Gene Expression Networks Underlying Retinoic Acid–Induced Differentiation of Human Retinoblastoma Cells

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Purpose. To understand the genetic regulatory pathways underlying the retinoic acid (RA) induction of cone arrestin, gene array technology and other molecular tools were used to profile global gene expression changes in human retinoblastoma cells.

Methods. Weri-Rb-1 retinoblastoma cells were cultured in the absence or presence of RA for various periods. DNA microarray analysis profiled gene expression followed by real-time PCR and Northern and immunoblot analyses to confirm the change in expression of selected retinal genes and their gene products. Additional methodology included flow cytometry analysis, immunocytochemistry, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay.

Results. DNA microarray analysis of approximately 6800 genes revealed RA-induced upregulation of cone-specific genes and downregulation of rod-specific genes in Weri-Rb-1 cells. Other significantly upregulated mRNAs included chicken ovalbumin upstream promoter-transcription factor (COUP-TF1), retinoid X receptor (RXR)-γ, thyroid hormone receptor (TR)-β2, and guanylyl cyclase-activating protein (GCAP)-1. Real-time PCR and/or Northern blot analysis confirmed the expression changes of a subset of genes including the upregulation of a pineal- and retina-specific transcription factor, CRX. RA treatment also led to G_0/G_1 cell cycle arrest and increased both the intensity of human cone arrestin (hCAR)-immunoreactivity and the number of apoptotic cells. The cell-cycle–arrest stage correlated with the observed microarray results in which the RA treatment downregulated critical genes such as cyclins (cyclin E, cyclin D3) and cyclin-dependent kinases (CDK5, CDK10).

Conclusions. These data suggest that RA induces a subpopulation of retinoblastoma cells to differentiate toward a cone cell lineage while selectively leading other cells into apoptosis. (*Invest Ophthalmol Vis Sci.* 2003;44:996–1007) DOI:10.1167/iovs.02-0434

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Retinoic acid (RA) treatment of immortalized human retinoblastoma cells Weri- Rb-1 and Y79 dramatically induces the mRNA and protein expression of a cone-specific gene, cone arrestin (CAR), but not its rod-specific counterpart, rod arrestin.[1,2] Because of our interest in the visual developmental pathway, especially cone photoreceptors, and the limited availability of human tissue, the retinoblastoma cell lines were used as an in vitro model system for studying photoreceptor-specific gene regulation.[2] RA induces the specific expression of CAR in this cell line and regulates numerous pathways involved in the differentiation of other cell types. What effect does RA have on cell fate when the differentiation pathway is triggered in these immortalized retinoblastoma cells?

With the advent of DNA microarray technology, the simultaneous study of global gene expression patterns of human retinal genes is feasible. Also, these new advances provide an efficient means of gaining critical insights into the expression, regulation, and potential function of genes that may contribute to human photoreceptor development, for which information is not currently available. To this end, we used commercially prepared microarrays (huGene FL; Affymetrix, Santa Clara, CA) to examine the expression of 6800 known genes in Weri-Rb-1 retinoblastoma cells and their RA-induced, differentiated progeny. These gene expression profiles are highly reproducible and represent modifications of a variety of molecular markers, including genes involved in phototransduction pathways; those expressing transcription factors, growth factors and their receptors, and cytokines and their receptors; and those serving as intracellular signal-transduction modulators and effectors. Our investigation was focused on the rod and cone photoreceptor-specific genes and the evaluation of the parameters of cell cycle, differentiation, and apoptosis of Weri-Rb-1 cells treated with RA. Moreover, our attention was also concentrated on the potential role of retina-specific transcription factors in the developmental cell fate when treated with RA.

Materials and Methods

Cell Culture and Treatment

Weri-Rb-1 and Y79 retinoblastoma cells (American Type Culture Collection, Rockville, MD) were maintained and treated with either all-*trans* RA (ATRA) or the drug vehicle dimethyl sulfoxide (DMSO), as previously described.[2]

Microarray Probe Preparation, Hybridization, and Scan

The RNA samples were processed as recommended by the manufacturer (Affymetrix). Briefly, total RNA was isolated from Weri-Rb-1 cells treated with either 10 μM of ATRA or DMSO (control) for 3 hours, 48 hours, or 7 days, using extraction reagent (Trizol; Life Technologies, Inc., Gaithersburg, MD). Reverse transcription was performed on 10 μg total RNA with the use of a kit and an oligo(dT)$_{24}$-anchored T7 primer (SuperScript Choice System; Life Technologies, Inc.). After second-strand synthesis, the double-stranded cDNA was cleaned up by extraction with phenol-chloroform-isoamyl alcohol and recovered by ethanol precipitation. A transcript labeling kit (BioArray HighYield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY) was

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TABLE 1. Sense and Antisense Primers for Amplification of Targeted Messages with Real-time PCR

mRNA Target	Sense (5′) Primers	Antisense (3′) Primers	Amplicon Size (bp)
ARR3 (hCAR)	5′-GCACAATTATTAGACCGGG-3′	5′-GCCTTTCCGCGTAAAC-3′	218
PDE6C	5′-CTGCCTCAGGTAGTGCT-3′	5′-GGTATGCCGTTCCGGG-3′	386
OPNIMW	5′-CCTTCCAGTTTATGAACCGGC-3′	5′-GGCGGGATGGGTAGGAG-3′	158
GNAT2	5′-AGGTTACGCAGGGGGA-3′	5′-CGTGCGCCACTGCATC-3′	324
PDE6H	5′-CCACCCCACGCAAAGG-3′	ACAGGGCAAAAGCAACCA	265
GNGT1	5′-AAGAACGATCTGGCGAGG-3′	5′-ACCACACCCCAATTTTTATTAAAAGTTTAC-3′	247
CNCG	5′-ATGGCAGCTACTTCGGTG-3′	5′-TTCCGCTCCAGGTCCC-3′	431
GCAP1	5′-AGGCTTAGCTCGCCTCT-3′	5′-AATGCAAAGCGCACAAGT-3′	375
COUP-TF1	5′-CATGTCTGCCGACCGC-3′	5′-ACCTACCAAACGGACGAAG-3′	328
RXRG	5′-GGACGATAAGGAAGGACC-3′	5′-GGGGAATACGCTTGGC-3′	372
THRB	5′-GTATGGGGCTGGAGAA-3′	5′-GTCCAAGTCAGAGTCCT-3′	304
CRX	5′-TGGCACGATTGTGACC-3′	5′-GTTTCACTCTTGTTGCCC-3′	272
OTX2	5′-TAAGCAACCGCCTTACG-3′	5′-GCACTTAGCTCTTCGATT-3′	289
NRL	5′-CGCGATCTCTACAAGGC-3′	5′-TTTGCGCGGTCATACA-3′	212
NR2E3	5′-AGGAAACTATCTCTCGGT-3′	5′-TTCCCTATGGTCTTGCG-3′	280
β-Actin	5′-CTTCCCCTCCATCGTGGG-3′	5′-GTGGTACGGCCAGAGGCG-3′	355

used for the production of hybridizable biotin-labeled RNA targets by in vitro transcription from T7 RNA polymerase promoters. The cDNA prepared from total RNA was used as a template in the presence of a mixture of unlabeled ATP, CTP, GTP, and UTP and biotinylated CTP and UTP. In vitro transcription products were purified (RNeasy Mini Kit; Qiagen, Valencia, CA) to remove unincorporated NTPs and fragmented by incubation at 94°C for 35 minutes. The fragmented sample cRNA (complementary RNA) was stored at −20°C until the hybridization was performed.

The following steps were then performed at the Children's Hospital LA Microarray Core Facilities. Gene microarray chips (huGene FL GeneChips; Affymetrix) containing 6800 genes were used to profile mRNA expression. The biotinylated cRNA (10 μg/chip) was hybridized for 16 hours at 40°C to a set of oligonucleotide arrays in a hybridization system (GeneChip Fluidics Station 400; Affymetrix). After hybridization, the microarray underwent a series of stringency washes and was stained with streptavidin-conjugated phycoerythrin. Probe arrays were scanned with a confocal laser scanner (Affymetrix).

