Functional Genomic Analysis Reveals Cross-talk between Peroxisome Proliferator-activated Receptor γ and Calcium Signaling in Human Colorectal Cancer Cells

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Activation of PPARγ in MOSER cells inhibits anchorage-dependent and anchorage-independent growth and invasion through Matrigel-coated transwell membranes. We carried out a longitudinal two-class microarray analysis in which mRNA abundance was measured as a function of time in cells treated with a thiazolidinedione PPARγ agonist or vehicle. A statistical machine learning algorithm that employs an empirical Bayesian implementation of the multivariate Hotelling T2 score was used to identify differentially regulated genes. Hotelling T2 scores, MB statistics, and maximum median differences were used as figures of merit to interrogate genomic ontology of these targets. Three major cohorts of genes were regulated: those involved in metabolism, DNA replication, and migration/motility, reflecting the cellular phenotype that attends activation of PPARγ. The bioinformatic analysis also inferred that PPARγ regulates calcium signaling. This response was unanticipated, because calcium signaling has not previously been associated with PPARγ activation. Ingenuity pathway analysis inferred that the nodal point in this cross-talk was Down syndrome critical region 1 (DSCR1). DSCR1 is an endogenous calcineurin inhibitor that blocks dephosphorylation and activation of members of the cytoplasmic component of nuclear factor of activated T cells transcription factors. Lentiviral short hairpin RNA-mediated knockdown of DSCR1 blocks PPARγ inhibition of proliferation and invasion, indicating that DSCR1 is required for suppression of transformed properties of early stage colorectal cancer cells by PPARγ. These data reveal a novel, heretofore unappreciated link between PPARγ and calcium signaling and indicate that DSCR1, which has previously been thought to function by suppression of the angiogenic response in endothelial cells, may also play a direct role in transformation of epithelial cells.

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor family of transcription factors (1–4), is expressed as multiple splice variants, which arise because of alternative promoter utilization (5–8). PPARγ2 is abundant in adipocytes (9, 10), whereas PPARγ1 is more widely distributed in the body, with the highest levels of expression reported in adipocytes and colonic epithelium (8, 11, 12). PPARγ plays an important role in adipocyte differentiation (9, 13) and controls important differentiated functions in intestinal epithelial cells (14). However, the physiological role of PPARγ in the colon is largely unknown or has been inferred from studies with transformed colonic epithelial cells. PPARγ is activated by dietary long chain polyunsaturated fatty acids (9, 15–19), and it is plausible that regulation of lipid metabolism is one of the major physiological roles of PPARγ in the gut. Moreover, diets that are rich in such lipids are known to suppress experimental colon carcinogenesis in rodents (20–23), and epidemiological data suggest that such diets also reduce colon cancer risk in humans (24–26). These observations are consistent with the hypothesis that activation of PPARγ results in suppression of colon carcinogenesis.

The ability of PPARγ to suppress experimental colon carcinogenesis in rodents has been unambiguously demonstrated. Several groups have shown that thiazolidinedione drugs, which activate PPARγ, suppress azoxymethane-induced pre-neoplastic lesions and tumors in the colons of rats and mice (27–30), whereas whole animal hemizygous knock-out of PPARγ promotes colon tumor formation in azoxymethane-treated mice (31). Two early studies reported that thiazolidinediones may have a slight promotive effect on colon tumor formation in APC+/Min mice (32, 33); however, more recent studies...
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reported no such effect (34, 35), and it has recently been shown that biallelic knock-out of PPARγ in colonic epithelial cells promotes colon carcinogenesis in APC+/Min mice (36). These data strongly indicate that PPARγ blocks colon carcinogenesis in rodents. The situation in humans is less clear.

Although loss-of-function mutations have been reported in human colon cancers (37), the frequency with which such mutations occur appears to be low (38). PPARγ expression is inhibited in ulcerative colitis (39, 40) and in acromegaly (41); both of these conditions are associated with increased risk of colon cancer, suggesting that a decrease in PPARγ expression may be a colon cancer risk factor. A small phase II clinical trial using a weak PPARγ agonist (troglitazone) showed no objective response among patients with metastatic colon cancer who had failed standard therapy (42). However, a number of human colon cancer cell lines express PPARγ (43), and thiazolidinediones inhibit growth of some of these cell lines in culture and in vivo (44–50).

