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2,3,7,8-tetrachlorodibenzo-*p*-dioxin suppresses the growth of human colorectal cancer cells *in vitro*: Implication of the aryl hydrocarbon receptor signaling

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Abstract. Human colorectal cancer is the third most common cancer disease with a 5-year survival rate of 55% in USA in 2016. The investigation to identify novel biomarker factors with molecular classification may provide notable clinical information to prolong the survival of patients with colorectal cancer. The aryl hydrocarbon receptor (AHR) binds the AHR nuclear translocator in the cytoplasm of various types of cells, including liver cells, and then binds to the xenobiotic responsive element on various genes. AHR was initially discovered via its ligand, the polychlorinated hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The present study was undertaken to determine whether TCDD, an agonist of AHR signaling, impacts the growth of RKO human colorectal cancer cells *in vitro*. Treatment with TCDD (0.1-100 nM) revealed suppressive effects on colony formation and proliferation of RKO cells, and stimulated death of these cells with subconfluence. These effects of TCDD were abolished by pretreatment with CH223191, an inhibitor of AHR signaling. Western blot analysis demonstrated that TCDD treatment decreased AHR levels and elevated cytochrome P450 family 1 subfamily A member 1 (CYP1A1) levels, indicating a stimulation of AHR signaling. TCDD treatment caused an increase in nuclear factor-κB p65 and β-catenin levels, although it did not have an effect on Ras levels. Notably, TCDD treatment increased the levels of p53, retinoblastoma, p21 and regucalcin, which are depressors of carcinogenesis. Additionally, action of TCDD on cell proliferation and death were not revealed in regucalcin-overexpressing RKO cells, and regucalcin overexpression depressed AHR signaling associated with CYP1A1 expression. Thus, AHR signaling suppresses the growth of colorectal cancer cells, indicating a role as a significant targeting molecule for colorectal cancer.

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Introduction

Intestinal homeostasis is maintained by complex interactions between intestinal microorganisms and the gut immune system, and dysregulation of gut immunity may cause inflammation and tumorigenesis (1). Interaction between epithelial cells and stromal cells, including leukocytes and fibroblasts, is considered to be pivotal for tumorigenesis and cancer progression (1). Adenocarcinoma colorectal cancer is a predominant malignancy located in the colon and rectum, and it has been proposed to arise from a subpopulation of self-renewing tumor stem cells located within the tumor microenvironment (1,2). Colorectal cancer is the third most common cancer diagnosed in USA in 2016 (3,4) and its 5-year survival rate remains poor at 55% (4). Colorectal cancer is a heterogeneous group of diseases, and its prognosis remains poor in spite of the development of novel therapeutic strategies (5-8), and its molecular classification is notable (5-9). The identification of novel biomarker targets is proposed to result in prolonged survival of patients with colorectal cancer (10).

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor, which is located in manifold types of cells (11,12). AHR forms a heterodimer with the AHR nuclear translocator, which is transcriptionally active after binding to xenobiotic responsive elements in various genes, including the cytochrome P450 family 1 subfamily A member 1 (CYP1A1) gene (11,12). The AHR was initially discovered in the process that investigates its binding to polychlorinated aromatic hydrocarbons, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (11,12). Numerous AHR ligands, as AHR agonists, have been identified, including synthetic and environmental chemicals, and naturally-occurring dietary and endogenous compounds (13-18). AHR signaling has been regulated through various signaling factors, including nuclear

factor-κB (NF-κB) p65, and it appears to serve an important role in the regulation of diverse cellular and biological processes (19). The canonical target genes for AHR are well known in cytochrome P450 isoforms (CYP1A1, CYP1A2 and CYP1B1), which are implicated in the metabolic pathway of xenobiotics and endogenous compounds located in tissues and cells (20,21). The AHR signaling-dependent pathway is also implicated in manifestation of chemically-induced toxicity and carcinogenesis, which are induced through the production of free radicals and conversion of pro-carcinogens to ultimate genotoxic carcinogens via metabolism that is mediated by cytochrome P450 enzymes (20,21). Furthermore, AHR ligands are involved in various pathologies in humans, resulting in toxic processes, including tumor promotion, immunosuppression and teratogenicity with disorder of the fine homeostatic regulations of cell functions (22-26).

The physiological role of AHR in the absence of exogenous ligand may serve a pivotal role in the regulation of cell function, compared with cellular impacts caused by its binding of exogenous ligand (27). Mice, which express a constitutively active AHR, exhibited a promoted development of hepatocarcinogenesis (28). Notably, AHR signaling may be demonstrated to serve a role of a depressor in the development of hepatocarcinogenesis (29). Furthermore, AHR signaling has been demonstrated to adjust liver repair and regeneration, and its signaling suppresses tumorigenesis by modulating the actions of stem-like cells and β-catenin signaling (30,31). Recently, it was demonstrated that TCDD treatment represses the proliferation and promotes the death of human liver cancer HepG2 cells *in vitro*, and that the exhibition of these effects was implicated in AHR signaling associated with various signaling factors, including NF-κB p65 (32).

The AHR is expressed and characterized in human colon adenocarcinoma cells, including RKO cells (33-35), and has been demonstrated to regulate the expression levels of CYP1A1 (36) and CYP1A2 (37) in colorectal cancer cells *in vitro*. The role of AHR thus has been reported in colon cancer cells (38). Notably, the AHR suppressed intestinal carcinogenesis in $Apc^{Min/+}$ mice following natural ligand treatment *in vivo* (39). Furthermore, the AHR is associated with tumor prevention by regulating gut immunity in normal intestinal tissues, and it is involved in growth suppression of tumor cells of $Apc^{Min/+}$ mice (16). Thus, the AHR may serve a repressive role in the development of colorectal cancer. However, the regulatory role of AHR signaling in the proliferation and death of human colorectal cancer cells is poorly understood. Therefore, this was investigated in RKO colorectal cancer cells *in vitro*. It was demonstrated that TCDD treatment suppresses the growth and proliferation, and stimulates the death of RKO cells, via AHR signaling. The observations indicated that the activation of AHR signaling serves a suppressive role in the development of human colorectal cancer, revealing a potential novel role of AHR as a target molecule in carcinogenesis.