Data Analysis

Hybridization data from text files were imported to a spreadsheet (Excel; Microsoft, Redmond, WA). Data analysis was performed with software developed by the microarray manufacturer (Genechip Analysis; Affymetrix) and the multiples of change between the hybridization intensities of RA-treated and control samples were obtained.

Real-Time PCR

Three micrograms of total RNA from Weri-Rb-1 cells treated with either 10 μM of ATRA or DMSO (control) for 48 hours or 7 days were reverse transcribed with 200 U reverse transcriptase (Superscript II RNase H^-; Invitrogen, San Diego, CA) in a volume of 20 μL, with 100 μM random primers (Invitrogen) used according to the manufacturers' instructions. The quantification of the selected 15 genes by real-time PCR was performed on a fluorescence detection system (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany). Oligonucleotide primers were designed using the accompanying software (LightCycler Probe Design Software; Roche Molecular Biochemicals). The nucleotide sequences of the primers used are shown in Table 1. The optimal PCR reactions for all investigated genes were established with a kit (LightCycler Fast Start DNA Master SYBR Green I Kit; Roche Molecular Biochemicals), according to the manufacturer's instructions. Annealing temperatures and $MgCl_2$ concentrations were optimized to create a one-peak melting curve. In addition, the PCR reactions were recovered after each PCR analysis and amplicons were checked by agarose gel electrophoresis for a single band of the expected size.

Real-time PCR mix was prepared as follows (to the indicated end-concentration): 14.2 μL water, 0.8 μL $MgCl_2$ (2 mM), 1 μL forward primer (0.5 μM), 1 μL reverse primer (0.5 μM), and 2 μL of master mix from the kit (LightCycler–Fast Start DNA Master SYBR Green I; Roche Molecular Biochemicals). Nineteen microliters of the PCR mix was placed in the glass capillaries and a 1 μL volume of cDNA was added as a PCR template. Capillaries were closed, centrifuged, and placed into a cycling rotor. A four-step experimental run protocol was used: a denaturation program (10 minutes at 95°C), an amplification and quantification program repeated 40 times (15 seconds at 95°C, 45 seconds at 55°C or at 57°C for some genes, 60 seconds at 72°C, with a single fluorescence measurement), a melting curve program (45°C for 30 seconds with a continuous fluorescence measurement), and a cooling program down to 40°C.

The reaction product was characterized by the point during cycling when amplification of PCR products was first detected (crossing point) with the manufacturer-provided software (LightCycler, ver. 3.5; Roche Molecular Biochemicals). The results are presented as the ratios of mRNA expression in treated cells in relation to the amount present in untreated cells and are normalized to an internal control gene, β-actin, for different mRNA input and reverse transcript efficiencies.

Northern Blot Analysis

Northern blot analysis was performed as previously described.[1,2] Probes used for hybridizations were prepared by RT-PCR with RA-treated or untreated Weri-Rb-1 cell cDNA as a template and verified by DNA sequencing.

Immunoblot Analysis

Immunoblot analysis was performed as described.[2] The immobilized proteins were detected on the membrane with an enhanced chemiluminescence kit with affinity-purified anti-bovine CRX peptide polyclonal antibody[3] or anti-human thyroid hormone receptor (TR)-β2 (N-16) peptide goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1: 1000 and 1:100 dilutions, respectively. Anti-rabbit (Bio-Rad Laboratories, Richmond, CA) or anti-goat (Santa Cruz Biotechnology) secondary antibodies were used at 1:10,000 and 1:5,000 dilutions.

Determination of Cell Cycle Stage by Fluorescence-Activated Cell Sorting

Cellular DNA content was analyzed with a flow cytometry system (FACScan; BD Bioscience Immunocytometry Systems, San Jose, CA) at the Norris Cancer Center Core Flow Cytometry Facility of the Keck School of Medicine of the University of Southern California (USC). Briefly, treated or untreated Weri-Rb-1 cells were harvested and washed, fixed in cool 70% ethanol for at least 2 hours, and then incubated with 1 mL propidium iodide/Triton X-100 staining solution

with RNase A for either 15 minutes at 37°C or 30 minutes at room temperature. For each cell population at least 10,000 cells was analyzed by flow cytometry. The proportion in G_0/G_1, S, and G_2/M phases was estimated by using the cell cycle analysis software (Cell Quest; BD Bioscience).

Immunocytochemistry

Weri-Rb-1 cells from suspension culture were gently dissociated and plated onto poly-D-lysine eight-well chamber slides (Cellware; BD Bioscience) at 3×10^5 cells/mL and maintained for 24 hours before the addition of RA (10 μM) or DMSO. Medium was changed and chemicals were reapplied every 2 days. After treatment for 5 days, medium was removed, and the cells were processed for immunofluorescent staining with the affinity-purified anti-hCAR polyclonal peptide antibody LUMIF,[1] and a Cy3-conjugated goat anti-rabbit secondary antibody, as described.[3] The slides were mounted and photographed with a confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany).[3]

Analysis of Apoptosis

To detect apoptosis in individual cells, Weri-Rb-1 cells were seeded onto poly-D-lysine eight-well chamber slides and treated as described earlier in the immunocytochemistry study. After a 48-hour treatment with either RA or DMSO, cells were fixed in 4% paraformaldehyde in PBS for 25 minutes at 4°C, rinsed with PBS and permeabilized for 5 minutes on ice in 0.2% Triton X-100 in PBS. Apoptotic cells were visualized by means of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay,[4] with an apoptosis detection system (Apoptosis Detection System; Promega), according to the manufacturer's instructions. After labeling with fluorescein-12-dUTP, the slides were rinsed with 2× SSC, and propidium iodide (PI) was added to stain all cells. The slides were then mounted and photographed. Apoptotic cells were quantified by counting cells labeled with fluorescein-12-dUTP in 10 random fields per condition under a microscope. The total number of apoptotic cells was divided by the total number of cells stained with propidium iodide in the same field, yielding the percentage of apoptotic cells within each specified field.

Results

RA Induction of Global Transcriptional Profile Changes in Weri-Rb-1 Cells

To determine whether induction of cone arrestin by RA treatment is accompanied by transcriptional alterations of other visual transduction genes in retinoblastoma cells, we conducted studies using oligonucleotide array technologies (Affymetrix) to monitor gene expression changes in Weri-Rb-1 cells treated with either ATRA or DMSO (control). To ensure reproducibility of the microarray results, each experiment was repeated twice. Genes that were either up- or downregulated were defined as those signals that showed at least a twofold difference between RA- and DMSO-treated cells.[5–7] In a total of 6800 genes examined, with the 3-hour treatment only 55 were upregulated and 17 were downregulated. With the 48-hour treatment, 506 genes were upregulated and 552 genes were downregulated, and with the 7-day treatment 310 genes were upregulated and 154 were downregulated. A complete list of these up- and downregulated genes is available on our Web site (http://eyesightresearch.org). Representative up- and downregulated genes are shown in Tables 2 and 3, respectively, with their GenBank accession numbers (http://www.ncbi.nlm.nih.gov/Genbank; GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

RA stimulated numerous cone-specific genes, not only cone arrestin, but also genes for cone phosphodiesterase (PDE)-α subunit, green cone pigment gene, and cone transducin alpha subunit, after either 48 hours or 7 days of treatment. Guanylyl cyclase-activating protein (GCAP)-1, which has a higher expression level in cones than in rods,[8] increased by 2.2-fold after 3 hours of RA treatment and continued to increase with longer treatment. TRβ2, which is essential in the development of green cone photoreceptors,[9] increased by 7.2-fold after 48 hours, but decreased 5-fold after 7 days of RA treatment. The retinoic acid receptor (RXR)-γ was upregulated after both 48 hours (3.3-fold) and 7 days (2.8-fold) of RA treatment. Expression of chicken ovalbumin upstream promoter-transcription factors (COUP-TF1) increased 4.4-, 16.2-, and 13.6-fold after 3 hours, 48 hours, and 7 days of RA treatment, respectively.