Taken together, the data indicate that PPARγ is a colon cancer suppressor, but that the effects of this receptor are manifest either in normal colonic epithelial cells or in some very early stage of transformation of such cells. Because PPARγ is a transcription factor, it is likely that the tumor-suppressive effects of this receptor are because of changes in gene expression and that the mechanism of action of PPARγ might be inferred from a functional genomic study of the effects of thiazolidinediones on early stage colon cancer cells. The observations described below result from such a study in which we used the high affinity PPARγ agonist RS5444 (14, 51–53) to activate PPARγ in MOSER cells. These cells have sustained an APC loss-of-function mutation followed by loss of heterozygosity of the wild-type APC allele; however, MOSER cells retain TGFβ sensitivity (54, 55) and have wild-type K-Ras, indicating that they represent a late adenoma or early adenocarcinoma stage. Furthermore, PPARγ inhibits transformed properties of MOSER cells (56), making them an ideal model to study the mechanism whereby this receptor inhibits early steps in transformation of colonic epithelial cells.

We carried out a longitudinal two-class experiment in which we analyzed gene expression as a function of time in RS5444-treated and control MOSER cells. The principal advantage of a two-class experiment of this sort is the ability to use statistical analysis of changes in gene expression over time to minimize type I statistical error, which is a major problem in the analysis of microarrays data from single time point experiments or longitudinal one-class experiments in which expression is measured only in treated cells (57). Analysis of changes in gene expression as a function of time after activation of PPARγ has the potential to identify early responses, which may be causal, and perhaps to infer regulatory connections between genes that are induced at different times after activation of PPARγ. Although the amount of information that one obtains from a two-class analysis of this sort is great, statistical analysis of a large, multivariate data set presents some interesting challenges that do not apply to one-class analyses. Here we report the application of an empirical Bayesian statistical machine learning algorithm to carry out a multivariate statistical analysis and to identify PPARγ targets in early stage colon cancer cells.

Ontologic analysis of such targets indicates that the pattern of gene expression that one observes is consistent with the cellular phenotype obtained upon activation of PPARγ. However, one entirely unanticipated pathway emerged from this analysis. Our data indicate that PPARγ regulates a number of components of the calcium/calmodulin/calcineurin signaling axis. Our studies have focused upon one critical component in this pathway, Down syndrome critical region 1 (DSCR1, which is also known and MCIP1, Adapt78, and calcipressin 1).

DSCR1 is an endogenous calcineurin inhibitor (58–60) and is known to block calcineurin-mediated dephosphorylation/activation of the cytoplasmic component of the nuclear factor of activated T cells (NFATc) family (58, 60, 61). DSCR1 isoform 4 is induced by NFATc and serves as a feedback inhibitor to inactivate calcineurin and inhibit nuclear uptake of NFATc (58, 60, 62–65). It has recently been reported that DSCR1 is a tumor suppressor (66), and the observation that DSCR1 is induced by PPARγ suggested to us that DSCR1-mediated inhibition of calcineurin activity and NFATc signaling might play a role in PPARγ-mediated inhibition of transformation. We report here that DSCR1 is necessary for PPARγ inhibition of proliferation and invasion. This study therefore establishes a novel, heretofore unappreciated link between PPARγ, calcium signaling, NFATc, and transformed properties of early stage colon cancer cells. The critical node in this pathway is DSCR1, which has previously been thought to function primarily as an angiogenesis inhibitor. Our data indicate that DSCR1 may also directly affect early events in transformation.

MATERIALS AND METHODS

Reagents—The thiazolidinedione RS5444 was provided by Sankyo Ltd., Tokyo, Japan (51). RS5444 is a high affinity PPARγ agonist that has been shown to regulate growth and motility of intestinal epithelial as well as thyroid cancer cells (14, 52, 53). Rosiglitazone (49653, Invitrogen) was purchased from ChemPacific. Cyclosporin A was purchased from Sigma. Cycloheximide was purchased from Calbiochem.

Cell Lines—MOSER S cells were maintained in mid-log phase growth in DMEM supplemented with 1% charcoal-stripped fetal bovine serum (CS-FBS) below 75% confluence. RS5444 was dissolved in Me2SO, and cells were treated with 10 μM RS5444 unless otherwise indicated. Control cells were treated with an equivalent volume of Me2SO equal to a final concentration of 0.1% v/v.

