All of the experiments were conducted using the highest standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

### Liver samples

Fifteen-day old $Rb^{fl/fl}$ and $Rb^{fl/fl}$ *Alb-*$Cre^{+/0}$ littermates were administered a single i.p. injection of the genotoxic hepatocarcinogen DEN (Sigma) dissolved in saline at a dose of 20 mg/kg body weight; littermates used as controls were inoculated with an equal volume of saline. This protocol resulted in the four groups of mouse livers analyzed in this work: *RB-positive, normal*, are livers from $Rb^{fl/fl}$ mice inoculated with saline; *RB-positive, tumors* are livers from $Rb^{fl/fl}$ mice inoculated with DEN; *RB-negative, normal* are livers from $Rb^{fl/fl}$*Alb-*$Cre^{+/0}$ mice inoculated with saline; and *RB-negative, tumors* are livers from $Rb^{fl/fl}$*Alb-*$Cre^{+/0}$ mice inoculated with DEN. Mice were euthanized 9 months post-DEN exposure. Immediately after euthanasia, livers were excised, weighed and photographed to facilitate scoring of surface liver lesions. Visible surface tumors were excised from both $Rb^{fl/fl}$ (normal) and $Rb^{fl/fl}$*Alb-*$Cre^{+/0}$ animals, immediately frozen in liquid $N_2$ and stored at −80 °C until use. Genotypes were determined by standard PCR of tail DNA and deletion or retention of the *Rb* gene was confirmed by genotyping the mouse livers themselves. Characterization of these tumors has been reported elsewhere (Mayhew et al. 2005; Mayhew et al. 2007; Srinivasan et al. 2007).

### cDNA sample preparation

RNA was isolated from frozen liver tumor and normal tissues using Triazol. cDNA was synthesized by reverse transcription of 1 μg of total RNA in a total volume of 20 μl containing 1X reverse transcriptase buffer (Invitrogen), 25 μg/ml oligo-$dT_{12–18}$ primer (Invitrogen), 0.5 mM dNTP mix (GeneChoice), 10 mM dithiothreitol (Invitrogen), 5 mM $MgCl_2$, 20 U of RNase inhibitor (RNasin, Promega), and 100 U of SuperScript™ II RNase $H^-$ reverse transcriptase (Invitrogen). Samples were denatured and annealed to the primer for 10 min at 70 °C, and reverse-transcribed for 1 hour at 42 °C. Before amplification, the reverse transcriptase was inactivated by heating to 70 °C for 15 minutes and RNA was hydrolyzed by incubation with 2 U RNase H (Invitrogen) at 37 °C for 20 minutes. The resulting cDNA products were diluted in a final volume of 200 μl and a 2 μl aliquot was used as template for subsequent quantification by real-time PCR amplification.

### Real-time PCR

Primers for cDNA amplification are shown in Table 1. Amplification of β-actin cDNA in the same samples was used as an internal control for all real-time PCR amplification reactions. PCR product specificity was confirmed using melting curve analyses and subsequent polyacrylamide gel electrophoresis. PCR reactions were conducted in duplicate in a total

volume of 25 μl containing SYBR Green PCR Master Mix (Applied Biosystems), and 0.1 μM of each primer. Amplification was performed on an ABI 7500 (Applied Biosystems) where the reaction was heated to 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing-elongation at 60 °C for 60 seconds. Detection of the fluorescent product was carried out during the elongation period, and emission data were quantified using threshold cycle ($C_t$) values. $C_t$ values were determined in duplicate, averaged and normalized to values for β-actin amplification of the same sample ($\Delta C_t$).

### CpG island analysis in the mouse Ahr promoter

A 1,500-nucleotide segment of the mouse *Ahr* promoter, from coordinates −1295 to +205 and containing the transcription start site, was analyzed with the Methprimer software program (http://www.urogene.org/methprimer) for the presence of CpG islands. A 705-nucleotide segment was identified as a CpG island using an observed/expected CpG ratio of 0.6, a minimum length island of 200 nucleotides and a minimum G+C content of 50%. A 395-nucleotide fragment was used for further analysis.