A dramatic contrast was observed in visual genes associated with components of the rod phototransduction pathway. All three subunits of rod transducin (α, β, and γ) and the rod photoreceptor cGMP-gated channel were each downregulated significantly, as shown in Table 3. Cone rhodopsin-sensitive cGMP 3′5′-cyclic phosphodiesterase gamma subunit (PDE6H), which is expressed in blue cone photoreceptor cells, was also among those downregulated genes. Adenylyl cyclase type II was downregulated dramatically after both 48 hours (55.1-fold) and 7 days (71.5-fold) of RA treatment. It is notable that transducin-like enhancer protein-1, a human protein homologous to *Drosophila groucho* (Gro) that affects neuronal fate through negative regulation of gene expression,[10,11] decreased 2.2-, 30-, and 4.9-fold after a 3 hours, 48 hours, and 7 days of RA treatment, respectively. Major histocompatibility complex (MHC) class I, previously identified in differentiated Y79 cells after RA treatment[12] and essential in central nervous system development,[13] increased 2.9-fold after 3 hours and 6.2-fold after 48 hours.

Another prominent observation of our study is that numerous cell cycle regulatory proteins, including cyclins, cyclin-dependent kinases (cdks), cdk inhibitors (CKIs), and the E2F families of proteins, which constitute a network of interacting factors that govern exit from or passage through the mammalian cell cycle, were downregulated by RA treatment, whereas the retinoblastoma (Rb) family members Rb1 and Rb-related protein p107 were upregulated after 48 hours of RA treatment. In contrast, the DNA fragmentation factor-45 α-subunit and caspase 8, which are coupled with apoptosis,[14,15] were increased 17.6-fold and 3.2-fold, respectively, after 48 hours.

Rb is a tumor suppressor involved in the transcriptional regulation of genes required for the G_1-to-S phase transition of the cell cycle,[16] and the mutation of this gene is the cause of hereditary retinoblastomas.[17] Whereas all normal tissues and many tumor cells express an Rb mRNA of 4.7 kb, retinoblastomas were found either to have no Rb expression or to have Rb transcripts of reduced size (4.0 kb).[17] Weri-Rb-1 has a homozygous deletion of the entire Rb gene, whereas Y79 contains a partial deletion of one allele and an uncharacterized mutation in the other.[18–20] In our experimental system, the upregulated Rb mRNA in Weri-Rb-1 cells by RA treatment is most likely a cross reaction with other Rb-related genes that are upregulated by RA.

Validation of Array Results by Real-Time PCR

Eleven genes with significant expression changes by microarray analysis, including those expressed in either cone or rod photoreceptors, were selected for confirmation of their relative expression by real-time quantitative PCR. Because of the importance of photoreceptor-specific transcription factors in the regulation of rod and cone photoreceptor differentiation, we also determined the relative change in expression of CRX, a homeobox gene[3,21,22]; OTX2, another member of the homeodomain-containing transcription factors, which is highly homologous to CRX and expressed in brain and retina[23–25];

TABLE 2. Up-regulated Gene Expression by RA Treatment in Weri-Rb-1 Cells

Increase (Change Multiple)	GenBank Accession No.	Gene	Function
3 Hours			
37.4	L24564	Ras associated with diabetes (RAD)	GTP-binding protein
4.4	X12795	V-erba–related ear-3 protein, COUP-TF1 (NR2F1)*	Nuclear orphan receptor
2.9	M16714	MHC class I (HLA-E)	CNS development and plasticity
2.8	M18185	Gastric inhibitory polypeptide (GIP)	Potent stimulator of insulin secretion
2.5	X63575	Plasma membrane calcium ATPase (ATP2B4)	Calcium transporting
2.2	L36861	Guanylate cyclase–activating protein (GCAP1)	Phototransduction regulation
48 Hours			
59.2	X60486	H4 histone family, member G (H4FG)	DNA replication
48.8	M90360	A kinase (PRKA) anchor protein 13, Ht31 (AKAP13)	PKA pathway downstream regulation
47.6	L11672	Zinc finger protein 91 (HTF10)	Transcription regulation
40.5	M37457	ATPase, Na+/K+ transporting, alpha3 chain (ATP1A3)	Energy providing
17.6	U91985	DNA fragmentation factor-45, alpha subunit (DFFA)	Trigger DNA fragmentation during apoptosis
16.9	X04434	Insulin-like growth factor 1 receptor (IGF-IR)	Regulation of proliferation and differentiation
16.2	X12795	V-erba related ear-3 protein, COUP-TF1 (NR2F1)*	Nuclear orphan receptor
10.6	U50079	Histone deacetylase, HD1 (HDAC1)	Core histones deacetylation
10.2	M18185	Gastric inhibitory polypeptide (GIP)	Potent stimulator of insulin secretion
9.6	U37431	Homebox A 1 (HOXA1)	Cell differentiation, embryogenesis
9	J02902	PP 2A, subunit A, alpha isoform (pr65) (PPP2R1A)	Protein dephosphorylation
8.7	L36861	Guanylate cyclase–activating protein (GCAP1)	Phototransduction regulation
7.5	X66945	Fibroblast growth factor receptor 1 (FGFR1)	Limb induction
7.2	X74497	Thyroid hormone receptor, beta-2 (THRB)†	Nuclear receptor
6.7	L39064	Interleukin-9 receptor (IL9R)	Cytokine receptor
6.6	M28983	Interleukin-1, alpha (IL1A)	Cytokine
6.2	M94880	MHC class 1 (HLA-E)	CNS development and plasticity
6.1	U03626	Cone arrestin (ARR3)†	Cone phototransduction
5.3	M19989	Platelet-derived growth factor, alpha chain (PDGFA)	Growth factor
5	L24564	Ras associated with diabetes (RAD)	GTP-binding protein
5	M15400	Retinoblastoma 1 (RB1)	Tumor suppressor
4.9	S66431	Retinoblastoma-binding protein-2 (RBP2)	Interaction with RB1
4.1	M31994	Cytosolic aldehyde dehydrogenase (ALDH1)	Retinoid metabolic regulator
3.8	L14812	Retinoblastoma-related protein (p107) (RBL1)	RB family member
3.6	X04571	Epidermal growth factor (EGF)	Neuroprotective role in the retina
3.5	M14764	Nerve growth factor receptor (NGFR)	Growth factor receptor
3.3	U38480	Retinoid X receptor-gamma (RXRG)*	Nuclear receptor
3.2	X98172	Caspase 8, apoptosis-related cysteine protease (CASP8)	Apoptosis
2.8	L14778	PP2B, catalytic subunit, alpha isoform (PPP3CA)	Protein dephosphorylation
2.8	Z18859	Cone transducin alpha subunit (GNAT2)†	Cone phototransduction
2.6	L07590	PP 2A, subunit B, B′-pr72/pr130 (PPP2R3)	Protein dephosphorylation
2.6	D50640	Phosphodiesterase 3B, cGMP inhibited (PDE3B)	Fat metabolism
2.2	M61176	Brain-derived neurotrophic factor (BDNF)	Survival of neuronal populations
2.1	U89326	Bone morphogenetic protein receptor, type 1B (BMPR1B)	Growth regulator of CNS
2.1	Z69030	PP2A, subunit B, B′ gamma isoform (PPP2R5C)	Protein dephosphorylation
2	X78520	Chloride channel (CLCN3)	Neuronal cell function
7 days			
27.4	L24564	Ras associated with diabetes (RAD)	GTP-binding protein
19	M18185	Gastric inhibitory polypeptide (GIP)	Potent stimulator of insulin secretion
16.6	U37431	Homebox A 1 (HOXA1)	Embryogenesis and cell differentiation
16.5	D10495	Protein kinase C, delta type (PRKCD)	Signal transduction
15.9	X98833	Sal (Drosophila)-like 1, Hsal1 (SALL1)	Transcription factor
14.6	L36861	Guanylate cyclase–activating protein (GCAP1)	Phototransduction regulation
13.6	X12795	V-erba related ear-3 protein, COUP-TF1 (NR2F1)*	Nuclear orphan receptor
11.3	U31973	Cone alpha′ PDE (PDE6C)†	Cone phototransduction
9.9	U67733	PDE2A3 (PDE2A)	Hydrolyze cyclic nucleotides
8	K03494	Green cone pigment gene 1 (OPN1MW)†	Cone phototransduction
7.7	M16707	H4 histone family, member 2 (H4F2)	DNA replication
7.5	Z80783	H2B histone family, member L (H2BFL)	DNA replication
7.2	M86528	Neurotrophin 5 (NTF5)	Survival of neurons
6.6	M57466	MHC class II (HLA-DPB1)	Immunoglobulin superfamily
6.5	U03626	Cone arrestin (ARR3)†	Cone phototransduction
5.3	L39064	Interleukin-9 receptor (IL9R)	Cytokine receptor
5.1	Z18859	Cone transducin alpha subunit (GNAT2)*	Cone phototransduction
3.8	L15388	G-protein–coupled receptor kinase (GRK5)	Protein phosphorylation
3.5	X00129	Retinol binding protein-4, interstitial (RBP4)	Defect causes night blindness
3.5	S62028	Recoverin (RCV1)	Phototransduction
3.4	M19989	Platelet-derived growth factor alpha chain (PDGFA)	Growth factor
3.2	M74587	Insulin-like growth factor–binding protein 1 (IGF-BP1)	Cell growth and proliferation
3.1	U18991	Retinal pigment epithelium–specific 65 kDa (RPE65)	Defect in retinal dystrophy
2.9	Z46632	3,5-cAMP-specific phosphodiesterase (PDE4C1)	Cyclic nucleotide metabolism
2.8	U38480	Retinoid X receptor-gamma (RXRG)*	Nuclear receptor
2.6	X59770	Interleukin-1 receptor, type 2 (IL1R2)	Cytokin receptor
2.5	X77197	Chloride channel (CLCN4)	Signal transduction
2.4	M14764	Nerve growth factor receptor (NGFR)	Growth factor receptor

