Modulation of transcription by the peroxisome proliferator-activated receptor δ–binding RNA aptamer in colon cancer cells

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Abstract
Peroxisome proliferator-activated receptor δ (PPAR-δ), one of three PPAR subtypes, is a lipid-sensing nuclear receptor that has been implicated in multiple processes, including inflammation and cancer. To directly establish the role of PPAR-δ in colon cancer development and progression, we selected high-affinity RNA aptamers and expressed them in several colon cancer cell lines. Nuclear-expressed aptamers efficiently inhibited PPAR-δ–dependent transcription from a synthetic peroxisome proliferator response element–driven luciferase reporter. PPAR-δ–specific aptamers suppressed transcription from natural promoters of vascular endothelial cell growth factor-A and cyclooxygenase-2. Moreover, vascular endothelial cell growth factor-A and cyclooxygenase-2 mRNA levels were significantly reduced by the PPAR-δ–specific aptamers in colon cancer cells. Most significantly, HCT116 colon cancer cells with high-level expression of PPAR-δ–specific aptamers exhibited a striking loss of tumorigenic potential. Further study on these RNA aptamers could provide an opportunity to modulate PPAR-δ–mediated colon cancer development and progression. Taken together, our results establish an important role for PPAR-δ in transcription of tumorigenic genes, which can be specifically modulated by high-affinity RNA intramers in colon cancer cells. The RNA intramers may be further developed as specific inhibitors for cancer therapeutic strategies.

Introduction
Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that play central roles in regulating metabolic pathways. Three PPARs (PPAR-α, PPAR-γ, and PPAR-δ) have so far been identified; each isotype displays a tissue-selective expression pattern, and have been proposed to have distinct roles in inflammation and cancer development (1–3). Evidence linking PPAR-δ to colon cancer development has emerged from studies on the homozygous deletion of PPAR-δ in colon cancer cell lines, and the activation of PPAR-δ by a selective synthetic agonist and endogenous activator in APCmin/+ mice (4, 5). The evidence is consistent with the proposal that PPAR-δ represents an attractive target for the treatment and prevention of colorectal cancer (6).

Because APC/β-catenin/Tcf signaling is the main signaling pathway for the development of colon cancer, concerted efforts have been devoted towards understanding the relationship between APC/β-catenin/Tcf signaling and PPAR-δ signaling. PPAR-δ has been reported to be the transcriptional target of the APC/β-catenin/Tcf pathway and the target of nonsteroidal anti-inflammatory drugs (7). Considering the cross-regulation of APC/β-catenin/Tcf and members of the nuclear receptor family, functional interaction between β-catenin/Tcf and PPAR-δ in the nucleus has also been proposed (8, 9). Whatever the mechanism, the PPAR-δ transcription factor clearly interacts with APC/β-catenin/Tcf signaling in colon cancer cells.

Inflammatory mediator signaling involving prostaglandins and cyclooxygenase (COX) is also critical for colorectal cancer development and progression. Because the connection between COX-2 and APC/β-catenin/Tcf signaling is important in cancer (10), cross-talk between PPAR-δ and COX-2 may also have a profound impact on many types of cancer (11–13). Positive feedback loop of PPAR-δ and prostaglandin E2 might be related to the coordinated up-regulation of PPAR-δ and COX-2 in colorectal carcinogenesis. For example, PPAR-δ is known to induce transcription of COX-2 mRNA in colon cancer (14–16).

Vascular endothelial growth factor-A (VEGF-A) is a prime therapeutic target for various cancers. With regard to the role of PPAR-δ in tumor progression, it is interesting to note that cross-talk between PPAR-δ and VEGF-A is critical for cancer progression, and that PPAR-δ acts as the transcription factor for the gene encoding VEGF-A (17, 18). Because VEGF and COX proteins are important regulators for the normal-to-inflammation switch, as well as in cancer initiation and progression, modulation of PPAR-δ transcriptional...
activity might provide a unique opportunity to simultaneously regulate these two critical transcripts (19). Because expression of VEGF-A mRNA is regulated by multiple signaling pathways involving β-catenin/Tcf and PPAR-δ, inhibition of VEGF-A mRNA may collaboratively involve both signaling inhibitors (20, 21).