Materials and methods

Materials. TCDD (>99.99% purity) was obtained from Dow Chemicals Co. (Midland, MI, USA), and it was dissolved in dimethyl sulfoxide (DMSO) and stored in the dark at -20˚C until use. Dulbecco's modified Eagle's medium (DMEM; including 4.5 g/l glucose, L-glutamine and sodium pyruvate) and antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin; P/S) were obtained from Corning Life Sciences (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Omega Scientific Inc. (Tarzana, CA, USA). 2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide (CH223191) was purchased from Selleck Chemicals (Houston, TX, USA), and it was dissolved in 100% DMSO. Caspase-3 inhibitor, crystal violet, and all other chemicals were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Human colorectal cancer cells. RKO epithelial cells, which originated from male adult patients with colorectal carcinoma, were used in the present study. This cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). RKO cells were cultured in DMEM including 10% FBS and 1% P/S.

Assay of colony formation of RKO cells. RKO cells ($1x10^3$ cells/well per 2 ml of medium in 6-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone in an atmosphere containing 5% CO_2 at 37˚C in the presence of vehicle (1% DMSO) or TCDD (1 or 10 nM) for 5 days, when visible clones formed on the plates (40,41). Following the culture, the dishes were washed with PBS (3 times with 2 ml) and fixed with 100% methanol (adding 0.5 ml per well) for 20 min at room temperature, and then washed 3 times with PBS (2 ml). The colonies were stained with crystal violet. Crystal violet solution (0.5%, dissolved in 20% methanol) was added to the fixed cells for 30 min at room temperature. Thereafter, stained cells were washed 5 times with PBS (2 ml). After washing, the plates were air-dried for 2 h at room temperature. The colonies (including >50 cells) were counted under a microscope (x10; Nikon Corporation, Tokyo, Japan) using a cell counter (Line Seiki H-102P; Line Seiki Co., Ltd., Tokyo, Japan). Data are represented as numbers of colonies per well.

Transfection of regucalcin cDNA/pCXN2 into RKO cells. To generate the regucalcin-overexpressing RKO cells, the RKO wild-type cells were transfected with empty pCXN2 vector (Addgene, Inc., Cambridge, MA, USA; 600 μg/ml) or pCXN2 vector (Addgene, Inc.; 600 μg/ml) expressing a cDNA encoding the human full-length (900 bp) regucalcin (regucalcin cDNA/pCXN2) (42,43). For transfection, the RKO cells ($1x10^5$/well per ml of DMEM) were grown on 24-well plates to reach subconfluency. Regucalcin cDNA/pCXN2 (1 μg/well) or empty pCXN2 vector (1 μg/well) alone was transfected into the RKO cells using the synthetic cationic lipid Lipofectamine® reagent, according to the manufacturer's protocols (Promega Corporation, Madison, WI, USA) (43). Following overnight incubation after transfection, Geneticin (600 μg/ml G418; Sigma-Aldrich; Merck KGaA) was added to the culture wells to select transfectants, and the cells were cultured in an atmosphere containing 5% CO_2 at 37˚C for 3 weeks to produce transfected cells. Subsequently, the transfected cells were plated with limiting dilution to isolate transfectants using 96-well plates. Surviving clones were isolated, transferred to 35-mm dishes, and grown in DMEM without Geneticin.

The transfectant clones 1 and 2 exhibiting stable expression of regucalcin were then obtained. The levels of regucalcin expressed in two clones were assayed using western blot analysis, and those exhibited an elevation expression of 7.4- or 10.9-fold in clones 1 or 2, respectively, compared with wild-type cells, respectively, as depicted in Fig. 6A. Therefore, clone 2 was used in the subsequent experiments.

Assay of cell proliferation. To determine the effect of TCDD on cell proliferation, the RKO wild-type cells ($1x10^5$/ml per well) were cultured using a 24-well plates in DMEM, containing 10% FBS, 1% P/S and 1% fungizone, in the presence of vehicle (1% DMSO) or TCDD (0.1, 1, 10 or 100 nM) in an atmosphere containing 5% CO_2 and 37˚C for 3 or 7 days (44). In separate experiments, the RKO wild-type cells or transfectants ($1x10^5$/ml per well) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone with or without vehicle (1% DMSO), TCDD (1, 10 or 100 nM), or CH223191 (1 or 10 μM) with or without TCDD (10 nM) in an atmosphere containing 5% CO_2 and 37˚C for 3 days. The RKO cells were then detached from each culture dish to determine cell number using a cell counter.

Assay of cell death. To determine the effect of TCDD on cell death, the RKO wild-type cells ($1x10^5$/ml per well) were cultured using 24-well plates in DMEM, containing 10% FBS, 1% P/S, and 1% fungizone, in the absence of TCDD in an atmosphere containing 5% CO_2 and 37˚C for 3 days in order to reach subconfluence. The cultured cells at subconfluency were incubated in the presence of vehicle (1% DMSO) or TCDD (0.1, 1, 10 or 100 nM), with or without the caspase-3 inhibitor (10 μM) in the presence of either vehicle or CH223191 (1 or 10 μM) for 24 h in an atmosphere containing 5% CO_2 at 37˚C (45). In other experiments, the RKO-wild-type cells or transfectants ($1x10^5$/ml per well) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone in the absence of TCDD in an atmosphere containing 5% CO_2 and 37˚C for 3 days. After reaching subconfluence, the cells were incubated in the presence of vehicle (1% DMSO), TCDD (1, 10 or 100 nM), or CH223191 (1 or 10 μM) with or without TCDD (10 nM) for 24 h in an atmosphere containing 5% CO_2 at 37˚C (45). Cells were then detached from each culture well to determine cell number using a cell counter.

Counting of cell number. To detach cells attached on each well after culturing in order to assay the proliferation and death of RKO cells, culture dishes were incubated for 2 min at 37˚C with the addition of a solution (0.1 ml per well) of 0.05% trypsin plus EDTA in Ca^{2+}/Mg^{2+}-free PBS, and then cells were detached through pipetting after the addition of DMEM (0.9 ml) containing 10% FBS and 1% P/S into the wells (44,45). The medium containing the suspended cells (0.1 ml) was mixed with 0.1 ml of 0.5% trypan blue staining solution (44,45). The number of viable cells with viability was counted under a microscope (x10; Olympus MTV-3; Olympus Corporation, Tokyo, Japan) using a Hemocytometer plate (Sigma-Aldrich; Merck KGaA) and a cell counter (Line Seiki H-102P; Line Seiki Co., Ltd.). The mean of two counts was calculated for each dish. The number of cells is presented as number per well of the plate.