* Steroid/thyroid receptor superfamily transcription factors.
† Cone-specific genes.

Table 3. Downregulated Gene Expression by RA Treatment in Weri-Rb-1 Cells

Decrease (Change Multiple)	GenBank Accession No.	Gene	Function
3 hours			
−6.5	M20469	Clathrin, light polypeptide (CLTB)	Component of coated vesicles and pits
−2.2	M99435	Transducin-like enhancer protein-1 (TLE1)*	Transcriptional repression
48 hours			
−55.1	L21993	Adenylate cyclase, type II (brain) (ADCY2)	Membrane-bound, calmodulin-insensitive
−50.2	L11329	Dual specificity phosphatase2, PAC-1 (DUSP2)	Mitogenic signal transduction
−43.3	X68879	Empty spiracles (Drosophila) homologue-1 (EMX1)	Cell fate specificity in the developing CNS
−36.8	X04828	G-protein alpha–inhibiting activity polypeptide-2 (GNAI2)	Transmembrane signaling modulator
−31	S62027	Rod transducin gamma-1 subunit (GNGT1)†	Rod phototransduction
−30	M99435	Transducin-like enhancer protein 1 (TLE1)*	Transcriptional repression
−21.9	L13720	Growth arrest–specific protein 6 (GAS6)	Cell proliferation
−19.7	L05624	MAP kinase kinase 1 (MAP2K1)	Protein phosphorylation
−19.6	X80343	Cdk5, regulatory subunit 1(p35) (CDK5R1)	Neuron-specific activator of cdk5/TPKII
−19.4	M24899	Thryoid hormone receptor alpha (THRA)*	Nuclear receptor
−19.2	X57129	H1 histone family, member 2 (H1F2)	Basal repression of gene expression
−18	AC002076	G-protein gamma-1 subunit, rod (GNGT1)†	Rod phototransduction
−17.2	D16227	Hippocalcin-like 1 (HPCAL1)	Rhodopsin phosphorylation
−15.1	D86968	MAP3K4	Activation of CSBP2, P38 and JNK MAPK pathways
−14.4	Z11695	p38 (MAPK1)	Cell cycle
−13.4	D45399	Cone rhodopsin-sensitive cGMP 3′5′-cyclic phosphodiesterase, gamma (PDE6H)†	Blue cone phototransduction
−12.2	M20469	Clathrin, light polypeptide (CLTB)	Component of coated vesicles and pits
−12	J05633	Integrin beta 5 (ITGB5)	Cell adhesion
−11.9	J00277	C-Ha-ras1 proto-oncogene (HRAS)	Oncogenesis, intrinsic GTPase activity
−11.6	S37730	Insulin-like growth factor–binding protein 2 (IGF-BP2)	Cell proliferation
−11.1	M83667	C/EBP delta (CEBPD)*	Immune response
−11	L32976	MAP3K11	Regulator of JNK pathway
−10.8	X78342	Cyclin-dependent kinase, CDC2-like (CDK10)	Cell cycle
10.1	M96684	Purine-rich element binding protein A (PURA)	Initiation of DNA replication
−9.3	L23959	E2F-related transcription factor DP-1 (TFDP1)†	Cell cycle and DNA replication
−9	M58603	NF-kappa-B p105 subunit (NFKB1)†	Immune response
−8.7	U48707	Protein phosphatase 1 inhibitor (PPP1R1A)	Control intracellular cAMP level
−8.5	X12534	Member of RAS oncogene family (RAP2A)	Oncogen
−7.7	U90551	H 2A histone family, member L (H2AFL)	Replication dependent
−6.9	M24398	Parathymosin (PTMS)	Immune response
−6.7	U53174	Cell cycle checkpoint control protein (RAD9)	DNA repair
−6.7	U20734	Jun B proto-oncongene (JUNB)	Oncogenesis
−6.6	M19720	L-myc (MYCL1)	Oncogenesis
−6.4	M74093	Cyclin E (CCNE1)	Cell cycle
−6.3	S78085	Programmed cell death 2 (PDCD2)	Cell proliferation
−6.1	Y10659	Interleukin-13 receptor, alpha 1 (IL13RA1)	Cytokine receptor
−5.3	L37882	Frizzled (Drosophila) homologue 2 (FZD2)	Signal transduction
−4.7	U57317	P300/CBP-associated factor (PCAF)	Cell cycle
−4.6	M81933	Cell devision cycle 25A (CDC25A)	Cell cycle control
−3.9	M36430	Transducin beta-1 subunit (GNB1)†	Identical to rod cell transducin
−3.5	M99436	Transducin-like enhancer protein-2 (TLE2)*	Transcriptional repression
−3.5	X68277	Dual-specificity (tyr/thr) phosphatase-1 (DUSP1)	Dephosphorylation of MAP kinase ERK2
−3.4	U31556	E2F transcription factor-5 (E2F5)*	Cell proliferation
−3.4	U22398	Cdk-inhibitor-1C (p57K1P2) (CDKN1C)	Cell proliferation
−3.4	X83493	Fas/apo-1 (CD95) (TNFRSF6)	Apoptosis
−3.2	X85753	Cyclin-dependent kinase 8 (CDK8)	Cell cycle
−2.8	U67932	cAMP phosphodiesterase (PDE7A2)	Signal transduction
−2.8	U18062	TFIID subunit TAFII55 (TAF2F)	TATA box–binding protein
−2.8	M92287	Cyclin D3 (CCND3)	Cell cycle
−2.5	S76965	Protein kinase inhibitor alpha (PKIA)	Inhibitor of cAMP-dependent protein kinase
−2.5	M12174	Ras homologue gene family, member B (ARHB)	Signal transduction pathway
−2.5	U13737	Caspase-3	Apoptosis
−2.1	M36429	Transducin beta-2 subunit (GNB2)	Transmembrane signaling regulation
7 days			
−71.5	L21993	Adenylate cyclase, type II (brain) (ADCY2)	Calmodulin-insensitive, cAMP formation
−31.7	S62027	Rod transducin gamma-1 subunit (GNGT1)†	Rod phototransduction
−14.8	L33477	Br-cadherin (CDH12)	Cell adhesion
−10.2	M27318	Interferon alpha-4 (IFN-alpha-M1)	Inflammation
−9.1	U68111	Protein phosphatase inhibitor-2 (PPP1R2)	Inhibitor of protein-phosphatase 1
−5	X74497	Thyroid hormone receptor, beta-2 (THRB)*	Nuclear receptor
−4.9	M99435	Transducin-like enhancer protein (TLE1)*	Transcriptional repression

TABLE 3. (*continued*). Downregulated Gene Expression by RA Treatment in Weri-Rb-1 Cells

