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Transforming Growth Factor-β_1 Coregulates mRNA Expression of Aryl Hydrocarbon Receptor and Cell-Cycle-Regulating Genes in Human Cancer Cell Lines

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Transforming growth factor (TGF)-β_1 down-regulates mRNA expression of the aryl hydrocarbon receptor (AhR) and of AhR-inducible genes in A549 cells. Here, we describe a dose-dependent inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced cytochrome P450 (CYP) 1A1, CYP1B1 and NADPH-quinone-oxidoreductase (NMO-1) mRNA expression as well as TCDD-induced 7-ethoxyresorufin-O-deethylase (EROD) activity in MDA-MB 231 cells. The AhR mRNA expression was not affected by TGF-β_1. TGF-β-responsiveness was investigated by examining the effect on the expression of responsive genes. While TGF-β_1 up-regulates mRNA expression of TGF-β_1 and TIEG (TGF-β-inducible early gene) as well as luciferase activity of a responsive reporter plasmid in both cell lines, a down-regulation of c-myc and cyclin A mRNA expression was only found in A549 cells. Furthermore, TGF-β_1 inhibits only cell proliferation of A549 but not of MDA-MB 231 cells. The results show a coregulation of mRNA expression of AhR and cell-cycle regulating genes, and further indicate that the AhR may be involved in regulation of cell proliferation.

Transforming growth factor (TGF)-β_1 is a multifunctional cytokine that belongs to a superfamily of paracrine-acting peptides known to elicit a variety of biological activities, including effects on cell proliferation, cell adhesion, cell migration, immunomodulation, and regulation of extracellular matrix composition. The effects of TGF-β_1 on gene expression are bipartite, and both up- and down-regulation of TGF-β_1-sensitive genes has been observed (1, 2). Signalling of TGF-β_1 is mediated by two transmembrane serine-threonine kinase receptors which act in common to propagate both negative and positive regulatory signals (3, 4, 5). Although an interaction of TGF-β receptors with the immunophilin FKBP12, the farnesyltransferase α subunit and a member of the MAPKKK family has been described (5, 6, 7, 8), the precise mechanisms of downstream signalling by TGF-β_1 are only poorly understood.

Recently, we reported that TGF-β_1 inhibits 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced cytochrome P450 (CYP) 1A1, CYP1B1 and NADPH:quinone oxidoreductase (NMO)-1 mRNA expression as well as CYP1-associated 7-ethoxyresorufin-O-deethylase (EROD) enzyme activity in human lung cancer A549 cells. Beside mRNA of TCDD-responsive genes, TGF-β_1 also inhibits expession of a TCDD-responsive luciferase reporter plasmid (9, 10). CYP1A1, CYP1B1 and NMO-1 genes belong to the Ah gene battery which consists of xenobiotic metabolizing enzymes of phase I and phase II. TCDD inducibility of genes of Ah gene battery is mediated by the cytosolic aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, and its heterodimeric partner protein Arnt (AhR nuclear translocator) (11, 12, 13). The inhibition of TCDD-induced gene expression by TGF-β_1 in A549 cells is accompanied by down-regulation of AhR but not of Arnt mRNA expression. From the results it was concluded that TGF-β_1 induces rapid transcription and translation of an as yet unknown negative trans-acting factor which directly regulates expression of AhR and genes of Ah gene battery (10).

In order to further characterize the mechanism of AhR mRNA down-regulation as well as cell specificity of TGF-β_1-mediated responses, we analyzed the interference of TGF-β_1 with TCDD-induced gene expression

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Abbreviations: AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; CYP, cytochrome P450; ER, estradiol receptor; EROD, 7-ethoxyresorufin-O-deethylase; NMO-1, NADPH:quinone oxidoreductase-1; PAI-1, plasminogen activator inhibitor-1; RT-PCR, reverse transcription-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TGF-β, transforming growth factor-β; TIEG, TGF-β-inducible early gene.

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in estradiol receptor (ER)-negative human breast cancer MDA-MB 231 cells. This cell model has been used to study TGF-β responses (14), and TCDD-responsiveness of these cells has also previously been shown (15, 16). The results provide additional clues that down-regulation of AhR expression is not required for inhibition of TCDD-induced gene expression, and suggest for an involvement of AhR in regulation of cell proliferation.