Western blot analysis. To determine levels of various proteins expressed in RKO cells, wild-type RKO cells or regucalcin-overexpressing cells were plated in 100x21 mm dishes at a density of $1x10^6$ cells/dish in 10 ml DMEM containing 10% FBS, 1% P/S and 1% fungizone, and then cultured in the presence of vehicle (1% DMSO) or TCDD (10 nM) in an atmosphere containing 5% CO_2 and 37˚C for 3 days. After culturing, the cells were washed three times with ice-cold PBS and removed from the dish by scraping after the addition of cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) supplemented with inhibitors of protease and protein phosphatase (Roche Diagnostics, Indianapolis, IN, USA). The collected lysates were centrifuged at 17,000 x g at 4˚C for 10 min, to prepare fractions including the cytoplasm and endoplasmic reticulum of RKO cells. The concentrations of protein in aforementioned supernatants were assayed using the Bio-Rad Protein Assay Dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin (Bio-Rad Laboratories, Inc.) as standard. The aforementioned supernatant from cell lysate was stored at -80˚C until use for western blot assay. Samples of 40 μg supernatant protein were applied to each lane and were separated using SDS-PAGE (12%). After electrophoresis, the gel was transferred onto PVDF membranes for immunoblotting with specific antibodies. The membranes were blocked with SuperBlock®T20 blocking buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 60 min at room temperature. Polyclonal AHR antibody sheep IgG was obtained from R&D Systems, Inc. (cat. no. AF6697; Minneapolis, MN, USA; dilution 1:500). Antibodies for other signaling proteins, including CYP1A1 (cat. no. sc-25304; dilution 1:1,000), NF-κB p65 (cat. no. sc-109; dilution 1:1,000), β-catenin (cat. no. sc-39350; dilution 1:1,000) and p53 (cat. no. sc-126; dilution 1:1,000) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; dilution 1:1,000), and Ras (cat. no. 14429; dilution 1:1,000), β-actin (cat. no. 3700; dilution 1:1,000), retinoblastoma (Rb; cat. no. 9309; dilution 1:1,000) and p21 (cat. no. 2947; dilution 1:1,000) were purchased from Cell Signaling Technology, Inc.. Rabbit anti-regucalcin antibody was provided from Abcam (Cambridge, MA, USA; cat. no. ab213459; dilution 1:1,000), and it was used as described previously (42,43,46). For immunoblotting with the aforementioned specific antibodies, the membranes were incubated with each primary antibody overnight at 4˚C, followed by horseradish peroxidase-conjugated secondary antibody (cat. nos. sc-2005 or sc-2305 for mouse and rabbit, respectively; Santa Cruz Biotechnology, Inc.; dilution 1:2,000) for 60 min at 4˚C. A total of 3 blots from independent experiments were scanned on an Epson Perfection 1660 Photo scanner, and the bands were quantified using ImageJ2 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation. Comparisons between two groups were performed using a Student's t-test. Furthermore, multiple comparisons were performed using one-way analysis of variance with Tukey-Kramer multiple comparisons post hoc test for parametric data as indicated. $P<0.05$ was considered to indicate a statistically significant difference.

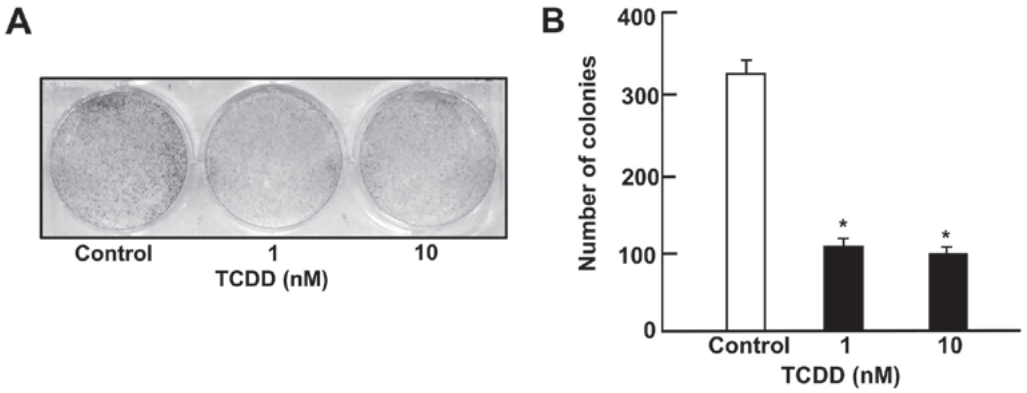

Figure 1. TCDD suppresses colony formation in RKO human colorectal cancer cells *in vitro*. Cells ($1x10^3$ cells/well) were seeded into 6-well plates and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone in the presence of vehicle (1% dimethyl sulfoxide) or TCDD (1 or 10 nM) for 5 days when visible clones formed. The colonies were washed with PBS, fixed with methanol and then stained with 0.5% crystal violet. (A) Stained cells are presented as images (x10), and (B) the colonies containing >50 cells were counted under a microscope. *P<0.001, vs. control. One-way analysis of variance and Tukey-Kramer post hoc test were used. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

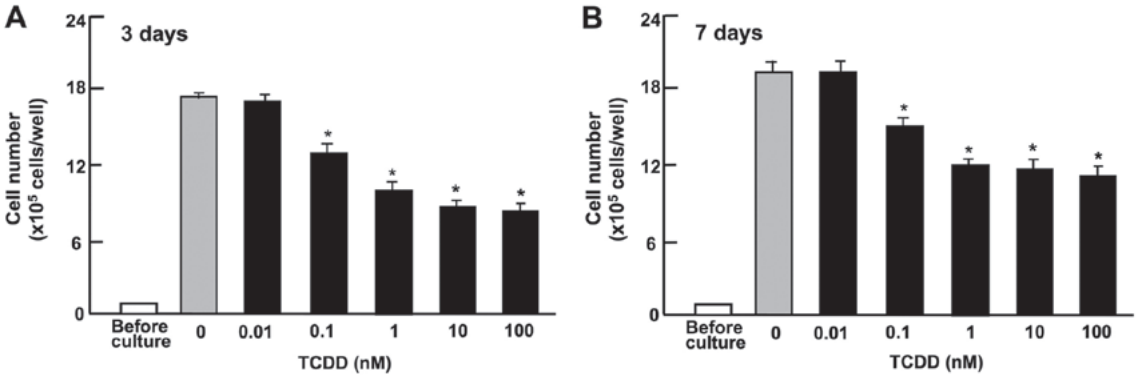

Figure 2. TCDD suppresses the proliferation of RKO human colorectal cancer cells *in vitro*. The cells ($1x10^5$ cells/well in 24-well plates) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone in the presence of vehicle (1% dimethyl sulfoxide) or TCDD (0.01-100 nM) for (A) 3 or (B) 7 days. After culture, the numbers of attached cells were counted. Data are presented as mean ± standard deviation obtained from 8 wells of 2 replicate plates per dataset using different dishes and cell preparations. *P<0.001, vs. 0 nM TCDD. One-way analysis of variance and Tukey-Kramer post hoc test were used. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Results

TCDD represses colony formation of RKO cells. The effects of TCDD on colony formation of RKO human colorectal cancer cells *in vitro* was investigated. Visible clones of RKO cells were formed by culture for 5 days (Fig. 1). Subsequently, RKO cells were cultured in the presence of TCDD (1 or 10 nM). The number of colonies with >50 nuclei was significantly decreased by treatment with TCDD (1 or 10 nM) as depicted in Fig. 1A and B. Thus, TCDD exhibited a suppressive effect on the colony formation of RKO cells.