Decrease (Change Multiple)	GenBank Accession No.	Gene	Function
7 days (*continued*)			
−4.5	D14826	cAMP-responsive element modulator-2 (CREM)*	Cell proliferation
−4.4	M21574	Platelet-derived growth factor receptor, alpha chain	Growth factor receptor
−4.1	L00058	c-Myc (MYC)	Oncogenesis
−3.7	M87507	Caspase 1	Apoptosis
−3	Y10659	Interleukin-13 receptor alpha-1 (IL13RA1)	Cytokine receptor
−2.8	M24748	Thryoid hormone receptor alpha (THRA)*	Nuclear receptor
−2.6	S42457	Rod photoreceptor cGMP-gated channel (CNCG)†	Rod phototransduction
−2.6	Y08639	Transcription factor ROR-beta (RORB)*	Orphan nuclear receptor
−2.5	U16799	ATPase, Na+/K+ transporting, beta-1 subunit	Cell adhesion
−2.5	M61176	Brain-derived neurotrophic factor (BDNF)	Nerve growth factor
−2.5	U57629	Retinitis pigmentosa GTPase regulator (RPGR)	Guanine nucleotide-releasing factor

* Transcription factors.
† Rod-specific genes.

neuroretinal leucine zipper (NRL)[26–29]; and NR2E3, a photoreceptor-specific nuclear receptor.[30–32] The quality of the real-time PCR products is shown in Figure 1, and the relative multiples of change in expression of these genes as determined by microarray, real-time PCR, and Northern blot analyses are presented in Table 4.

The overall concordance of trends between the two techniques was 77% (e.g., an increase or decrease in gene expression seen by microarray in 48 hours and 7 days compared with real-time PCR). For those genes with results that agreed between the two experiments, 71% of these results indicated larger change multiples by real-time PCR than those identified by array analysis. This concordance includes both genes determined to be significantly changed and those genes determined not to have significantly changed. CRX was slightly upregulated after both 48 and 72 hours of RA treatment, which is consistent with the results of Northern blot analysis (Table 4 and later discussion). However, the expression of OTX2, NRL, and NR2E3 did not change after RA treatment of the Weri-Rb-1 cells, as shown by real-time quantitative PCR (Table 4).

Confirmation of CRX and TRβ2 Upregulation by RA

CRX is a homeobox gene specifically expressed in the photoreceptors of the developing and mature retina and is crucial in rod and cone photoreceptor differentiation.[21,22] Previously, we identified potential CRX elements in the cone arrestin promoter.[2,34] Because the microarrays (huGene FL; Affymetrix) did not contain the CRX gene, Northern blot analysis was used to examine CRX gene regulation by RA in Weri-Rb-1 and Y79 cells. A CRX-specific cDNA fragment, which is outside the homeodomain, was radioactively labeled and used as a probe for Northern blot analysis. As shown in Figure 2A, the expression of both the 3.9- and the 2.5-kb mRNA of CRX was induced dramatically by 6-day RA treatment in both cell types. To analyze the dynamics of this induction, a dose–response and time-course analysis of CRX mRNA expression was analyzed after RA treatment of Weri-Rb-1 cells. Figure 2B shows that RA enhanced CRX mRNA levels in a dose-dependent manner and that the maximal effect was achieved with 3 μM RA. In the time-course experiments, the expression of CRX mRNA was elevated above basal levels after 4 days of exposure to RA (Fig. 2C). Immunoblot analysis confirmed that the immunoreactive CRX protein was upregulated after 6 days of RA treatment (Fig. 2D). Immunoreactive NRL, a rod-specific transcription factor, was below detectable levels in these cells, whether

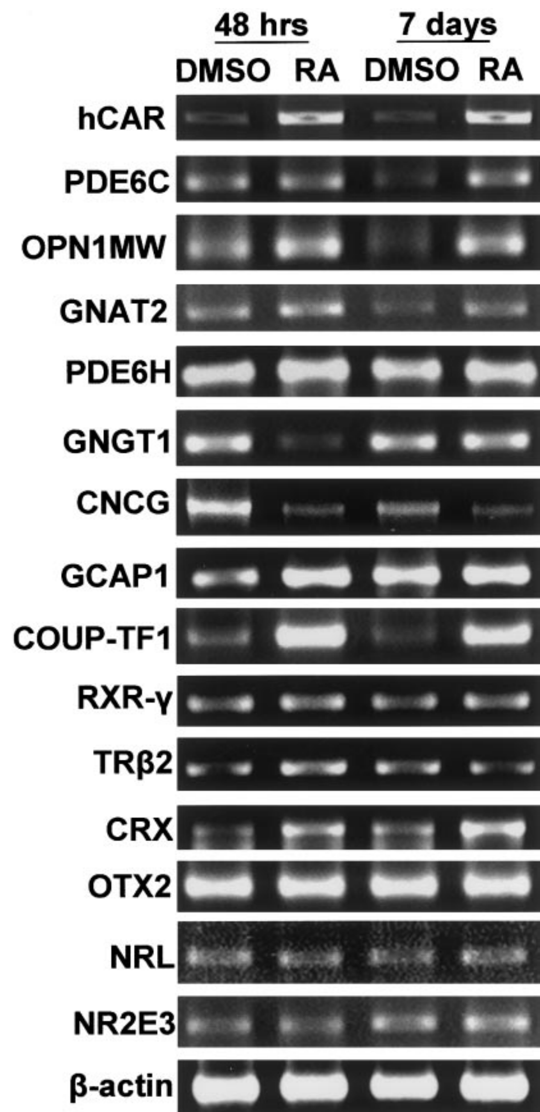

FIGURE 1. Representative real-time quantitative PCR analysis of gene expression changes in RA or DMSO (control) treated Weri-Rb-1 cells. β-actin was used as a normalization control for real-time PCR.

Table 4. Validation of Array-Based Gene Expression Profile by Real-Time PCR or Northern Blot Analysis

Gene	Array (48 h, 7 d)	Real-Time PCR (48 h, 7 d)	Northern Blot (48 h, 7 d)	Cell or Tissue Type
ARR3 (hCAR)	+6.1, +6.5	+4.21 ± 1.09, +7.12 ± 0.82	+16, +16	Pineal, cone
PDE6C	NC, +11.3	+1.52 ± 0.08, +18.51 ± 3.98	ND	Pineal, cone
OPN1MW	NC, +8	+5.39 ± 0.55, +14.09 ± 2.12	ND	Cone
GNAT2	+2.8, +5.1	+1.88 ± 0.005, −1.67 ± 0.20	ND	Pineal, cone
PDE6H	−13.4, NC	+1.11 ± 0.10, −0.12 ± 1.99	ND	Blue cone
GNGT1	−31, −31.7	−108.91 ± 11.75, −29.87 ± 4.21	ND	Pineal, rod
CNCG	NC, −2.6	+2.16 ± 0.29, −10.25 ± 2.45	ND	Rod
GCAP1	+8.7, +14.6	+4.85 ± 1.77, +22.10 ± 4.59	ND	Cone<<<rod*
COUP-TF1	+16.2, +13.6	+215.11 ± 9.05, +32.52 ± 13.69	ND	Developing CNS
RXRG	+3.3, +2.8	+2.43 ± 0.89, +0.23 ± 0.95	+4.5, +2.5	Cone<<<rod*
THRB	+7.2, −5	+2.11 ± 1.25, −2.15 ± 0.14	+1.4, −1	Cone<<<rod*
CRX	NA	+2.91 ± 1.65, +3.86 ± 1.96	+1, +3.3	Pineal, cone, rod, INL
OTX2	NA	+0.68 ± 1.62, −1.12 ± 2.30	ND	Pineal, cone, rod
NRL	NA	+1.71 ± 0.78, −0.78 ± 2.09	ND	Rod
NR2E3	NA	−1.32 ± 0.24, −0.06 ± 1.25	ND	Cone, rod
β-Actin	NC	+1.12 ± 0.04, +0.89 ± 0.13	NC	Wide expression
IRBP	NC	NA	NC[33]	Pineal, cone, rod
S-antigen	NC	NA	NC[33]	Pineal, rod

Array and Northern blot data are multiples of change, and PCR data are the mean multiple of change ± SEM of three to four assays. NA, not available; NC, no change; ND not done; CNS, central nervous system; INL, inner nuclear layer.