Although controversy exists on the direct role of PPAR-δ during colon cancer development (22–24), PPAR-δ is still an attractive target for the treatment and prevention of neoplastic diseases due to its critical roles for expression of VEGF-A and COX-2 mRNA. In this study, we generated a RNA aptamer against PPAR-δ and expressed it in the cells as a nuclear localized aptamer (25). The RNA aptamer inhibited the transcriptional activity of PPAR-δ-dependent VEGF-A and COX-2 target genes at the mRNA level. Moreover, dual expression of the novel RNA aptamer for PPAR-δ in conjunction with a previously characterized RNA aptamer for β-catenin (26) greatly reduced transcription from promoters activated by PPAR-δ and β-catenin. Most significantly, the novel aptamer reduced the tumor-forming potential of HCT116 human colorectal carcinoma cells. The present observations suggest that the RNA aptamer is potentially useful as a tool for obtaining further insight into the functional properties of nuclear PPAR-δ.

Materials and Methods

Plasmids and Reagents

pUC19 vectors containing PPAR-α and PPAR-δ cDNA inserted in the BamHI site and pCR2.1 vector with the PCR product of PPAR-γ were kindly provided by Dr. Young Mee Kim Pak (Kyung Hee University, Seoul, Korea). The mamalian expression clone for PPAR-δ was cloned into the pCMV-Tag2 vector. The PPAR responsive element (PPRE) 3×-TK reporter was a gift from Dr. Heurian Lee (Ulsan University School of Medicine, Seoul, Korea). The OT luciferase reporter was from Dr. Ramesh A. Shivdasani (Dana-Farber Cancer Institute, Boston, MA). The -891 COX-2 natural promoter-driven luciferase reporter (27) was kindly provided by Dr. Kenneth K. Wu (University of Texas Health Science Center, San Antonio, TX). The VEGF-A promoter (+950 to +450) in the pGL3 reporter (28) was a gift of Dr. Jin-Su Kim (Seoul National University, Seoul, Korea), and the 1.9-kb VEGF promoter (+805 to +1000; ref. 20) was a gift of Dr. Daniel C. Chung (Harvard Medical School, Boston, MA). The pU6 vector was modified from pTZU6+27 to incorporate a zeocin-resistance gene, allowing the selection of stable pU6-aptamer clones (26). The PPAR-δ agonist GW501516 was purchased from Alexis (Lausen, Switzerland).

Cell Culture, Transfection, and Luciferase Assay

Human colorectal carcinoma HCT116, and adenocarcinoma SW480 and DLD-1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum. For PPAR-δ activation, cells were seeded into the wells of a 6-well plate (Corning, New York, NY) and incubated overnight. After 8 h of serum starvation, GW501516 (5 μmol/L) was added into the medium, and cells were cultured for 24 h. Total RNA was prepared and analyzed by reverse transcriptase-PCR and real-time PCR. For reporter transfection, cells were cotransfected with a corresponding luciferase reporter and pCMV-β-galactosidase with the use of Lipofectamine (Invitrogen, Carlsbad, CA), and treated with either vehicle or GW501516. Firefly luciferase activity was determined with the use of a luciferase assay system (Promega, Madison, WI) with a GloMax 20/20 luminometer (Turner Biosystems, Sunnyvale, CA). Relative luciferase activity was normalized to the activities from cotransfected β-galactosidase or Renilla luciferase reporters. Student’s t test was done to obtain P-values.