TCDD suppresses the proliferation of RKO cells. To determine the effect of TCDD on cell growth, RKO cells were cultured in 24-well plates in the presence of TCDD (0.01-100 nM) for 3 or 7 days. The cells reached subconfluency after culturing for 3 days, and they reached confluency at 4-7 days of culture. Thus, cell growth was suppressed by the treatment with TCDD (0.1-100 nM) for 3 (Fig. 2A) or 7 (Fig. 2B) days.

TCDD stimulates the death of RKO cells. Subsequently, the effect of TCDD on the death of RKO cells *in vitro* was investigated. The cells were cultured for 3 days to reach subconfluency, and then exposed to TCDD (0.01-100 nM) for a further 24 h. Treatment with TCDD (0.1-100 nM) resulted in a decrease of attached cells (Fig. 3A and B), indicating that cell death is induced. In separate experiments, RKO cells that had reached subconfluency after culture for 3 days were incubated with a caspase-3 inhibitor (10 μM) and TCDD. The reduction of cell number caused by the treatment with TCDD (1 or 10 nM) was prevented in the presence of the inhibitor of caspase-3. Activation of caspase-3 is demonstrated to induce DNA fragmentation associated with apoptosis (45). TCDD-induced cell death may be due to activation of caspase-3, which is known to induce DNA fragmentation associated with cell death (41). However, this remains to be elucidated using other methods.

Involvement of AHR signaling in the proliferation and death of RKO cells. To characterize the TCDD-induced repression of proliferation and promotion of death of RKO cells, the cells were cultured in the treatment with CH223191, a suppressor of AHR signaling (47). CH223191 (1 or 10 μM) did not have a significant effect on the proliferation or death of RKO

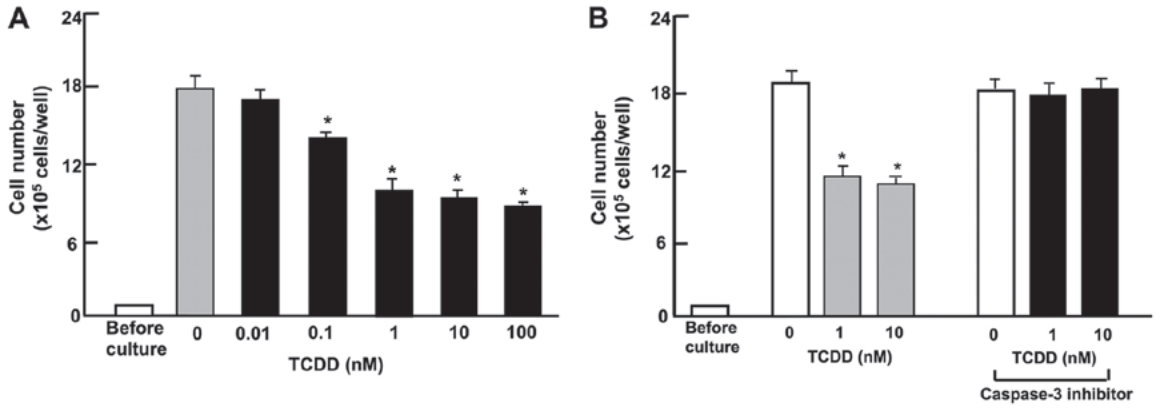

Figure 3. TCDD stimulates the death of RKO human colorectal cancer cells *in vitro*. (A) Cells (1x10^5 cells/well in 24-well plates) were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone for 3 days to reach subconfluency, and the cells were then cultured in DMEM as aforementioned in the presence of vehicle (1% DMSO) or TCDD (0.01-100 nM) for 24 h. (B) After culture for 3 days, the cells were cultured in the presence of vehicle (1% DMSO) or TCDD (1 or 10 nM) for 24 h with or without caspase-3 inhibitor (10 μM). The numbers of attached cells were then counted. Data of cell number are presented as mean ± standard deviation obtained from 8 wells of 2 replicate plates per dataset using different dishes and cell preparations. *P<0.001 vs. 0 nM TCDD. One-way analysis of variance and Tukey-Kramer post hoc test were used. DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

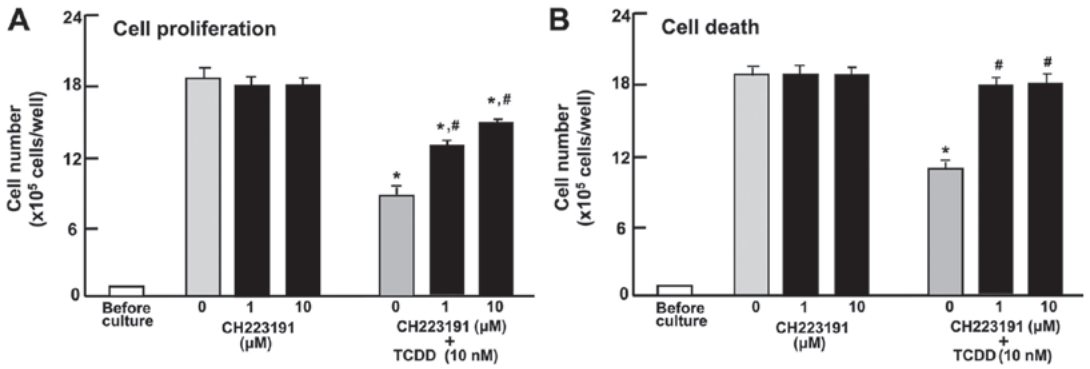

Figure 4. The AHR is involved in mediating the effects of TCDD on the proliferation and death of RKO human colorectal cancer cells. (A) The cells (1x10^5 cells/per well in 24-well plates) were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone containing vehicle (1% DMSO) or AHR inhibitor, CH223191 (1 or 10 μM), with or without TCDD (10 nM) for 3 days. (B) The cells (1x10^5 cells/per well in 24-well plates) were cultured in DMEM as aforementioned for 3 days, and once the cells reached subconfluency, they were cultured in DMEM as aforementioned in the presence of vehicle (1% DMSO) or CH223191 (1 or 10 μM). After 1 h, TCDD (10 nM) was added into the medium containing vehicle (1% DMSO) or CH223191 (1 or 10 μM), and the cells were cultured for a further 23 h. The number of attached cells were then counted. Data are presented as mean ± standard deviation obtained from 8 wells of 2 replicate plates per dataset using different dishes and cell preparations. *P<0.001 vs. 0 μM CH223191. #P<0.01 vs. 0 μM CH223191 + 10 nM TCDD. One-way analysis of variance and Tukey-Kramer post hoc test were used. AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide.