### DNA methylation analysis

The methylation status of the mouse *Ahr* promoter was determined by PCR amplification of sodium bisulfite-treated genomic DNA followed by cloning and DNA sequencing, as described (Schnekenburger, Peng, and Puga, 2007). Genomic DNA was extracted using standard procedures and 750 ng of each DNA sample underwent sodium bisulfite modification using a bisulfite modification kit (Active Motif MethylDetector™) following the manufacturer's recommendations. Specific primers for PCR amplification were designed using the same MethPrimer software program. Several primer sets were designed such that the target sequences did not contain any CpG dinucleotide, thus allowing for amplification of both unmethylated and methylated DNA. Primers were tested for their ability to yield high-quality sequencing reactions. PCR products were quality controlled by agarose gel electrophoresis. Inclusion of restriction enzyme sites in the PCR primers allowed for cloning of the amplification products in the pGEM4Z vector (Promega). For each DNA sample, a minimum of 10 clones were analyzed by sequencing.

### Statistical analyses

Statistical analyses of RT-PCR data was performed using SigmaStat 2.03. Group comparisons were made using a two-tailed Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

Comparison of mRNA levels of RB-positive tumors (n = 4) with RB-positive normal livers (n = 3) showed significant changes in the expression of a number of genes. mRNA levels of *Ahr*, *Cdkn2d* (p19) and *Tgfb1* in tumor tissue were decreased to 30%, 50% and 40%, respectively, of their levels in normal liver. On the other hand, mRNA levels of *Cdkn1a* (p21), *Cdkn2c* (p18), *Pai1* (Serpine-1), *Tgfb2* and *Tgfb3* were elevated in RB-positive tumors by 4.9-, 4.3-, 3.2-, 10.5- and 11.3-fold, respectively, relative to their levels in normal liver (Table 2). In contrast, RB-negative tumors (n = 8) and RB-negative normal livers (n = 3) showed fewer differences, with *Ahr* mRNA being the only one showing significantly reduced levels (60%) in tumors and *Cdkn2c* (p18) and *Tgfb2* mRNAs increased by 6.5- and 8.6-fold, respectively, relative to their levels in RB-negative normal liver tissues. Expression of several other genes, including *Cdkn2d, Cyp1a1, Pai1* and *Tgfb3*, was also increased in RB-negative tumors, but the changes did not reach statistical significance (Table 2). Expression of *Trp53* (p53) was unchanged in tumors relative to normal tissues and *Dnmt1*and *Hdac1*, which were assayed for

their potential role in repression of AHR-regulated genes (Schnekenburger, Peng and Puga, 2007), also showed no significant changes between tumors and normal livers (Table 2).

Changes in DNA methylation have been directly linked to genomic instability and play an important role in tumorigenesis. In tumors and in cancer cell lines, aberrant methylation usually occurs at CpG islands, unmethylated in normal somatic cells and hypermethylated in tumor suppressor gene promoters, representing a major mechanism of gene inactivation in primary human tumors (Fraga et al., 2004; Esteller, 2007). A CpG island in the promoter of the human *AHR* gene has recently been found to be methylated in a panel of 19 tumor cell lines and in lymphocytes of one-third of acute lymphoblastic leukemia patients (Mulero-Navarro et al., 2006). This observation suggested the possibility that promoter hypermethylation might be a potential mechanism responsible for the down-regulation of *AHR* expression in our liver tumor samples. Accordingly, we used DNA sequencing of bisulfite-modified cloned PCR products to test this hypothesis. We sequenced a minimum of 10 clones from each tumor DNA sample but found no evidence of methylation (data not shown) in the corresponding CpG island of the mouse *Ahr* promoter (Fig. 1), indicating that hypermethylation was not the main cause of the decrease in *Ahr* expression in the mouse tumors analyzed.

Because of their role as tumor suppressors, the members of the two families of CDK inhibitors, the INK4 (p16, p15, p18 and p19) and the CIP/KIP (p21, p27 and p57) are often found in a silenced state in tumors of many different origins (Damo, Snyder, and Franklin 2005; Bai et al. 2007); however, recent evidence in pancreatic and lung tumors shows up-regulation, rather than repression of p18 (*Cdkn2c*) and argues against a pure tumor suppressor role for this gene (Joshi et al., 2007; Lindberg, Akerstrom, and Westin, 2007; Pei et al., 2007), whereas p19/ARF (*Cdkn2d*) is almost exclusively repressed in tumors (Lowe and Sherr, 2003; Canepa et al., 2007). Our results are consistent with those findings, showing increased expression of *Cdkn2c* in both RB-positive and RB-negative tumors, and decreased expression of *Cdkn2d* in RB-positive but not in RB-negative tumors. Possibly, in the absence of RB, repression of one of the main inhibitors of its phosphorylation is less essential for tumor suppression.