MATERIALS AND METHODS

Materials. TCDD (purity ≥ 99 %) was obtained from Ökometric (Bayreuth, Germany). Recombinant human TGF-β_1, and chemicals for EROD assay were supplied by Sigma (Taufkirchen, Germany), M-MLV-reverse transcriptase, and TRIzol total RNA preparation kit by Gibco-BRL (Eggenstein, Germany), oligo $(dT)_{15}$ primer, and DNase I by Boehringer-Mannheim (Mannheim, Germany), deoxynucleotide triphosphates, and RNase inhibitor by Pharmacia (Freiburg, Germany), Taq DNA polymerase, transfectam, pRL-SV40, and dual luciferase assay system by Promega (Mannheim, Germany), and [α-^{32}P]dCTP by ICN (Costa Mesa, CA). Media for cell cultures were purchased from Sigma (Taufkirchen, Germany), and medium supplements from Seromed (Berlin, Germany). The p800.luc reporter plasmid was kindly donated by Drs. X.-H. Feng (UCSF, CA) and D.J. Loskutoff (La Jolla, CA).

Cell culture and treatment. The human breast cancer MDA-MB 231 and the human lung cancer A549 cell lines were a kind gift from Dr. C. Knabbe (UKE, Hamburg, Germany). Cells were maintained under standard conditions in supplemented DMEM, and nearly confluent monolayers were treated with TCDD and TGF-β_1 as described (9, 10).

Growth inhibition studies. To determine the influence of TGF-β_1 on cell proliferation, 50,000 cells were seeded into small culture flasks (25 cm^2) and treated for 1 d to 4 d with 100 pM TGF-β_1, control cells received the solvent. Cells were harvested by treatment with trypsin, and cell numbers were determined using a Coulter counter (Coulter, Krefeld, Germany).

EROD activity. For determination of EROD activity, cells were harvested in ice-cold Tris/sucrose (10 mM / 25 mM, pH 7.4) collected by centrifugation, and homogenized in 1 ml of Tris/sucrose. EROD activity was determined spectrofluorometrically as previously described using a Jobin Yvon spectrofluorometer (Jobin Yvon, Grasbrunn, Germany) (16).

RNA preparation and RT-PCR. RNA preparation and radioactive RT-PCR were performed as previoulsy described (10). Briefly, total RNA was isolated with TRIzol RNA isolation kit followed by digestion with DNase I. Subsequently, 1 μg of total RNA was reverse transcribed with M-MLV reverse transcriptase. PCR amplifications were performed with the following profiles: 4 min at 94°C before the first cycle, 1 min for denaturation at 94°C, 1 min for primer annealing, 1 min for extension at 72°C, and 7 min at 72°C after the last cycle. Primer sequences were FP: 5′-CAT TTT GTT TGA CTC CAC CTT and RP: 5′-GGC TCT TTT CTT CCT CTT TGA for TIEG (17), and FP: 5′-ATT GGT CCC TCT TGA TTA and RP: 5′-GTG ATG TCT GGC TGT TTC for cyclin A (18). The other primer sequences were taken from published sources (10). The following annealing temperatures and cycle numbers were used for amplification of gene-specific transcripts: β-actin: 60°C, 19 cycles (541 bp); AhR: 61°C, 25 cycles (578 bp); Arnt: 65°C, 25 cycles (225 bp); c-myc: 61°C, 25 cycles (380 bp); CYP1A1: 60°C, 26 cycles (146 bp); CYP1B1: 63°C, 24 cycles (360 bp); NMO-1: 68°C, 20 cycles (269 bp); TGF-β_1: 60°C, 22 cycles (161 bp); TIEG: 57°C, 26 cycles (155 bp); and cyclin A: 58°C, 28 cycles (601 bp). Linearity of amplification was controlled by different cycle numbers for one cDNA concentration. PCR products were analyzed on 10% (w/v) polyacrylamide gels, and gels were dried and autoradiographed. For semiquantitative analyses, respective bands were quantified using a OmniMedia gel scanner (Millipore, Überlingen, Germany).

Transfection experiments. For transient transfections MDA-MB 231 cells (1.5×10^5 cells per well) and A549 cells (2×10^5 cells per well) were seeded onto 6-well plates and maintained overnight. The cells were then incubated for 6 h with the DNA / liposome mixture (2.5 μg p800.luc reporter plasmid (19), 0.1 μg pRL-SV40 control plasmid, and 12.5 μg transfectam per well), and subsequently maintained in fresh DMEM / 5% (v/v) basal medium supplement overnight. Cells were then treated as indicated, followed by cell lysis in 250 μl passive lysis buffer. Luciferase activities in cell lysates were determined with the dual luciferase assay system in a Berthold LB 96 P Luminometer (Berthold, Weiterstadt, Germany).