cells (Fig. 4A and B). The repressive effect of TCDD (10 nM) on the proliferation and the promoting effect of TCDD (10 nM) on the death of RKO cells were significantly blocked by CH223191 (1 or 10 nM; Fig. 4A or B). The effects of TCDD on cell proliferation were not completely blocked by the inhibitor (Fig. 4A); however, the promoting effects of TCDD on cell death were completely blocked (Fig. 4B). These results indicate that the effects of TCDD on the proliferation and death of RKO cells are partially mediated by AHR signaling.

TCDD enhances the levels of proteins associated with tumor suppression in RKO cells. To characterize the mechanism of TCDD action, and determine whether or not TCDD treatment regulates the levels of key transcription factors, western blot analysis was used. AHR and CYP1A1 mRNAs were previously reported to be expressed in RKO cells *in vitro* (34,35). It was demonstrated that the levels of AHR and CYP1A1 were altered by TCDD in RKO cells (Fig. 5A and B). Notably, treatment with TCDD (10 nM) significantly elevated the levels of NF-κB p65 and β-catenin, which are crucial transcription factors associated with cell signaling (32). Additionally, TCDD treatment significantly elevated the levels of p53, Rb, p21 and regucalcin, which are known as pivotal repressors of the growth of tumor cells (48,49) (Fig. 5C and D). TCDD (10 nM) did not significantly alter the level of Ras, which acts upstream in Akt signaling (32,49) (Fig. 5A and B).

The effects of TCDD are suppressed in the regucalcin-overexpressing RKO cells. Overexpression of regucalcin has been demonstrated to repress the enhanced proliferation and death of RKO cells *in vitro* (43). Therefore, the present study investigated whether the effects of TCDD were attenuated in regucalcin-overexpressing RKO cells *in vitro*. These cells exhibited increased levels of

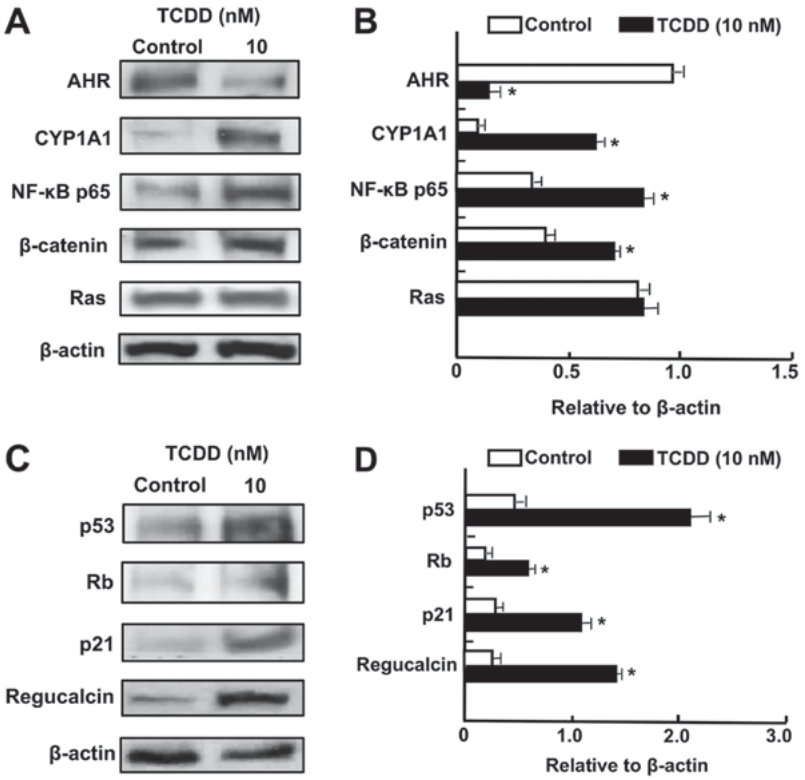

Figure 5. TCDD regulates the expression of proteins associated with AHR signaling in RKO human colorectal cancer cells *in vitro*. The cells (1x10^6 cells/dish) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone in the presence of vehicle (1% dimethyl sulfoxide) or TCDD (10 nM) for 3 days. Cell lysates were prepared and centrifuged, and 40 μg of the supernatant protein per lane were separated by SDS-PAGE and transferred to nylon membranes for western blotting using specific antibodies against various proteins as indicated. Data represent a typical figure of three independent experiments using different cell preparations, and also are presented as mean ± standard deviation. (A) Representative film image for cell signaling-associated proteins. (B) Relative to β-actin cell signaling-associated protein levels. (C) Representative film image of tumor suppressor proteins.(D) Relative to β-actin tumor suppressor proteins. *P<0.01 vs. control using Student's t-test. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHR, aryl hydrocarbon receptor; NF-κB, nuclear factor-κB; CYP1A1, cytochrome P450 family 1 subfamily A member 1; Rb, retinoblastoma.