* The expression in cones is much higher than in rods.

treated or untreated with RA, by immunoblot analysis (antisera generously provided by Anand Swaroop, Department of Ophthalmology & Visual Sciences and Eccles Institute of Human Genetics, University of Michigan, Ann Arbor, MI; data not shown). These data suggest that CRX may have a role in a later stage of retinoblastoma cell differentiation due to RA treatment.

To further confirm the gene regulation of TRβ2 by RA observed by the microarray analysis, both Northern and immunoblot blot analyses were performed. Northern blot from the CRX time-course experiment was reprobed with a TRβ2 cDNA probe, which revealed multiple human TRβ2 mRNAs of 2.4, 2.5, 5.2, and 6.6 kb in Weri-Rb-1 cells. The expression of TRβ2 mRNA slightly decreased during the initial 12 hours, but increased above basal levels after 24 hours of RA treatment. It reached its maximum induction of 1.7-fold over the control by 5 days (120 hours) and thereafter decreased (Figs. 3A, 3B). Immunoblot analysis of the TRβ2 protein from this experiment determined a single band of 60 kDa in Weri-Rb-1 cells. TRβ2 protein levels increased after 4 days of RA treatment and remained constant until day 8 (Fig. 3C).

RA Induction of G_0/G_1 Cell Cycle Arrest

To verify the microarray observation that RA inhibits cell cycle progression, flow cytometry analysis determined the cell cycle phases. Representative examples depicting the effect of RA treatment on distribution of the cell cycle phases in the retinoblastoma cell line are shown in Figure 4. Treatment with 10 μM RA for 2 days resulted in the accumulation of cells in the G_0/G_1 phase (42%), compared with the control (DMSO)-treated cells (26%). A concurrent decrease in the percentage of cells in the G_2/M phase was observed in RA-treated cells (23%) compared with control cells (44%). The G_0/G_1 accumulation and G_2/M phase depletion were also present in cells treated with RA for 3 (52% vs. 20%) and 7 days (42% vs. 27%), respectively. No significant changes in the percentage of cells within S-phase were observed.

RA Induction of CAR-Immunoreactive Staining in Weri-Rb-1 Cells

Previously, we have shown by using immunoblot analysis that RA upregulates CAR protein levels in both Weri-Rb-1 and Y79 cells.[1,2] To further examine the CAR protein expression pattern in RA-treated Weri-Rb-1 cells, we performed an immunocytochemical study. In related work, CAR has been identified in all cone photoreceptor cells and thus is an excellent hallmark for cones.[1] As shown in Figure 5, treatment of Weri-Rb-1 cells with 10 μM of RA for 5 days increased the staining intensity with a CAR-specific antibody compared with the control (DMSO-treated) cells, suggesting that RA induces the subset of retinoblastoma cells to differentiate toward cone cell lineage.

RA Induction of Apoptosis in a Subpopulation of Retinoblastoma Cells

The effect of RA on apoptosis in Weri-Rb-1 cells was also analyzed by TUNEL assay. As shown in Figure 6, the percentage of apoptotic cells increased in RA-treated cultures by 48 hours, compared with DMSO-treated control groups. An average of 16% of apoptotic cells was observed in control groups, in contrast to 35% in RA-treated cultures ($P < 0.05$; unpaired *t*-test; $n = 3$). These cumulative data, together with the immunocytochemical data, suggest that both an increase in conelike cells and a decrease in other cellular subtypes by apoptosis play a role during RA treatment, resulting in a net increase or decrease in the expression level of cone- and rod-specific visual pathway genes, respectively.

Discussion

For many years, the vitamin A metabolite RA has been known to have profound effects on development, cellular proliferation and differentiation, and tumor growth and invasion. RA is known to play an important role during embryonic development in the generation of several organs and systems, including the retina in the eye[35–42] and the nervous system.[43–45] In vitro, RA also plays a prominent role in regulating the transition from the proliferating precursor cell to the postmitotic differentiated cell.[46,47] The wide-ranging effects of RA on cellular proliferation and migration have made it a useful chemotherapeutic agent in the treatment of many types of cancer.

In prior work, we examined the molecular mechanisms involving both the mRNA transcription and the protein translation of cone arrestin expression that are dramatically in-

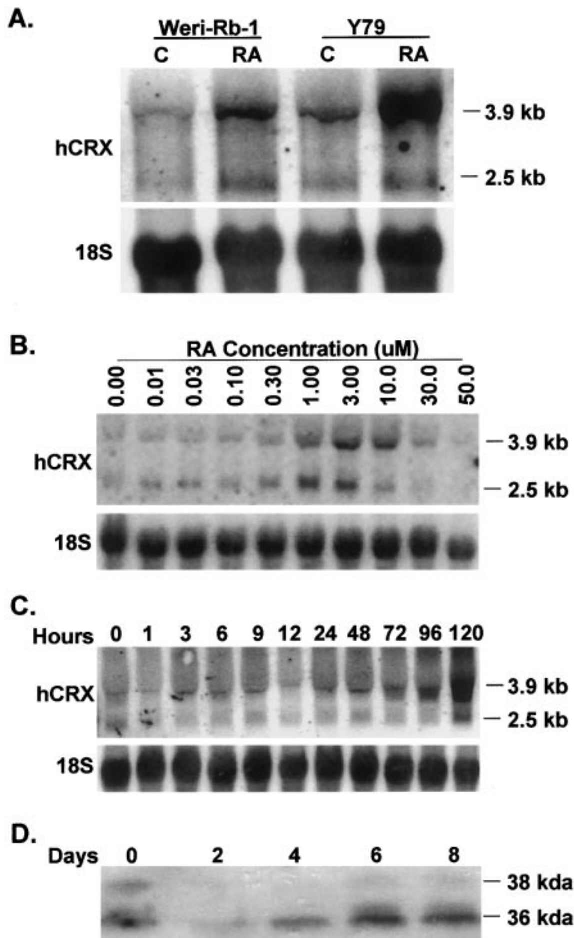

FIGURE 2. Upregulation of human CRX (hCRX) expression by RA treatment of Weri-Rb-1 or Y79 cells. (**A**) A representative Northern blot for hCRX expression in both Weri-Rb-1 and Y79 cells. Total RNA (20 μg) from either treated (RA, 10 μM RA treatment for 6 days) or untreated (C, control) cells was electrophoresed in a denaturing agarose gel, transferred to a membrane, processed, and hybridized with the appropriate radiolabeled cDNA probe. To quantitate gel loading and transfer efficiency, the membranes were striped and reprobed with a radiolabeled 18S probe. (**B**) A representative Northern blot for hCRX mRNA expression in Weri-Rb-1 cells treated with increasing concentrations of RA for 6 days. (**C**) A representative Northern blot for hCRX expression in Weri-Rb-1 cells with different duration of RA treatment. (**D**) Immunoblot analysis of hCRX expression in Weri-Rb-1 cells treated with RA for the indicated times.

creased with RA treatment of retinoblastoma cells.[2] Because RA induces hCAR expression in retinoblastoma cells through the upregulation of RXRγ, which is mainly localized to the cone photoreceptors in the human retina, it would be interesting to know whether RA induction of hCAR is accompanied by transcription alterations of other cone-specific genes. With oligonucleotide array-based expression-profiling technology (Affymetrix), we observed that the expression pattern of a broad network of genes was modulated in a time-dependent manner by RA treatment. In addition, with RA treatment, the retinoblastoma cell line underwent G_0/G_1 cell cycle arrest and displayed conelike genetic differentiation and selective apoptosis.