RESULTS

TGF-β_1 inhibits dose-dependently TCDD-induced gene expression in MDA-MB 231 cells. TCDD induces CYP1A1, CYP1B1 and NMO-1 mRNA expression in ER-negative human breast cancer MDA-MB 231 cells, with a maximum response at a concentration of 1 nM TCDD (16). Preincubation of MDA-MB 231 cells for 2 h with 0.5 - 250 pM TGF-β_1 followed by an additional treatment for 24 h with 1 nM TCDD led to a dose-dependent inhibition of TCDD-induced expression of the three mRNA species examined (Fig. 1A). The maximum effect was found at a concentration of 100 pM TGF-β_1. The calculated IC_{50} values for inhibition of CYP1A1, CYP1B1 and NMO-1 mRNA expression were approximately 10 pM TGF-β_1. In contrast to CYP1A1, which was completely inhibited under the described experimental conditions, the mRNA levels of CYP1B1 and NMO-1 were reduced only to 50 percent. TGF-β_1 treatment also led to a reduced constitutive CYP1A1 and CYP1B1 mRNA expression detectable by RT-PCR at higher cycle numbers in MDA-MB 231 cells (data not shown).

The inhibitory effect of TGF-β_1 on CYP1 was confirmed on enzyme level by determining EROD activity in MDA-MB 231 cells. TCDD (1 nM) induced the EROD activity to 1.67 ± 0.25 pmol resorufin/mg/min which was down-regulated to 0.82 ± 0.13 and 0.54 ± 0.06 pmol resorufin/mg/min in cells cotreated with 10 and 50 pM TGF-β_1, respectively.

Influence of TGF-β_1 on AhR and Arnt mRNA expression. The heterodimeric nuclear AhR/Arnt complex mediates TCDD-induced gene expression of responsive genes. To find out whether inhibition of TCDD-induced gene expression is at least in part due to down-regulation of AhR or Arnt mRNA expression, the effect of TGF-β_1 on these mRNAs was studied. The results shown in Fig. 1B point out that neither AhR nor Arnt mRNA expression were influenced in cells treated with 0.5 to 250 pM TGF-β_1.

Effect of TGF-β_1 on mRNA expression of TGF-β_1-responsive genes. To demonstrate TGF-β-respon-

siveness, the mRNA expression of TGF-β-responsive genes was determined in MDA-MB 231 cells, treated for 2 h, 8 h, and 24 h with 100 pM TGF-β_1. The mRNA levels of both TGF-β_1 and TIEG were elevated by TGF-β_1 (Fig. 2A). While mRNA expression of TIEG seems to be transient with a maximum after 2 h, the TGF-β_1 mRNA increased time-dependently. In contrast, TGF-β_1-treatment did not influence expression of c-myc and cyclin A mRNAs (Fig. 2A). In comparison, TGF-β_1 also induced the expression of TGF-β_1 and TIEG mRNAs in A549 cells, whereas the mRNA expression of c-myc and cyclin A was inhibited (Fig. 2B).