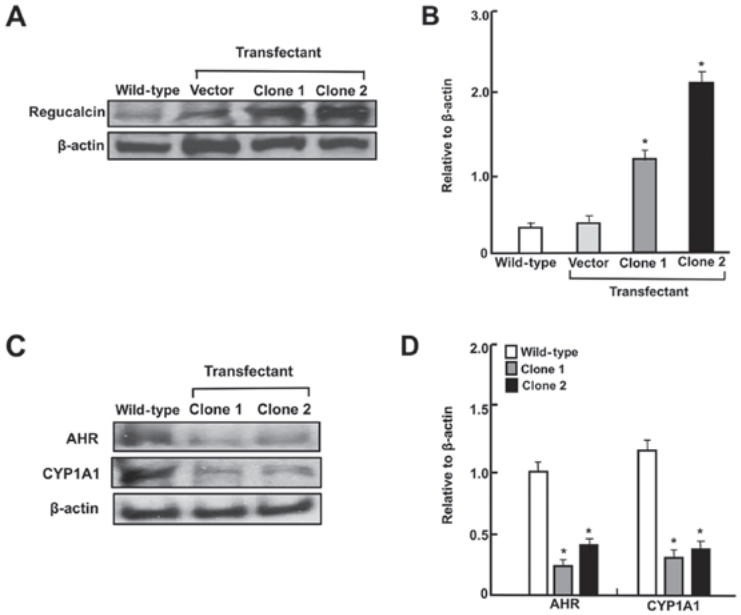

Figure 6. AHR and CYP1A1 levels are suppressed in regucalcin-overexpressing RKO human colorectal cancer cells *in vitro*. The wild-type RKO cells or regucalcin-overexpressing RKO cells (1x10^6 cells/per dish) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone in the presence or absence of vehicle (1% dimethyl sulfoxide) for 3 days. After culture, resulting cell lysates were centrifuged, and 40 μg of the supernatant protein per lane were separated by SDS-PAGE and transferred to nylon membranes for western blotting using specific antibodies as indicated. Data represent a typical figure obtained from three independent experiments using different cell preparations, and also are presented as mean ± standard deviation. (A) Representative film image for regucalcin. (B) Relative to β-actin regucalcin level. (C) Representative film image of AHR and CYP1A1. (D) Relative to β-actin AHR and CYP1A1 levels. *P<0.01 vs. wild-type using one-way analysis of variance and Tukey-Kramer post hoc test. AHR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 family 1 subfamily A member 1.

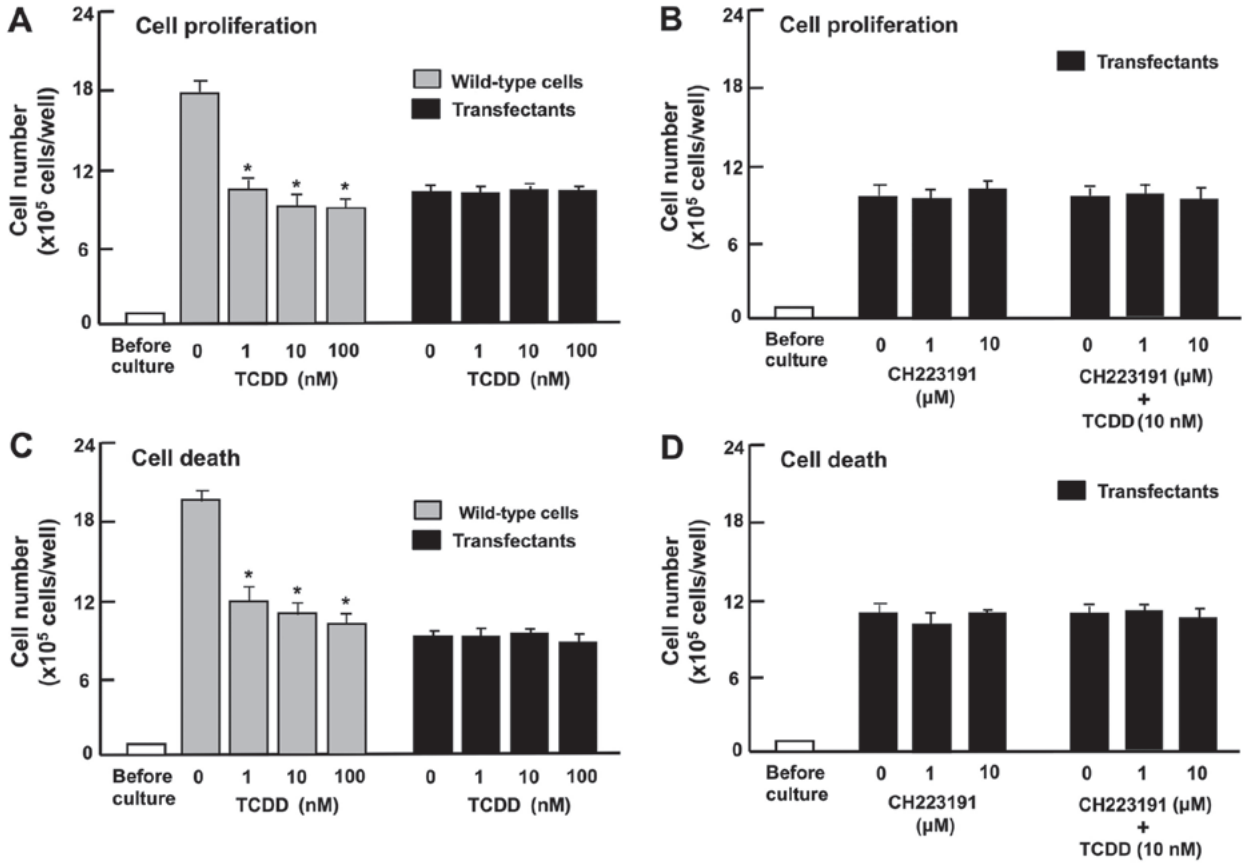

Figure 7. The effects of TCDD on the proliferation and death of RKO human colorectal cancer cells are attenuated by the overexpression of regucalcin *in vitro*. Wild-type cells or transfectants (1x10^5 cells/per well of 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone in the presence of (A) vehicle (1% DMSO) or TCDD (1, 10 or 100 nM), or (B) vehicle (1% DMSO) or CH223191 (1 or 10 μM) with or without TCDD (10 nM). In separate experiments, the wild-type cells or transfectants (1x10^5 cells/per ml of well) were cultured in DMEM as aforementioned for 3 days, and upon reaching subconfluence, the cells were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone in the presence of (C) vehicle (1% DMSO) or TCDD (1, 10 or 100 nM), or (D) vehicle (1% DMSO) or CH223191 (1 or 10 μM) with or without TCDD (10 nM) for 24 h. After culture, the numbers of attached cells were counted. Data are presented as mean ± standard deviation obtained from 8 wells of 2 replicate plates per dataset using different dishes and cell preparations. *P<0.001 vs. 0 nm TCDD in wild-type cells. One-way analysis of variance and Tukey-Kramer post hoc test were used. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; P/S, penicillin/streptomycin.

regucalcin (Fig. 6A and B). Notably, regucalcin overexpression significantly suppressed CYP1A1 and AHR levels in RKO cells (Fig. 6C and D).