Cloning and Expression of Glutathione S-transferase–PPAR Fusion Proteins

Plasmids with PPAR-α, PPAR-δ, or PPAR-γ inserts were excised with BamHI (PPAR-α and PPAR-δ) or SalI (PPAR-γ), and ligated to pGEX4T-1 vectors (Pharmacia, New York, NY). Recombinant PPAR-α–glutathione S-transferase (GST), PPAR-δ–GST, and PPAR-γ–GST fusion proteins were produced in BL21 (DE3) cells. Cells were incubated with 0.1 mmol/L isopropyl-β-D-thio-galactopyranoside at 30°C for 4 h and lysed in extraction buffer consisting of 50 mmol/L Tris-HCl (pH 8.5), 100 mmol/L NaCl, 1 mmol/L EDTA, protease inhibitor cocktail, and 1 mg/mL lysozyme on ice for 30 min. N-lauryl sarcosine was added to 1%, followed by incubation on ice for 15 min. After sonication in four 20-s bursts, 1% Triton X-100 was added, followed by incubation at 4°C for 15 min. After centrifugation, glutathione-Sepharose 4B beads (Amerham, Piscataway, NJ) were incubated with the lysate for 2 h at 4°C before washing thrice with the extraction buffer. Bound proteins were eluted by the addition of elution buffer [10 mmol/L reduced glutathione, 50 mmol/L Tris-HCl (pH 8.0), and 100 mmol/L NaCl] for 2 h at 4°C. The eluted proteins were dialyzed extensively against PBS and quantified by Bradford assay (Bio-Rad, Hercules, CA).

In vitro RNA Selection, Aptamer Construction, and Determination of RNA Binding

A RNA library of random 50-nucleotide sequences (1 × 1012 different molecules; IDT, Coralville, IA) was used as starting material for systematic evolution of ligands by exponential enrichment procedure (26). In each cycle, RNA was preincubated with GST protein in binding buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1 mmol/L MgCl2, 0.5 mmol/L dithiothreitol], GST-bound RNA was discarded, and RNA bound to GST–PPAR-δ was selected with glutathione-Sepharose 4B beads (Amerham). After 13 cycles, selected sequences were cloned into pUC19 (pUC19 aptamer), and 40 clones were sequenced. To test for in vitro RNA binding, RNA was radiolabeled, incubated with GST fusion proteins, and assayed by RNA electrophoretic mobility shift assay, as described previously (29, 30).

Construction and Expression of RNA Intramer

The design and principles of intramer cloning have been previously described (25, 26, 31). To clone the aptamer sequence into pTZU6+27, DNA was amplified from the pUC19 aptamer (#4, #5, and #6) with the primers U6-F1 (5′-TATGAGTCGACTAAGGAGCCCTGTTG-3′) and U6-R1 (5′-GACTCTAGAGGATCCCNG-3′). The PCR product

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was digested with *Sall* and *Xba*I, and cloned into the same sites of the pU6 vector to generate the pU6 aptamer. The level of expression of the RNA aptamer in the cells was estimated by reverse transcriptase-PCR with the use of the aptamer-specific primer sets U6-F1 and U6-R1, which produced a 144-bp PCR product. To measure the expression of RNA intramer #6, pU6–PPAR-δ aptamer #6 forward (5′-GGATCCGGTGTACCACAAAG-3′) and U6–PPAR-δ aptamer #6 reverse (5′-CTGTGTCACAGGGCCGATC-TATG-3′) primers were used.

**Reverse Transcriptase-PCR and Real-Time PCR**

To confirm the expression of the RNA intramer, total RNA was extracted from cells transfected with pU6–PPAR-δ aptamer #6 or pU6 vector with the use of TRIzol (Invitrogen). RNA was converted to cDNA with M-MuLV reverse transcriptase (Stratagene, La Jolla, CA) and used in the PCR reactions. The following PCR primers were used to test for the expression of mRNA: PPAR-δ forward (5′-GGTGAATGGCCTGCCTCCCTACAA-3′) and PPAR-δ reverse (5′-CACAGAATGATGGCCGCAATGAAT-3′) primers to generate a 381-bp PCR product; COX-2 forward (5′-TTCAAATGAGATTGTGGAAAAAT-3′) and COX-2 reverse (5′-AGATCATCTCTGCTGATGTATCTT-3′) primers to generate a 281-bp PCR product; and VEGF-A forward (5′-AGACCTCTGGTGCGATCTTC-3′) and VEGF-A reverse (5′-TGGATCCACATTTGTTTG-3′) primers to produce a 201-bp PCR product. All PCR products were analyzed through 1.5% agarose gel electrophoresis and ethidium bromide staining. Real-time PCR was done with a Rotor-Gene RG-3000A system (Corbett Research, Sydney, Australia). Reactions were amplified with the use of the selective primers described above and the SYBR Green 1 kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The PCR amplification cycle involved denaturation at 95°C for 5 s, annealing at 55°C for 10 s (PPAR-δ) or at 54°C for 10 s (VEGF-A), and extension at 72°C for 15 s. Quantification was carried out with the Rotor-Gene 6 software (Corbett Research). Relative levels of mRNA molecules were expressed as the ratio of comparative threshold cycle to internal control glyceraldehyde-3-phosphate dehydrogenase mRNA.