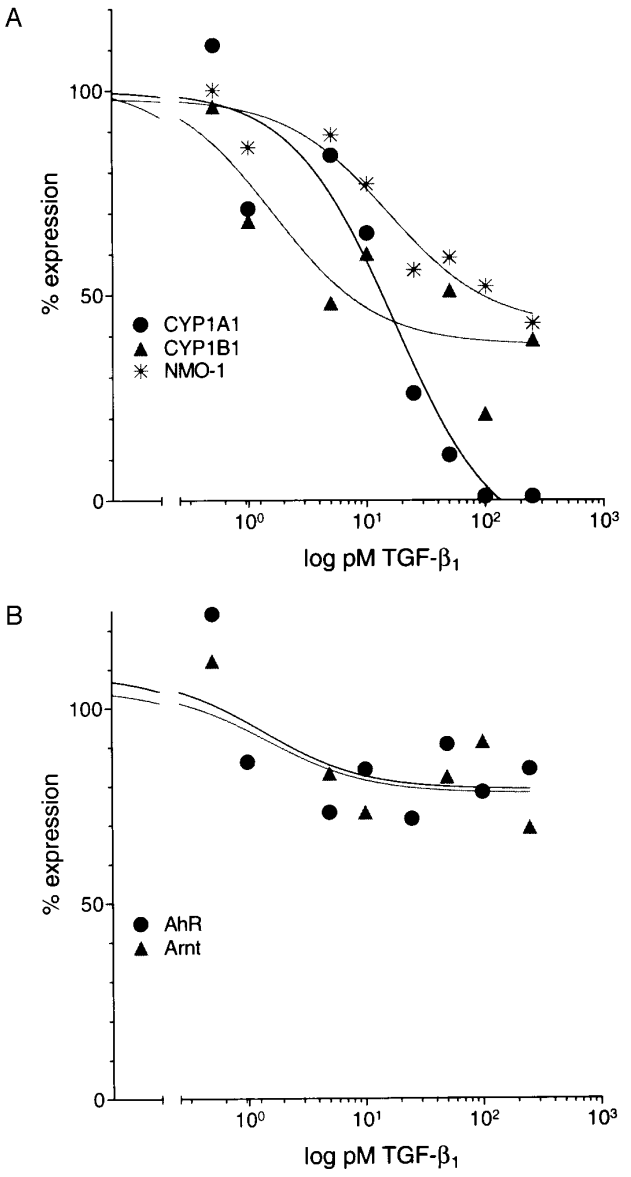

FIG. 1. Effect of TGF-β_1 on mRNA expression of TCDD-sensitve genes (A) as well as AhR and Arnt (B) in MDA-MB 231 cells. Cells were pretreated for 2 h with 0.5 - 250 pM TGF-β_1 and then cotreated for 24 h with 1 nM TCDD. Control cells received only TCDD. mRNA expression was detected by RT-PCR, the respective bands were scanned, and mRNA levels are given as relative intensities to β-actin as described in Materials and Methods. Typical results of three independent experiments are shown.