Subsequently, whether TCDD exhibits a repressive effect on proliferation and a promoting effect on cell death in the regucalcin-overexpressing RKO cells *in vitro* was investigated. Wild-type RKO cells or regucalcin-overexpressing cells were treated with TCDD (1, 10 or 100 nM). Proliferation of wild-type RKO cells was significantly repressed by regucalcin overexpression (Fig. 7A). However, treatment with TCDD (1, 10 or 100 nM), which suppressed the proliferation of wild-type RKO cells, did not exhibit a significant effect on the proliferation of transfectants with or without CH223191, an inhibitor of AHR signaling (Fig. 7B). Additionally, although treatment with TCDD (1, 10 or 100 nM) significantly stimulated the death of wild-type RKO cells (Fig. 7C), it did not have a significant effect on the death of transfectants with or without CH223191, an inhibitor of AHR signaling (Fig. 7D). These observations indicate that regucalcin overexpression depresses AHR-dependent repression of proliferation and promotion of death of RKO cells.

Additionally, the effects of TCDD (10 nM) on the levels of AHR, CYP1A1, p53, Rb and p21 in the regucalcin-overexpressing RKO cells were determined (Fig. 8A and B). TCDD treatment on transfectants cells did not appear to have a significant effect on CYP1A1 expression, since the effect of TCDD treatment on AHR-dependent CYP1A1 levels were depressed by regucalcin overexpression. Regucalcin overexpression has been demonstrated to increase the levels of p53, Rb and p21 in RKO cells (43) and other human cancer cells (50,51). Notably, the effects of TCDD in increasing p53, Rb and p21 levels were potentiated by regucalcin overexpression. Since TCDD treatment increased regucalcin levels in wild-type RKO cells (Fig. 5), the effects of TCDD on increasing the levels of p53, Rb and p21 in RKO cells are likely to depend, at least in part, on the elevation in levels of regucalcin.

Discussion

Human colorectal cancer is diagnosed as the third most common cancer type in USA in 2016 and its 5-year survival rate remains poor at 55%, in spite of the promotion of novel therapeutic strategies (3-8). Identification of novel biomarker targets may ultimately cause the prolongation of survival of patients with

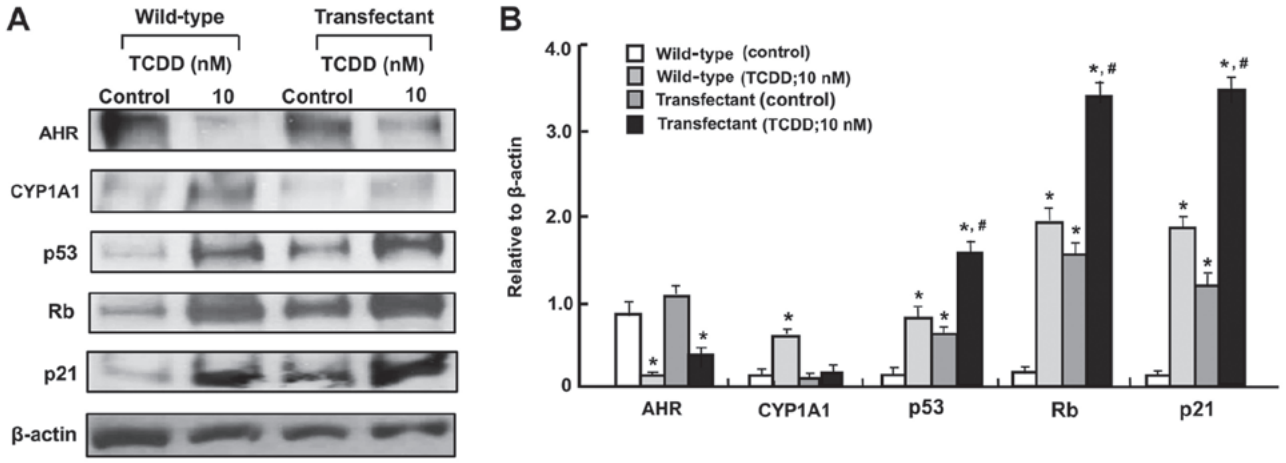

Figure 8. The TCDD-induced increase in CYP1A1 levels are suppressed in the regucalcin-overexpressing RKO human colorectal cancer cells *in vitro*. The wild-type RKO cells or regucalcin-overexpressing transfectants ($1x10^6$ cells/ dish) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone in the presence or absence of vehicle (1% dimethyl sulfoxide) or TCDD (10 nM) for 3 days and then cell lysates were centrifuged. Subsequently, 40 μg of the supernatant protein per lane were separated by SDS-PAGE and transferred to nylon membranes for western blotting using specific antibodies against the indicated proteins. Representative data from three independent experiments using different cell preparations are presented, and data are presented as mean ± standard deviation. (A) Representative film images of the TCDD effect. (B) Presented relative to β-actin of the TCDD effect. *P<0.01, vs. wild-type (control). $^\#$P<0.01, vs. wild-type (TCDD; 10 nM) or transfectant (control). One-way analysis of variance and Tukey-Kramer post hoc test were used. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Rb, retinoblastoma; AHR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 family 1 subfamily A member 1.