Cell differentiation is a coordinated process that includes cell cycle exit and tissue-specific gene expression.[48] The present study is of particular interest and significance because RA upregulated a subset of cone-specific genes during the differentiation of retinoblastoma cells. These results are consistent with the established concept that RA levels influence cell fate and mediate the differentiation of specific neuronal phenotypes during retinal histogenesis.[38,49] In cultured embryonic rat or fetal human retinas, RA promotes retinal progenitor cells to develop into rod photoreceptors.[39] Exogenous RA also promotes rod photoreceptor differentiation in rat retina in vivo when injected in pregnant rats at days 18 and 20 of gestation.[39] Moreover, application of RA to zebrafish embryos causes precocious rod photoreceptor development and inhibition of the maturation of cone photoreceptors.[50] The results presented in this article are in conflict with the published observations, in that RA induced retinoblastoma cells to differentiate into cone photoreceptors, but it inhibited rod gene expression. This discrepancy could be explained in at least three ways. First, the effect of RA on cell differentiation during embryonic development is probably tightly controlled by timing. All the published results have been obtained with embryonic retinas, but the retinoblastoma cell lines were developed from juvenile tumor tissues, which had passed the embryonic developmental stage. Indeed, a more recent observation reveals that RA induces rod photoreceptor-selective apoptosis during postnatal development of mouse retina.[51] Second, the retinoblastoma cells used in our study may differ from developing retinal cells in that certain diffusible induction factors normally found in the microenvironment of the embryo may be either missing or present in overabundance. A third alternative is the different cell–cell interaction involved in the RA-induced cell differentiation between normal retinal development and retinoblastoma cell differentiation. In normal retinal development, photoreceptors arise from a population of precursor cells that are multipotent and intrinsic in the retina. The first photoreceptors to form are the cones, with the rods appearing much later.[52]

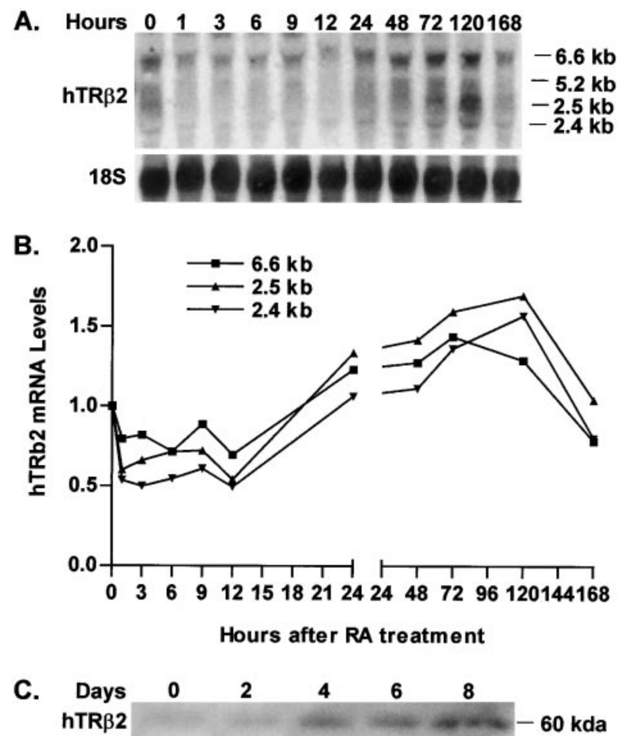

FIGURE 3. Detection of hTRβ2 mRNA and protein levels in Weri-Rb-1 cells. The cells were incubated with 10 μM RA for indicated times. (**A**) A representative Northern blot for hTRβ2 and 18S mRNA signals is shown. (**B**) Densitometric quantitation of hTRβ2 mRNA expression. Data are from two independent experiments. (**C**) Immunoblot analysis of hTRβ2 expression in RA-treated Weri-Rb-1 cells for the indicated times.

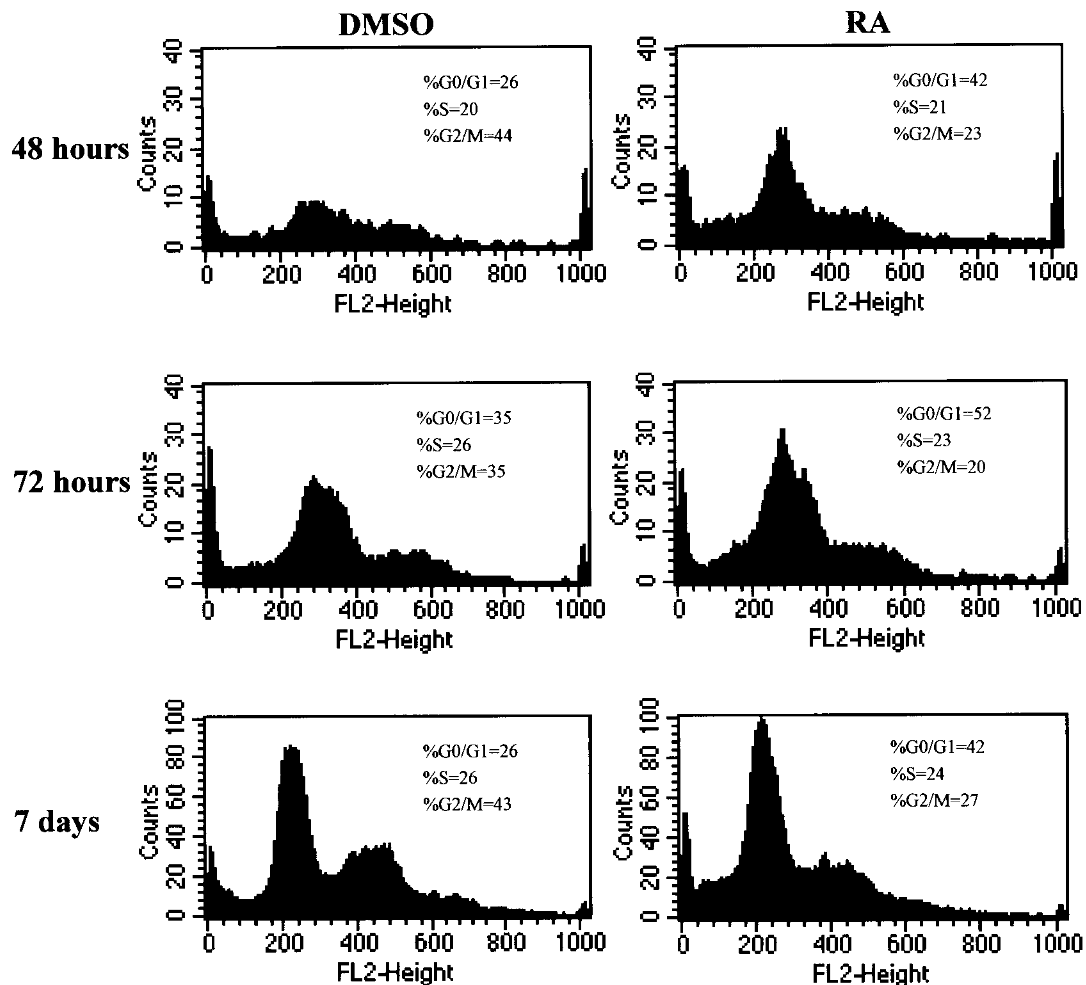

FIGURE 4. Flow cytometry analysis of the effect of RA treatment on the cell cycle stages in Weri-Rb-1 cells. Percentage of cells in G_0/G_1, S, and G_2/M phases of the cell cycle is shown in the *right upper corner*.

However, in retinoblastoma, neoplastic photoreceptors, mostly red/green–sensitive cones, and glia cells (Müller cells) constitute the overwhelming majority of the cells.[53] It seems likely that RA induces retinoblastoma cells along a red/green-cone pathway due to a red/green-cone–dominant microenvironment.

We have also shown that RA downregulates rod-specific genes during retinoblastoma cell differentiation. Given the example that RA induces rod-selective apoptosis during postnatal retinal development,[51] it easily led us to postulate that RA also induces the rodlike cell population in the heterogeneous retinoblastoma cell line to choose a cell death pathway. This assumption is supported by the increased expression of a DNA fragmentation factor, caspase 8, and the increased number of apoptotic cells in RA-treated Weri-Rb-1 cells compared with the DMSO-treated control.