**Soft Agar Colony Formation Assay**

HCT116 cells were cotransfected with pU6-aptamer #6 or pU6 vector, seeded onto a 35 mm diameter dish (5 × 10³ cells) in DMEM containing 10% fetal bovine serum, and incubated overnight. To prepare the bottom layer of 0.5% agarose and 10%/1 × DMEM, 1% melting agar and 20% fetal bovine serum/2 × DMEM were mixed well and poured into the 35-mm dishes. The bottom layer was solidified at room temperature for 10 to 15 min. After 15 d, colonies were enumerated from three independent plates, and the P-value was obtained by Student’s *t* test. Photographs were taken with a Zeiss Axiolab microscope (Carl Zeiss, Jena, Germany).

**Cell Proliferation Assay**

Equal numbers of HCT116 cells (3 × 10⁵) were transfected either with or without the minimized RNA aptamer #6 using Lipofectamine 2000 (Invitrogen) in 96-well plates. After incubation for 16 h, cells were serum starved and then treated with 1 μmol/L GW501516 for 16 h. Cell proliferation was examined by MTS assay with the use of the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer’s instructions. Absorbance at 490 nm is directly proportional to the number of viable cells in culture.

**Results**

**Selection of RNA Aptamers for PPAR-δ**

Because PPAR-δ has been implicated in colon cancer development (1–6), we developed RNA aptamers that could specifically modulate nuclear PPAR-δ functions in colon cancer cells. First, we isolated RNA aptamers that bind to GST–PPAR-δ fusion protein after 13 cycles of selection from the original RNA library, which did not show any detectable binding to the target protein (Fig. 1A). After cloning the RNA pool after 13 cycles of selection, individual RNA molecules were isolated, and 40 of the independent clones obtained were sequenced. Two distinct RNA sequence motifs were found, showing the most prominent consensus sequences in RNA aptamers #4, #5, and #6 (Fig. 1B). To confirm the binding of these RNA aptamers to the PPAR-δ target protein, RNA electrophoretic mobility shift assay was carried out. As shown in Fig. 1C, all three aptamers clearly bound to the recombinant PPAR-δ protein.

RNA aptamer #6 was selected for further study because it is predicted to have only one stable secondary structure by the mfold program. The protein binding specificity of RNA aptamer #6 was confirmed for its binding to the PPAR-δ protein compared with its binding to homologous PPAR-α and PPAR-γ proteins (Fig. 1D). By comparing the intensities of bound and unbound bands in Fig. 1D, we have estimated the approximate binding constant (Kd) for RNA aptamer #6 to PPAR proteins. We observed a striking binding specificity for RNA aptamer #6 (Kd = 20 nmol/L) without any detectable binding to other PPAR isoforms or GST protein (Fig. 1D). RNA binding specificity was also confirmed by competition with unlabeled RNA aptamer #6 (Fig. 1D).

**Suppression of PPAR-δ–Mediated Transcription by the RNA Aptamer**

Because previous experiments clearly showed the high affinity and specificity of PPAR-δ–binding RNA aptamers in vitro, we expected them to be effective in modulating PPAR-δ functions in cells as well. To directly assess this, aptamers were expressed with the use of the U6 promoter-driven (pU6-aptamer) vector, which expresses nuclear localized RNA (Fig. 2A). We confirmed the high expression level and prolonged stability of the RNA aptamers, as reported previously (26, 31).