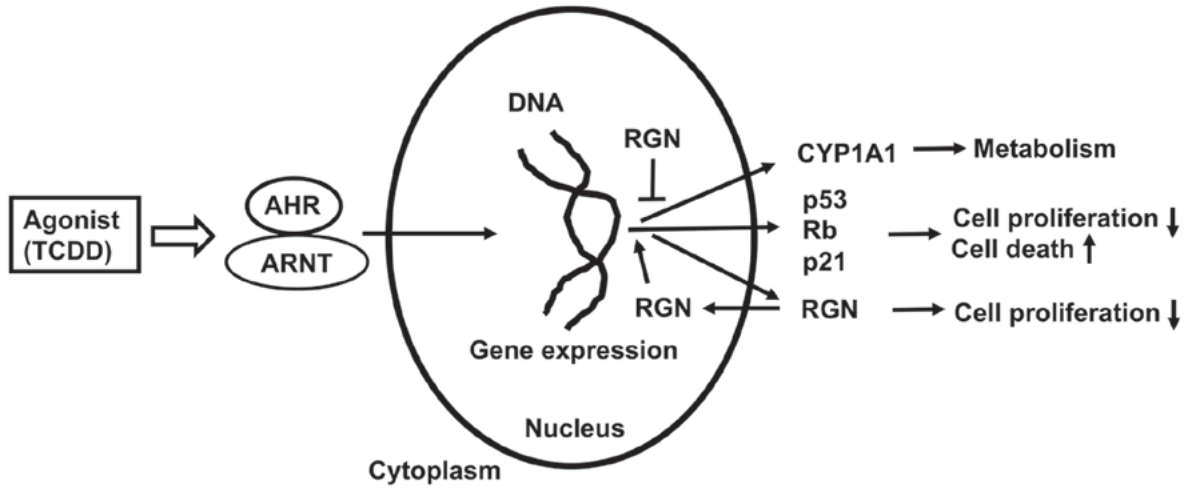

Figure 9. Schematic diagram of the mechanistic association between TCDD, AHR, CYP1A1, RGN and other molecules in RKO human colorectal cancer cells. TCDD activates AHR signaling by binding to ARNT. The complex is translocated into the nucleus and regulates expression of various genes. TCDD-activated AHR signaling enhances expression of various genes, including CYP1A1, RGN, p53, Rb and p21. Overexpressed RGN regulates the suppression of pathways of AHR signaling associated with CYP1A1, resulting in inhibition of metabolic pathways. Furthermore, overexpressed RGN enhances the expressions of p53, Rb and p21, which is increased via TCDD-activated AHR signaling, revealing a potential suppressive effect of cell proliferation and stimulatory effect of cell death. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Rb, retinoblastoma; AHR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 family 1 subfamily A member 1; RGN, regucalcin; ARNT, AHR nuclear translocator.

colorectal cancer (9). In the present study, it was demonstrated that TCDD treatment suppresses the growth and proliferation, and stimulates the death, of RKO human colorectal cancer cells. These effects of TCDD were demonstrated to be blocked by the treatment with CH223191, an inhibitor of AHR signaling (47), indicating that the action of TCDD is at least partially mediated through the AHR signaling pathway. The observations thus demonstrate that the enhanced AHR signaling serves a suppressive role in the development of human colorectal cancer cells.

To investigate the mechanism of action of TCDD, it was first demonstrated that the AHR and CYP1A1 proteins are present in RKO cells, consistent with previous studies, demonstrating that these mRNAs are expressed in these cells *in vitro* (34,35). In the present study, TCDD treatment was demonstrated to be caused a reduction of AHR levels and an elevation of CYP1A1 levels in the cytosol, including endoplasmic reticulum of RKO cells. TCDD treatment has been demonstrated to enhance the translocation of cytoplasmic AHR into the nucleus and increases CYP1A1 expression (11,12,32). Notably, TCDD treatment also elevated the levels of NF-κB p65 and β-catenin, which are crucial transcription factors implicated in the manifold process of cell signaling, and the levels of p53, Rb,

p21 and regucalcin, which are pivotal repressors of the growth of tumor cells (48,49). TCDD treatment did not change the level of Ras, which acts upstream in Akt signaling. β-catenin has been demonstrated to enhance regucalcin expression in HepG2 cells *in vitro* (52). It has also been reported that p53 modulates Hsp90 ATPase activity, which is implicated in AHR-dependent activation of gene expression (53). These signaling factors may be partially implicated in mediating the action of TCDD on the proliferation and death of RKO cells. Whether or not these molecules serve a role in the expression of the AHR gene remains to be elucidated.

Furthermore, it was determined that the effects of TCDD are attenuated in the regucalcin-overexpressing RKO cells. Overexpression of regucalcin has been demonstrated to repress the proliferation and death of RKO cells *in vitro* (43). Notably, regucalcin overexpression was demonstrated to decrease AHR and CYP1A1 levels in RKO cells, indicating that overexpressed regucalcin suppresses AHR signaling in RKO cells. Regucalcin has been indicated to translocate from the cytoplasm to nucleus in various types of normal and cancer cells, including liver and kidney (48,49), and it regulates the gene expressions of various proteins, including p53 and Rb, apparently acting as a novel transcriptional factor via binding nuclear DNA (48,49,54). Thus, it was considered that regucalcin serves a crucial role as a novel suppressor of AHR signaling. Notably, it was considered that TCDD treatment, which exhibits a repressive effect on the proliferation and a promoting effect on the death of wild-type RKO cells, did not have such effects in regucalcin-overexpressing RKO cells. These results support the view that AHR signaling is depressed by regucalcin overexpression in RKO cells *in vitro*.

Subsequently, whether the action of TCDD on the levels of AHR, CYP1A1, p53, Rb and p21 was attenuated in regucalcin-overexpressing RKO cells was investigated. Whereas TCDD treatment decreased AHR levels and increased CYP1A1 levels, these effects were determined to be depressed by regucalcin overexpression, indicating that AHR signaling activated by TCDD, an agonist, is suppressed by regucalcin overexpression. Notably, the effects of TCDD in increasing p53, Rb and p21 levels were demonstrated to be potentiated by regucalcin overexpression. Overexpression of regucalcin has been demonstrated to increase the levels of p53, Rb and p21 in RKO cells (43), and in other types of human cancer cells (50,51). Additionally, TCDD treatment increased regucalcin levels in wild-type RKO cells. These observations indicate that the action of TCDD in increasing the levels of p53, Rb and p21 in RKO cells are mediated, at least in part, via increases in regucalcin. Additionally, regucalcin overexpression suppressed the activation of AHR signaling associated with CYP1A1 expression. It is not known whether the deficiency of regucalcin enhances AHR signaling, although this remains to be elucidated using regucalcin siRNA. It is possible that the activation of AHR signaling enhances regucalcin gene expression, and that increased regucalcin suppresses AHR signaling pathways. This suppressive effect may result in inhibition of metabolic pathways associated with CYP1A1. The schematic diagram to demonstrate the mechanistic association between TCDD, AHR and regucalcin is depicted in Fig. 9. Collectively, AHR signaling may serve a crucial role in suppression of the growth of colorectal cancer cells, probably mediated via manifold molecules linked to tumor suppression.

In conclusion, the present study demonstrates that the agonist of AHR signaling, TCDD, suppresses the growth of human colorectal cancer cells and stimulates their death, via AHR signaling, probably as the result of stimulation of manifold molecules in regulating various signaling pathways. Therefore, targeting AHR signaling may cause an antitumor effect *in vivo*, providing a novel strategic tool for therapy of various cancer types.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MY conceived and designed the study. MY performed the experiment, and MY and OH discussed the data. MY wrote the manuscript, and OH reviewed and edited the manuscript. All authors read and approved the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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