Microarray analysis also revealed that RA treatment of Weri-Rb-1 cells resulted in an induction of expression of genes in the steroid-thyroid receptor superfamily, such as RXRγ, TRβ2, and COUP-TF1, plus a photoreceptor-specific homeobox gene, CRX. Previously, we identified that both Weri-Rb-1 and Y79 cells express all subtypes of RAR and RXR transcripts, and RXRγ is potentially the RA receptor subtype involved in human cone arrestin regulation by RA.[2]

TRβ2 is a member of the steroid-thyroid receptor superfamily that plays a key role in cone-specific gene expression. Thyroid hormone receptors are located in the developing chicken retina[54,55] with the TRβ2 isoform being expressed in cones.[54] Northern blot analysis revealed a prominent expression of TRβ2 mRNA in mouse eye development, which peaked at approximately embryonic day 17.5 and declined in the postnatal period, similar to the pattern in chick.[9] In recent mouse gene-knockout studies, deleting the THRB gene (encoding TRβ2) caused the selective loss of green (M) cones and a concomitant increase in S-opsin immunoreactive cones.[9] Our microarray and Northern blot data are consistent with this study, because the upregulation of TRβ2 is followed by the increase in expression of green cone pigment gene after RA treatment. Furthermore, the human cone arrestin promoter also contains an element (DR-4) that strongly resembles an authentic thyroid hormone response element (TRE).[2] These results suggest that TRβ2 may play an important role in driving cone-specific gene expression, both in developing retina and in retinoblastoma differentiation.

Several studies have suggested that COUP-TF may be a part of the retinoid signaling pathway, both in vivo and in cell culture systems.[56–58] Upregulation of COUP-TFI and COUP-TFII genes also occurs in differentiation programs of P19 embryonic carcinoma (EC) cells triggered by retinoic acid (RA).[59] The biological importance of COUP-TF in retina development was demonstrated with the *Drosophila* homologue seven-up (SVP). SVP is essential to specify photoreceptor subtype in the development of the compound eye.[60,61] Lu et al.[62] further demonstrated that in the mouse, bovine, or human rod arrestin

gene promoter, a DR-7 element (TGACCT of direct repeat with a 7-bp spacer), mediates the positive transcriptional effect of COUP-TF. The mouse retinal expression pattern of COUP-TF in embryonic day 14 coincided with the initial expression of the rod arrestin gene. No COUP-TF transcripts were detected in either adult retina or Weri-Rb-1 cells,[62] leading them to propose that COUP-TF may effectively regulate only the expression of the rod arrestin gene in the developing retina, whereas other factors regulate this gene in the adult retina and in retinoblastoma cells.[62] We demonstrated for the first time that RA rapidly induces expression of COUP-TF1 in Weri-Rb-1 cells, and the expression of COUP-TF1 is associated with retinoblastoma cell differentiation, implying that COUP-TF1 plays a crucial role, not only in the control and timing of rod-specific programs during retinal development, but also in cone-specific programs during the differentiation of cone photoreceptors.

CRX is the first major photoreceptor-specific homeodomain transcription factor to be identified.[21,22,63] Although CRX is unlikely to be the dominant transcription factor involved in expression of the cone arrestin and cone transducin alpha subunit (GNAT2) genes, despite the presence of three CRX binding sites in the cone arrestin promoter,[2] we could not exclude the green cone pigment gene from transactivation by CRX, because it was expressed only after 7 days of RA treatment in Weri-Rb-1 and was absent in $CRX^{-/-}$ mice.[64] The requirement for CRX in transcription of the cone opsin loci in mice supports the notion that the human red/green locus requires CRX activity at the CRX-binding element in the locus control region.[22]

In summary, our findings imply that RA mediates induction that coordinates cone-specific gene activation, rod-specific gene inactivation, and cell cycle arrest during the differentiation of retinoblastoma cells. The molecular events involved depend on the modulation of two main opposing switches that contain members of the steroid-thyroid receptor superfamily and cell cycle regulators. This may represent a general mechanism by which retinoids signal cell cycle control and cell fate biases that may have therapeutic implications in the pharmacologic triggering of growth suppression and destruction of tumor cells. Even more important, the fact that RA induces most of these cells to differentiate into a more conelike phenotype may someday be part of a viable option for therapeutic rescue of the cone photoreceptors before their destruction leads to blindness.

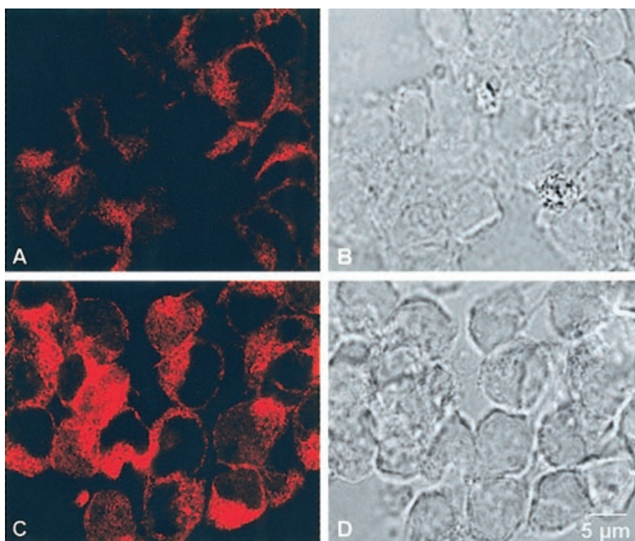

FIGURE 5. Enhanced hCAR expression in RA-treated Weri-Rb-1 cells detected by immunocytochemical analysis. Weri-Rb-1 cells were treated with RA (10 μM) or DMSO (control) for 5 days and processed for immunocytochemical studies with the hCAR polyclonal antibody LUMIF (1: 2000) and a Cy3-conjugated goat anti-rabbit secondary antibody. Slides were mounted and photographed with a confocal microscope. Fluorescent image of DMSO-treated (**A**) and RA-treated (**C**) Weri-Rb-1 cells; phase-contrast image of DMSO-treated Weri-Rb-1 cells (**B**), and RA-treated Weri-Rb-1 cells (**D**).

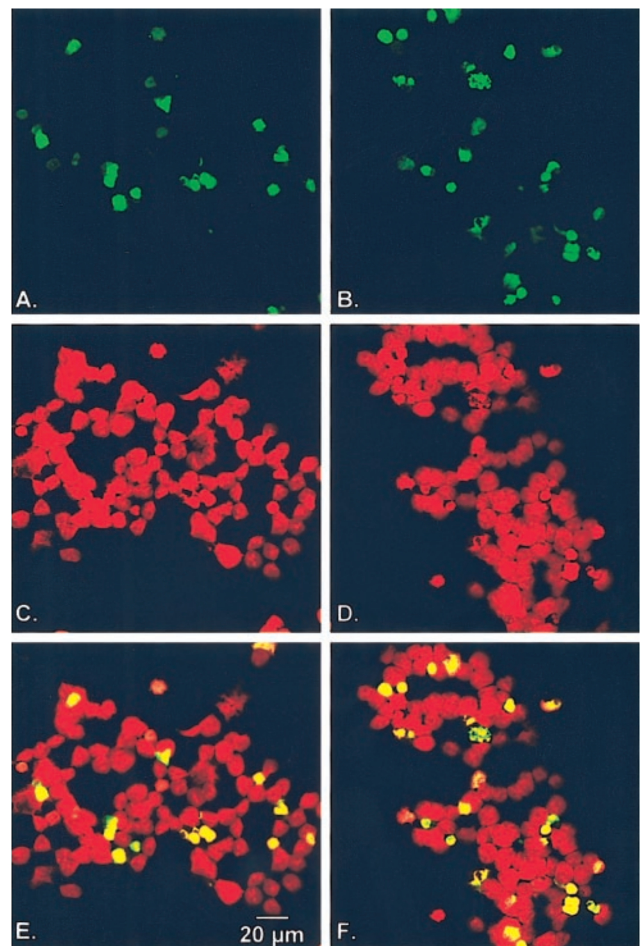

FIGURE 6. Apoptosis analysis of Weri-Rb-1 cells in the absence or presence of RA. Cells were treated with either RA (10 μM) or DMSO (control) for 48 hours, and the fragmented DNA of apoptotic cells was detected by catalytically incorporating fluorescein-12-dUTP at the 3′-OH DNA ends with the TUNEL principle. Slides were then mounted and photographed. The DNA strand breaks of apoptotic cells were labeled with fluorescein-12-dUTP (**A**, **B**, *green*), and cell nuclei were stained with PI (**C**, **D**, *red*). (**A**, **C**) DMSO-treated control cells. (**B**, **D**) RA-treated cells. (**E**) Overlay of (**A**) and (**C**); (**F**) Overlay of (**B**) and (**D**).

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