Because the main function of PPAR-δ is the activation of PPRE-dependent transcription, PPRE 3× luciferase reporter was utilized to test if the expressed RNA aptamers could inhibit transcription (Fig. 2A). We first screened three different colorectal cancer cell lines to measure their responses to the PPAR-δ specific agonist GW501516 with the use of the

1 http://mfold.bioinfo.rpi.edu/
PPRE 3× luciferase reporter. As shown in Fig. 2B, colorectal carcinoma HCT116 and adenocarcinoma SW480 cells responded fairly well to the PPAR-δ agonist GW501516; however, a relatively low-level activation of PPRE luciferase activity was observed in the DLD-1 cell line, suggesting a variable range of PPAR-δ activation depending on the cell type. To test the effect of RNA aptamers in activated cells, we first used SW480 cell lines because they showed the

**Figure 1.** Enrichment and biochemical characterization of PPAR-δ-binding RNA aptamers. A, RNA EMSA of original RNA library and the RNA library after 13 cycles of selection (SE-13). The [α-32P]UTP-labeled RNA library (40 pmol/L) was incubated with increasing amounts of recombinant GST–PPAR-δ protein and subjected to 4% polyacrylamide native gel electrophoresis. Lanes 1 to 8, PPAR-δ protein (0, 0.5, 10, 20, 40, 80, 160, and 320 nmol/L, respectively). Lanes 9 and 10, PPAR-δ protein (320 nmol/L) in the presence of nonlabeled original and SE-13 RNA (10× and 100×, respectively) as competitors (C). Bound and unbound RNA bands are indicated. B, sequences of PPAR-δ-binding RNA aptamers (Apt) #4, #5, and #6 from SE-13. C, RNA EMSA of RNA Apts #4, #5, and #6 to recombinant PPAR-δ protein. The [α-32P]UTP-labeled RNA Apts (40 pmol/L) were incubated with increasing amounts of GST–PPAR-δ proteins (10, 50, and 100 nmol/L). Lane 1, input RNA only; lanes 2 to 4, RNA Apt #4; lanes 5 to 7, RNA Apt #5; lanes 8 to 10, RNA Apt #6 binding to PPAR-δ protein. Bound and unbound RNA bands are indicated. D, RNA EMSA of RNA Apt #6 to recombinant PPAR proteins (left). The [α-32P]UTP-labeled RNA (40 pmol/L) was incubated with increasing amounts of GST–PPAR-δ proteins. Lanes 1 to 9, respectively, 0, 1, 5, 10, 20, 40, 80, 160, and 320 nmol/L; lanes 10 and 11, PPAR-δ protein (320 nmol/L) in the presence of nonlabeled RNA Apt #6 as competitor (10× and 100×, respectively); lanes 12 to 14, PPAR-α protein (80, 160, and 320 nmol/L, respectively); lanes 15 to 17, PPAR-γ protein (80, 160, and 320 nmol/L, respectively); lanes 18 to 20, GST protein (80, 160, and 320 nmol/L, respectively). Bound and unbound RNA bands are indicated. Right, relative binding of RNA Apt #6 to PPAR proteins. Percentages of RNA binding were calculated by densitometer scanning of bound and unbound bands from the gels (right). The graph was presented as increasing concentrations of the proteins. Approximated binding constants of RNA Apt #6 to PPAR proteins were estimated as the protein concentration at 50% binding. EMSA, electrophoretic mobility shift assay.
The highest activation of PPRE luciferase by GW501516. As shown in Fig. 2C, significant suppression of transcription was found for most of the pU6 aptamers; the suppression efficiency was exceptional for RNA aptamer #6 in adenocarcinoma SW480 cell lines. To confirm the inhibitory role of RNA aptamer #6 in other colorectal cancer cell lines, we also tested the effect of RNA aptamers in colorectal carcinoma HCT116 cells (Fig. 2D) as well as in DLD-a cells (data not shown). Regardless of the cell line, we consistently observed a significant inhibition of PPRE luciferase activity by RNA aptamer #6.

Reduction of PPAR-δ-Mediated VEGF-A and COX-2 mRNA Expression

Because PPAR-δ was shown to be involved in the activation of VEGF-A and COX-2 transcription, we tested the role of RNA aptamer #6 on the VEGF-A promoter luciferase reporter. In this experiment, we used HCT116 cell lines because they activate VEGF-A luciferase activity to a level similar to that of PPRE luciferase upon GW501516 treatment (Fig. 2B). As expected, dose-dependent inhibition of VEGF luciferase activity was clearly correlated with the expression of RNA aptamer #6 in HCT116 cells (Fig. 3A). We also observed a similar dose-dependent inhibitory effect of RNA aptamer #6 when the COX-2 promoter was used as reporter (data not shown). These data clearly suggested that PPAR-δ-binding RNA aptamer #6 modulated the transcriptional activity of PPAR-δ through repression of PPRE on the promoter.