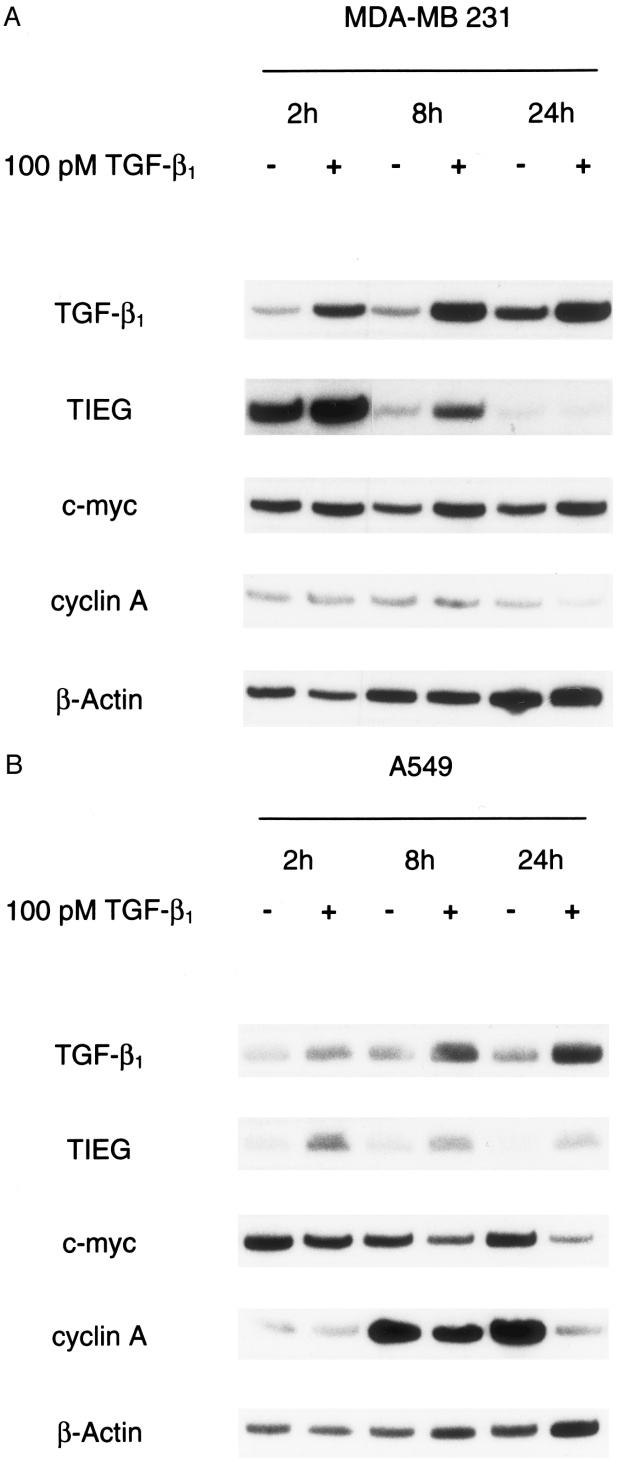

FIG. 2. Effect of TGF-β_1 on mRNA expression of TGF-β_1-responsive genes in MDA-MB 231 (A) and A549 cells (B). Cells were treated for 2 h, 8 h and 24 h with 100 pM TGF-β_1, total RNA was prepared and gene-specific transcripts for TGF-β_1, TIEG, c-myc, cyclin A and β-actin were detected by RT-PCR as described in Materials and Methods.

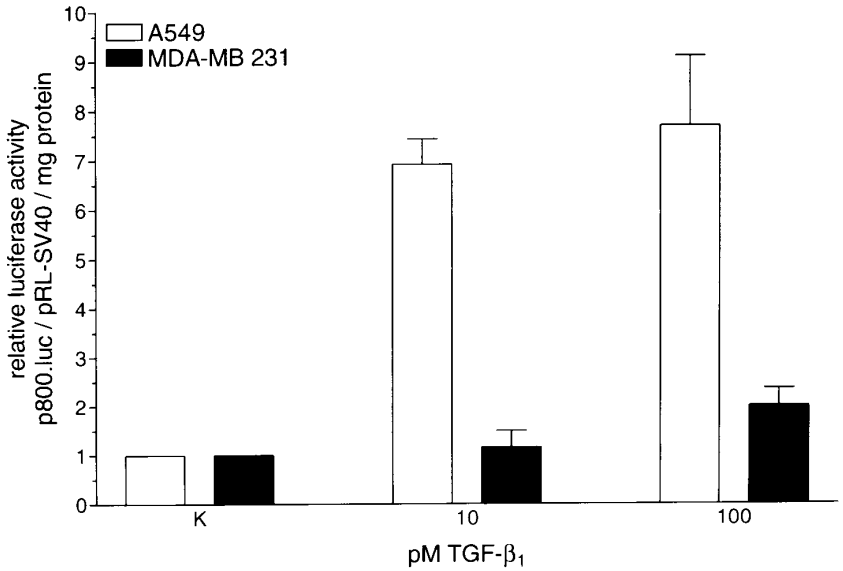

FIG. 3. Induction of luciferase activity from p800.luc by TGF-β_1 in A549 and MDA-MB 231 cells. Cells transiently transfected with p800.luc driven by the PAI-1 promotor were treated for 24 h with 10 pM or 100 pM TGF-β_1. Luciferase activities were determined as described in Materials and Methods, and were corrected by activities of cotransfected pRL-SV40 control plasmid and by the protein content of each well. Means ± SD of triplicate experiments are given.

TGF-β_1-responsiveness of the cell lines was further addressed by studying the expression of the TGF-β_1-inducible luciferase reporter plasmid p800.luc driven by the plasminogen activator inhibitor (PAI)-1 promoter. Treatment of transiently transfected A549 cells for 24 h with 10 and 100 pM TGF-β_1 led to a 6.9 ± 0.5-fold and 7.7 ± 1.4-fold induction of Luciferase activity, respectively. In MDA-MB 231 cells, treatment with 10 and 100 pM TGF-β_1 led to a 1.2 ± 0.3-fold and 2.0 ± 0.4-fold induction of luciferase activity, respectively, compared to controls (Fig. 3).

Effect of TGF-β_1 on cell proliferation of A549 and MDA-MB 231 cells. Because TGF-β_1 had different effects on expression of cell-cycle regulating genes in A549 and MDA-MB 231 cells, we studied the cell proliferation of adherent growing cells treated for 4 d with 100 pM TGF-β_1. TGF-β_1 inhibited proliferation of A549 cells to nearly 50% of solvent control after 4 d treatment (Table 1). In contrast, TGF-β_1 did not influence cell proliferation of MDA-MB 231 cells.