The TGF-β proteins are growth modulators involved in cell proliferation, apoptosis, differentiation, adhesion and migration with their growth inhibitory effects resulting from their ability to arrest cells in the $G_1$ phase of the cell cycle (Massague, Blain, and Lo, 2000; Siegel and Massague, 2003; Massague and Gomis, 2006). Reduced expression of TGF-β proteins or loss of their inhibitory effects have been linked to cell hyperproliferation and tumor progression (Massague, Blain, and Lo, 2000; Siegel and Massague, 2003; Massague and Gomis, 2006). In fact, TGF-β appears to have a dual role in tumorigenesis, acting as a tumor suppressor in the earlier tumor phases, and as a tumor promoter in the later phases (Derynck, Akhurst and Balmain, 2001; Ten Dijke et al., 2002). At that time, TGF-β is produced in high amounts in the tumor and it stimulates tumorigenesis by allowing tumor cells to escape immune surveillance (Hazelbag et al., 2002) and promoting angiogenesis (Zhang et al., 2006; Ten Dijke et al., 2002). Consistent with this dual role of TGF-β, we find a significant decrease of *Tgfb1* expression in the tumors and very large increases of *Tgfb2* and *Tgfb3* mRNA levels, with a concomitant increase in *Pai1* expression, in agreement with the regulatory role of *Tgfb* in *Pai1* transcription (Kutz et al., 2006). The *Pai1* gene encodes the plasminogen activator inhibitor serpine-1, whose expression increases filopodia formation and migration and is highly elevated in many invasive tumors, including breast, brain and gastric cancers (Rao et al., 1993; Chazaud et al., 2002; Lei et al., 2007).

*Ahr* expression was significantly down-regulated in both RB-positive and RB-negative liver tumors, although not by promoter hypermethylation. These findings are consistent with the hypothesis that the AHR may have tumor suppressor gene properties in these tumors and is in good agreement with recent observations that AHR functions as a tumor suppressor in prostate

carcinogenesis in mice (Fritz et al., 2007). A hypothesis to explain mechanistically these results is that in the absence of RB, cells with lower levels of AHR will have a greater probability of survival because they will escape RB/AHR repression of S-phase entry. On the other hand, in both RB-positive and RB-negative cells, the lower AHR levels will promote and increase in TGFβ, as we have recently shown (Chang et al. 2007), allowing tumor cells to escape immune surveillance and promoting angiogenesis. An important limitation of these studies is that they offer a snapshot of a dynamic process that began 9 months before, hence, the data may represent events happening at relatively late stage liver tumors, providing no clues about potential events that may have taken place early in the process and contributed to tumor promotion, as for example, reversal of *Tgfb* expression consistent with its dual role in tumorigenesis. Future work in this area will of necessity include analyses of earlier time points.

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## ABBREVIATIONS

ALDH3A1, aldehyde dehydrogenase-3A1
AHR, aryl hydrocarbon receptor
AHRE, aryl hydrocarbon receptor response element
AIP, AHR-interacting protein
ALL, acute lymphoblastic leukemia

ARA9, Ah receptor associated protein 9
ARNT, aryl hydrocarbon receptor nuclear translocator
bHLH, basic-region helix-loop-helix
CDK, cyclin-dependent kinase
DEN, diethylnitrosamine
DRE, dioxin response element
HCC, hepatocellular carcinoma
Hsp90, heat shock protein 90
MEM-α, minimal essential medium-α
NQO1, NAD(P)H-dependent quinone oxidoreductase
PAI-1, plasminogen activator inhibitor type-1
PAS, Period-Aryl hydrocarbon nuclear translocator-Simple-minded
RB, retinoblastoma
TCDD, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin
TGFβ, transforming growth factor-beta
XAP2, hepatitis virus X-associated protein-2
XRE, xenobiotic response element