Previous experiments showed a significant reduction of the transcriptional activity of PPAR-δ by RNA aptamer #6 on PPAR-δ-dependent promoters. Thus, we then tested whether RNA aptamer #6 could inhibit the expression of endogenous target genes, such as VEGF-A and COX-2, in HCT116 colon cancer cells, and found that RNA aptamer #6 reduced the expression of VEGF-A and COX-2 mRNAs in a dose-dependent manner (Fig. 3B and 3C). This finding

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Figure 2. Expression and effect of PPAR-δ-binding RNA Apts in colon cancer cells. A, diagrams of the PPRE ×3-TK luciferase reporter (PPRE-Luc) and the pU6-Apt cloning vector. PPRE-Luc plasmid has three synthetic PPRE sites in addition to the TK promoter. pU6-Apt has a PolIII-driven U6 promoter and two strong stems of the human U6 transcript (filled box) flanking RNA Apt sequences (cloned by SaI and XbaI restriction sites). pU6-Apt generates stably expressed RNA Apt in the cells. B, luciferase assay with PPRE-Luc in three colorectal cancer cell lines (HCT116, SW480, and DLD-1). Cells were cotransfected with PPRE-Luc (300 ng) and pCMV-β-galactosidase (150 ng). After 16 h, transfected cells were serum starved for 8 h, and GW501516 (5 μmol/L) was added for 20 h. Cells were harvested for luciferase assay. Three independent experiments and a statistical analysis were done. **, P < 0.01; *, P < 0.05. C, luciferase assay of PPRE-Luc in the absence (pU6 vector; lanes 1 and 2) or presence of pU6-Apt (#4, #5, and #6; lanes 3 to 5, respectively) in SW480 cells. Cells were cotransfected with PPRE-TK luciferase reporter (400 ng), RNA Apt (500 ng), and pCMV-β-galactosidase (150 ng). After 24 h, transfected cells were serum starved for 8 h, and GW501516 (5 μmol/L) was added for 24 h. Cells were harvested for luciferase assay. Three independent experiments and a statistical analysis were done. ***, P < 0.001. D, luciferase assay with PPRE-Luc in the presence of RNA Apt #6 in HCT116 cells. Cells were prepared and analyzed as described in C. Three independent experiments and a statistical analysis were done. ***, P < 0.001.
was consistent with the idea that the RNA aptamer can modulate the transcription of PPAR-δ target genes in colon cancer cells.

Reduction of β-Catenin–Mediated Transcription by RNA Aptamers

Previously, it was reported that PPAR-δ is the target of β-catenin (7), and PPAR-δ was proposed to modulate the transcriptional activity of β-catenin (9). Considering the complex functional interactions of PPAR-δ and β-catenin, it became of interest to determine whether PPAR-δ–binding RNA aptamer #6 could modulate the transcriptional activity of β-catenin. We used the OT luciferase reporter, which has three copies of the TCF/β-catenin responsive element, and

Figure 3. Reduction of PPAR-δ target gene expression by PPAR-δ–binding RNA Apt. A, luciferase assay with VEGF-A promoter Luc reporter (VEGF-A Luc) in the presence of PPAR-δ–binding RNA Apt #6 in HCT116 cells. Cells were cotransfected with VEGF-A Luc (0.3 μg) with pU6 vector (lanes 1 and 2; shown as horizontal line in Apt #6; 1 μg) or with increasing amounts of PPAR-δ Apt #6 (lanes 3 to 6; 0.2, 0.5, 0.7, and 1 μg). After 24 h, the cells were serum starved for 8 h, and cells were treated with GW501516 (5 μmol/L). After 24 h, cells were harvested for luciferase assay. Three independent experiments and a statistical analysis were done.* P < 0.05. B, RT-PCR analysis of VEGF-A and COX-2 mRNA. Cells were transfected with increasing amounts of the PPAR-δ–binding RNA Apt (#6; 0.2, 0.5, and 1 μg) or pU6 vector (U6, 1 μg). After 24 h, transfected cells were serum starved for 8 h, and cells were treated with GW501516 (5 μmol/L). After 24 h, transcript levels were measured by RT-PCR. M, mock-transfected cells. C, densitometer measurements of RT-PCR products from VEGF-A and COX-2 mRNA obtained as described in B. Three independent experiments were done. RT-PCR, reverse transcriptase-PCR.