DISCUSSION

Currently only a few hints on specific endogenous or exogenous regulators of AhR expression have been described in various *in vitro* systems. Serum, platelet-derived growth factor (PDGF), and a tyrosine-dependent phosphorylation induce the activity of an AhR promoter-driven reportergene in murine 3T3 fibroblasts (20). Simultaneous treatment of human HEL cells with TGF-β_1 and vitamin D3 stimulates AhR mRNA expression during monocyte differentiation (21), whereas a down-regulation of AhR mRNA expression has been described for retinoic acid in human HaCat cells (22). Tissue-specific distribution of AhR mRNA (23) as well as developmental expression of AhR (24) indicate for a specific transcriptional control of the AhR gene *in vivo*. The finding of tissue- and cell-specific regulation of AhR expression were supported by analyses of the expression of an AhR-promoter-driven reportergene in murine cell lines of different tissue origin (25). We previously described a down-regulation of AhR mRNA expression in A549 cells (10). In the present study we show, in accordance with our findings in A549 cells, that TGF-β_1 inhibits dose-dependently TCDD-induced and constitutive CYP1A1, CYP1B1 and NMO-1 mRNA

TABLE 1

Effect of TGF-β_1 on Cell Proliferation of A549 and MDA-MB 231 Cells

Time point	A549 (% of solvent control)	MDA-MB 231 (% of solvent control)
0 d	100	100
1 d	97.5 ± 11.6	105.3 ± 2.3
2 d	54.2 ± 1.1	101.7 ± 8.3
3 d	57.7 ± 0.6	104.9 ± 12.0
4 d	47.2 ± 2.5	100.6 ± 10.3

Note. 50,000 cells were seeded into small culture flasks, and treated for 1 d to 4 d with 100 pM TGF-β_1. The cell numbers were determined, and are given as percentage of solvent control. Mean ± SD values of triplicate experiments are given.

expression in MDA-MB 231 cells. The IC_{50} values of approximately 10 pM TGF-β_1 are in the same order of magnitude described in A549 cells. The inhibition of CYP1 mRNA is accompanied by inhibition of TCDD-induced EROD activity, which has recently been shown to be catalyzed by either CYP1A1 or CYP1B1 (26). However, in contrast to A549 cells, TGF-β_1 has no effect on AhR mRNA expression in MDA-MB 231 cells. These results provide additional evidence that down-regulation of AhR mRNA is not necessary for inhibition of CYP1 and NMO-1 gene expression.

It has been postulated that expression of both TGF-β type I and type II receptors is necessary to propagate both negative and positive regulatory signals (3, 4, 5), but uncoupling of these responses has also been observed. For example, a divergency of TGF-β_1 responses has been reported in cells either expressing a truncated or a non-glycosylated TGF-β type II receptor. In these cells TGF-β_1 induces expression of responsive genes like PAI-1, whereas the antiproliferative effect of TGF-β_1 was abolished (27, 28). We performed a series of experiments to analyze TGF-β_1 responsiveness of the cell lines examined. In both A549 and MDA-MB 231 cells, TGF-β_1 up-regulates expression of TGF-β-sensitive genes like TGF-β_1 and TIEG, a recently identified TGF-β-responsive gene (17). These results were confirmed by transfection studies with a TGF-β-responsive luciferase reporter plasmid. However, whereas TGF-β_1 inhibits cell proliferation and mRNA expression of cell-cycle regulating genes like cyclin A and c-myc in A549 cells, no effects were found on cell proliferation and cell-cycle regulating genes in MDA-MB 231 cells. In addition, our results indicate for cell-specific differences in the negative regulatory pathway of TGF-β_1, since, in contrast to A549 cells, TGF-β_1 down-regulates mRNA expression of AhR-responsive genes but not of cell-cycle regulating genes and of AhR itself in MDA-MB 231 cells.

Although the physiological function of the AhR is still unknown, it became increasingly likely in the last few years that the AhR may be involved in modulation of cell proliferation and differentiation (e.g. 22, 24, 29, 30). It is noteworthy that regulation of AhR mRNA expression by TGF-β_1 seems to be similar to that of cell-cycle regulating genes. The particulars of the crosstalk between AhR- and TGF-β_1-mediated cell-signalling pathways are unknown, but putative TGF-β-responsive, negative regulatory elements described in the 5′-flanking regions of AhR and CYP1A1 genes may be responsible for TGF-β_1-mediated down-regulation (10). The recently identified AhR interacting protein, which shares homologies to the immunophilins FKBP12 and FKBP52 (31, 32), could also be an appropriate candidate for mediating the interference between both signalling pathways. An interaction of the immunophilin FKBP12 with the cytoplasmatic domain of the TGF-β_1 type I receptor has been described (8).

In summary, we described that TGF-β_1 coregulates mRNA expression of AhR and cell-cycle regulating genes in human cancer cell lines. These results strengthen a hypothetical role of AhR in regulation of cell proliferation and differentiation.

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