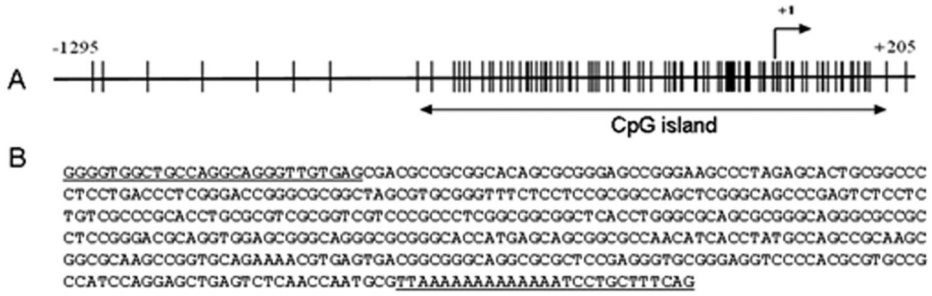


**Fig. 1.**

**(A)**. A 705-nucleotide segment of the mouse *Ahr* promoter from −556 to + 148 was identified as a CpG island using the Methprimer software program. **(B)**. A 395-nucleotide DNA fragment within this CpG island, from coordinates −245 to +148 and containing 56 CpG dinucleotides was selected for methylation analysis after bisulfite modification followed by PCR amplification with specific oligonucleotide primers (underlined), cloning and sequencing of a minimum of 10 clones from each liver DNA sample.

**Table 1**

Primer sequences fo cDNA amplification of selected genes

| Gene | Primer sequences |
|---|---|
| β-*Actin* | Forward: 5′- CATCCGTAAAGACCTCTATGCC -3′<br>Reverse: 5′- ACGCAGCTCAGTAACAGTCC -3′ |
| *Ahr* | Forward: 5′- GGCCAAGAGCTTCTTTGATG - 3′<br>Reverse: 5′- TGCCAGTCTCTGATTTGTGC - 3′ |
| *Cdkn1a (p21)* | Forward: 5′- GAACAGGGATGGCAGTTAGG - 3′<br>Reverse: 5′- AGTATGGGGTGGGGGAAAAG - 3′ |
| *Cdkn1b (p27)* | Forward: 5′- GGGTTCCAGCTTGTTGTGTT - 3′<br>Reverse: 5′- GGCCATTTTCCATCTCTGAA - 3′ |
| *Cdkn2b (p15)* | Forward: 5′- CCTTCCAAAACTTGAACCCTAC - 3′<br>Reverse: 5′- TCCCTTGCTATTTTACACCAC - 3′ |
| *Cdkn2c (p18)* | Forward: 5′- TGCGCTGCAGGTTATGAAACTTGG - 3′<br>Reverse: 5′- AACATCAGCCTGGAACTCCAGCAA - 3′ |
| *Cdkn2d (p19)* | Forward: 5′- GCCTTGCAGGTCATGATGTTTGGA - 3′<br>Reverse: 5′- ATTCAGGAGCTAGGAAGCTGACCA - 3′ |
| *Cyp1a1* | Forward: 5′- GTGTCTGGTTACTTTGACAAGTGG-3′<br>Reverse: 5′- ACATGGACATGCAAGGACA -3′ |
| *Dnmt1* | Forward: 5′- CTGACCGCTTCTACTTCCTC -3′<br>Reverse: 5′- TCCCTTTCCCCTTCCCTTTC -3′ |
| *Hdac1* | Forward: 5′- TTCCAACATGACCAACCAGA -3′<br>Reverse: 5′- GGCAGCATCCTCAAGTTCTC -3′ |
| *Nqo1* | Forward: 5′- ACCCCACTCTATTTTGCTCC -3′<br>Reverse: 5′- ACTTACTCCTTTTCCCATCCTC -3′ |
| *Pai1* | Forward: 5′- GTCTTTCCGACCAAGAGCAG -3′<br>Reverse: 5′- GACAAAGGCTGTGGAGGAAG -3′ |
| *Tgfb1* | Forward: 5′- CAACGCCATCTATGAGAAAACC-3′<br>Reverse: 5′- AAGCCCTGTATTCCGTCTCC-3′ |
| *Tgfb2* | Forward: 5′- CTCAACACACCAAAGTCCTC-3′<br>Reverse: 5′- ATCAAAACTCCCTCCCTCC-3′ |
| *Tgfb3* | Forward: 5′- CAGCCTACATAGGTGGCAAGAAT-3′<br>Reverse: 5′- ACCCAAGTTGGACTCTCTCCTCAA -3′ |
| *Trp53* | Forward: 5′- TGGAAGACTCCAGTGGGAAC - 3′<br>Reverse: 5′- TCTTCTGTACGGCGGTCTCT - 3′ |

**Table 2**

**Fold change of the expression of selected genes in tumor *vs* normal liver tissues of mice**

$\Delta C_T$ values were calculate using mRNA levels of β-actin as the normalization standard. The values shown are the mean ± S.D. of RB positive normal (n = 3) and tumor (n =4) liver samples and RB-negative normal (n = 3) and tumor (n = 8) liver samples. Fold-change was calculated by raising 2 to the power of $\Delta C_T^{normal}$ minus $\Delta C_T^{tumor}$; a value <1 indicates less mRNA in tumors than in normal tissues, whereas a value >1 indicates higher mRNA levels in tumors than in normal tissues. *P*-values were calculated using Student's *t* test and are considered significant if ≤ 0.05.