Figure 4. Inhibition of TCF/β-catenin–mediated transcription by RNA Apts. A, luciferase assay with synthetic TCF/β-catenin–driven firefly luciferase gene (OT reporter). DLD-1 cells were cotransfected either with 600 ng of pU6 vector (lane 1 or 5), or 200, 400, and 600 ng each of PPAR-δ Apt #6 (lanes 2 to 4, respectively), or β-catenin (β-cat) Apt (β-lanes 6 to 8, respectively). When both RNA Apts were transfected (lane 9), 300 ng each of two Apts were transfected. The Renilla luciferase reporter (50 ng) was also coexpressed. After 24 h, luciferase activities were measured, and relative luciferase activity was plotted. *, P < 0.05; **, P < 0.01. B, luciferase assay with PPRE-Luc. DLD-1 cells were cotransfected either with 600 ng of pU6 vector (lane 1 or 5), or 200, 400, and 600 ng each of PPAR-δ Apt #6 (lanes 2 to 4, respectively), or β-catenin (β-cat) Apt (β-lanes 6 to 8, respectively). When both RNA Apts were transfected (lane 9), 300 ng each of two Apts were transfected. The Renilla luciferase reporter (50 ng) was also coexpressed. After 24 h, luciferase activities were measured, and relative luciferase activity was plotted. **, P < 0.01; ***, P < 0.001.
coexpresses RNA aptamers for PPAR-δ, β-catenin, or both in DLD-1 adenocarcinoma cells (Fig. 4A). Among the colorectal cancer cell lines, DLD-1 cells were chosen for this experiment because they showed significant endogenous activation of PPRE and OT luciferase activities. We observed an interesting phenomenon in which PPAR-δ-binding RNA aptamer #6 significantly suppressed β-catenin-mediated transcription. The most prominent inhibition of transcription was found when both the aptamers for PPAR-δ and β-catenin were expressed together. Therefore, the combination of two aptamers could be more beneficial for the regulation of oncogenic transcription. A similar pattern of inhibition was shown when the PPRE reporter was used (Fig. 4B).

Modulation of Tumor-Forming Potential by RNA Aptamer

To test whether the RNA aptamer could suppress the transforming potential of PPAR-δ, HCT116 cells were used in a soft agar colony formation assay. A marked reduction of colony sizes was found in RNA aptamer expressing HCT116 cells (PPAR-δ Apt #6) compared with U6-vector-expressing cells (Fig. 5A). The number of colonies was also significantly reduced in cells expressing RNA aptamer #6 (Fig. 5B).

To further develop the RNA aptamer for therapeutic purposes, we analyzed the predicted structure of RNA aptamer #6 by the mfold program (Fig. 6A). Because it forms a distinct secondary structure, we designed a minimized version of the RNA aptamer with the same stem-loop structure (Fig. 6A). For the therapeutic application, the minimized aptamer #6 was synthesized with 2'-O methylated nucleotides to increase RNA stability (Fig. 6A). To confirm the effect of the minimized aptamer #6, we tested it on the GW501516-activated PPRE luciferase reporter and found it to be as effective as the full-length RNA aptamer #6 in the HCT116 cell (Fig. 6B). To confirm whether the minimized aptamer #6 could suppress the cellular proliferative effect, we carried out an MTS assay. HCT116 cells were treated with GW501516 after the addition of low-serum culture media containing 1% fetal bovine serum. As shown in Fig. 6C, the minimized aptamer #6 suppressed cell growth by about 20%. Similar results were obtained for other colorectal cancer cell lines (data not shown). Taken together, these results strongly suggest that the PPAR-δ-binding RNA aptamer #6 is effective in reducing the tumorigenesis of colon cells.

Discussion

Here, we report the selection and characterization of PPAR-δ-binding RNA aptamers in vitro, their expression in colon cancer cells, and their modulation of the transcriptional activity of PPAR-δ. Because PPAR-δ has important functions in regulating cell proliferation, survival, and apoptosis, it is proposed to be the focal point in the cross-talk between the signaling pathways leading to colorectal cancer development and progression (1–3, 32). Therefore, it might be reasonable to assume that the activation of PPAR-δ disturbances normal cell physiology depending on the cell types (33–37). There are many conflicting reports on the role of PPAR-δ in cell survival, apoptosis, and proliferation, and cancer development (38–41). Whatever the consequences of PPAR-δ activation, PPAR-δ seems to control the inflammatory switch, and balance cell survival and growth by regulating the PI3-kinase/Akt pathway. Therefore, disruption of PPAR-δ might lead to uncontrolled cell growth, which in turn could lead to the development of cancer in many tissues, such as colon, breast, and lung, and cholangiocarcinoma (33–37). Although controversy exists over the roles of PPAR-δ in cell physiology and pathology (38–41), a molecule that could modulate this process would be a useful agent for antitumor therapy because PPAR-δ-mediated transcription is an important regulator for carcinogenesis (1–3, 32). These studies provide a rationale for the development of conventional drugs that have been shown to reduce PPAR-δ-mediated transcription in colon cancer cells and small molecule
antagonists of β-catenin signaling that have been shown to inhibit PPAR-δ protein activity (42).

Emerging evidence suggests that cooperative interaction between Wnt, COX-2, and VEGF signaling can initiate cellular transformation and promote the progression of colorectal cancer (10, 13, 16). Wnt/β-catenin and PPAR-δ are the main players in the development of colorectal cancer, and Wnt signaling has been shown to interact with nuclear receptors (8). The complex regulatory network between PGE2 and Wnt signaling suggests the presence of a positive feedback

Figure 6. Design and effect of minimized RNA Apt #6. A, predicted secondary structure of RNA Apt #6 and minimized RNA Apt #6 (Mini-Apt #6). Gray, selected sequences; defined sequences are not shown. Numbers, the order from the 5' end of the RNA Apt. A portion of Mini-Apt #6 was altered to generate a more stable stem structure. B, luciferase assay of PPRE-Luc. HCT116 cells were cotransfected with PPRE-Luc and Renilla luciferase in the presence of 100 nmol/L of U6 vector, RNA Apt #6, or Mini-Apt #6. Relative luciferase activities are shown. **, P < 0.01. C, measurements of cell proliferation by MTS assay. HCT116 cells were transfected either with Mini-Apt #6 or transfection reagent (mock) only. After 16 h, transfected cells were serum starved for 8 h, and GW501516 (1 μmol/L) was added for 16 h. After incubation, the culture media were changed to fresh media containing 1% fetal bovine serum. Top, time points of the experiment. Cells were measured at 490 nm each day. ***, P < 0.01.
loop between them (14). We also observed the activation of VEGF and COX-2 mRNA expression by PPAR-δ and β-catenin. The exploitation of specific RNA aptamers for these two transcription factors provides a unique opportunity for the complete inhibition of two major signaling pathways in colon cancer development, thereby reducing the power of inflammation-induced tumorigenesis, as shown in Fig. 6.

VEGF is also an interesting target for cancer therapeutics due to its role in angiogenesis. Although the mechanism of VEGF transcription by PPAR-δ is largely unknown, GW501516 treatment clearly induces expression of VEGF-A mRNA. The presence of many TCF/β-catenin responsive elements in the promoter region suggests a role for β-catenin/Tcf in VEGF transcription; appropriately, we tested how the expression of VEGF could be regulated by PPAR-δ as well as by TCF/β-catenin. Because VEGF is also regulated post-transcriptionally, such as through alternative splicing or RNA stability, we expect that alternatively spliced transcripts of VEGF are also selectively regulated by aptamers. Currently, we show that the use of novel RNA aptamers for PPAR-δ and its combination with a previously characterized RNA aptamer for β-catenin may provide novel targeted therapeutics for cancer cell development. We also propose that the use of specific RNA aptamers in cells could be a valuable tool for dissecting the complex physical interactions between signaling molecules. Thus, we believe that aptamer technology can be developed as an emerging class of therapeutics and diagnostics. Nevertheless, further study of the mechanism by which the RNA aptamer inhibits PPAR-δ-mediated transcription is needed. The basic protocol that uses RNA aptamer selection and intramer expression described in this article may well be applicable to other proteins.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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