| | RB Positive Tumors | | | | RB Negative Tumors | | | |
|---|---|---|---|---|---|---|---|---|
| **Gene** | **$\Delta C_T$ Normal** | **$\Delta C_T$ Tumor** | **Fold Change** | ***p* value** | **$\Delta C_T$ Normal** | **$\Delta C_T$ Tumor** | **Fold Change** | ***p* value** |
| ***Ahr*** | **8.2 ± 0.1** | **10.0 ± 0.6** | **0.3 ± 0.2** | **0.004** | **8.8 ± 0.2** | **9.5 ± 0.2** | **0.6 ± 0.1** | **<0.001** |
| ***Cdkn1a (p21)*** | **12.5 ± 0.8** | **10.2 ± 1.4** | **4.9 ± 0.7** | **0.01** | 8.8 ± 3.1 | 8.3 ± 0.5 | 1.4 ± 1.0 | N.S. |
| *Cdkn1b (p27)* | 9.0 ± 0.3 | 9.7 ± 0.7 | 0.6 ± 0.3 | N.S. | 10.2 ± 1.0 | 9.4 ± 0.2 | 1.7 ± 0.3 | N.S. |
| *Cdkn2b (p15)* | 19.7 ± 3.6 | 19.7 ± 3.2 | 1.0 ± 2.0 | N.S. | 18.8 ± 2.0 | 19.1 ± 1.4 | 0.8 ± 0.8 | N.S. |
| ***Cdkn2c (p18)*** | **11.6 ± 0.2** | **9.5 ± 1.0** | **4.3 ± 0.4** | **0.02** | **12.2 ± 1.5** | **9.5 ± 0.6** | **6.5 ± 0.5** | **0.001** |
| ***Cdkn2d (p19)*** | **10.3 ± 0.3** | **11.4 ± 0.4** | **0.5 ± 0.2** | **0.01** | 12.4 ± 1.8 | 11.1 ± 0.4 | 2.5 ± 0.6 | N.S. |
| *Cyp1a1* | 14.2 ± 0.8 | 20.1 ± 4.7 | 0.0 ± 2.0 | N.S. | 20.3 ± 1.4 | 19.0 ± 2.3 | 2.5 ± 0.9 | N.S. |
| *Dnmt1* | 11.8 ± 0.2 | 12.2 ± 0.7 | 0.8 ± 0.3 | N.S. | 11.2 ± 0.5 | 11.0 ± 0.4 | 1.1 ± 0.2 | N.S. |
| *Hdac1* | 8.0 ± 0.4 | 8.4 ± 0.3 | 0.8 ± 0.2 | N.S. | 8.6 ± 0.4 | 8.2 ± 0.3 | 1.3 ± 0.2 | N.S. |
| *Nqo1* | 8.8 ± 0.7 | 9.4 ± 2.1 | 0.7 ± 0.9 | N.S. | 10.4 ± 1.4 | 10.6 ± 1.6 | 0.9 ± 0.7 | N.S. |
| ***Pai1*** | **12.6 ± 0.4** | **10.9 ± 1.0** | **3.2 ± 0.4** | **<0.001** | 12.1 ± 2.2 | 10.6 ± 1.4 | 2.8 ± 0.8 | N.S. |
| ***Tgfb1*** | **8.2 ± 0.1** | **9.4 ± 0.2** | **0.4 ± 0.1** | **<0.001** | 8.8 ± 0.5 | 8.4 ± 0.3 | 1.3 ± 0.2 | N.S. |
| ***Tgfb2*** | **14.4 ± 1.4** | **11.0 ± 0.5** | **10.6 ± 0.6** | **0.006** | **14.5 ± 1.1** | **11.4 ± 0.8** | **8.6 ± 0.4** | **<0.001** |
| ***Tgfb3*** | **18.6 ± 1.2** | **15.1 ± 1.0** | **11.3 ± 0.6** | **0.008** | 16.3 ± 1.3 | 15.3 ± 1.2 | 2.0 ± 0.6 | N.S. |
| *Trp53* | 6.7 ± 0.2 | 6.5 ± 0.6 | 1.1 ± 0.3 | N.S. | 6.3 ± 0.6 | 6.1 ± 0.3 | 1.1 ± 0.2 | N.S. |