Cell Proliferation Assays—MOSER cells were plated at 2 × 10^4 cells per 60-mm tissue culture plates in DMEM + 1% CS-FBS and allowed to attach overnight. Cells were then treated with 0.1% Me2SO, 10 μM RS5444, or 1 μM rosiglitazone. Cells were harvested 1, 3, and 5 days after treatment and counted using a Beckman Coulter counter (diameter = 8 μm).

For anchorage-independent proliferation assay, 2 × 10^5 MOSER cells were suspended in 0.75% SeaPlaque agar (FMC Corp., Philadelphia, PA) in DMEM supplemented with 10% CS-FBS, 1% penicillin/streptomycin/glutamine, 10 μM HEPES, 0.1% gentamycin, and 0.1% Me2SO or 10 μM RS5444. Suspended cells were layered over 0.75% agar base in the same medium. After 2 weeks, cells were fixed with 100% methanol. The agarose plates were dried on glass, and cells were stained...
with Giemsa Staining solution (J. T. Baker Inc.) for 20 min at room temperature. Stained colonies were washed with H2O, and a digital image was obtained using a Kodak 4800 photographic system with an EL Logic 100 camera and Gel Logic 100 digital imaging software. Camera zoom was set to 48.5 mm and F-stop to 12.5. Gel Logic 100 software was set to receive white light trans-illumination with an exposure time of 0.068 s. Images were digitally captured as 8-bit color TIFF files, converted into 1-bit files, and cropped to ensure no data outside the field were included in the file. All images were kept at the same size and resolution and imported into MATLAB as matrices. MATLAB then summed the number of bits over the entire matrix. The fraction of colonies represented as black spots on the TIFF image (denoted as 1s) divided by the entire area was scored as the percentage of colony coverage.

Colony Formation Assay—MOSER cells were treated with 0.1% Me2SO or 10 nM RS5444 for 24 h, and 2 × 10^5 cells were plated in 60-mm tissue culture dishes. The cultures were grown in media without RS5444 or Me2SO for 5 days. After media were removed, the cells were washed once with PBS and then fixed with ice-cold 100% methanol for 10 min at −20 °C. Fixed cells were washed once with PBS and stained with Giemsa for 20 min at room temperature. Stained colonies were washed once with PBS and quantified by the Kodak 4800/MATLAB imaging and quantification method described above.

Migration and Invasion Assays—MOSER cells treated with 0.1% Me2SO or 10 nM RS5444 for 24 h were harvested using trypsin, washed once with PBS, and suspended in serum-free DMEM containing 250 μg/ml trypsin, washed once with PBS, and then suspended in serum-free DMEM containing 5% CS-FBS. For migration assays, transwell chamber membranes (BD BioCoat) were coated with 15 μg/ml collagen I (Collagen Biomaterials, Vitrogen) for 30 min at 37 °C. 1 × 10^5 cells suspended in DMEM/BSA were added to the upper chamber, and DMEM containing 5% CS-FBS was added to the lower chamber. After incubating for 6 h at 37 °C, nonmigrating cells were removed from the upper chamber with a cotton swab, and cells that had migrated to the lower surface of the membrane were washed once with PBS and stained with 0.2% (w/v) crystal violet in 2% ethanol. Migration was quantified by counting cells per mm^2 using bright field optics. For invasion assays, cells were pretreated and harvested as described above. Matrigel invasion chambers (8-μm pore size, BD BioCoat) were re-hydrated according to the manufacturer’s instructions. 1 × 10^5 cells were added to the chamber and allowed to invade for 6 h. Cells that had invaded were stained as described above. Invasion was quantified by averaging the cell count per chamber.

Lentiviral shRNA Gene Silencing Assay—shRNA MISSION™ RNA interference lentiviral transduction particles were obtained through the partnership agreement between Sigma and the Mayo Clinic RNA Interference Technology Resource. The Invitrogen Viralpower lentiviral expression kit was used to produce lentiviral stock in the packaging cell line 293FT according to the manufacturer’s protocol. MOSER cells, no more than 70% confluent and growing in DMEM + 0.1% FBS, were infected with virus-containing media at a 1:10 dilution of virus to target cell media and 0.6 μg/ml Polybrene. 24 h post-infection, MOSER cells were re-fed with DMEM + 1% CS-FBS. 48 h post-infection, cells were harvested, and knockdown efficiency was assessed by quantitative real time PCR (qPCR).

Western Blotting, RNA Isolation, and Quantification—Western blotting was performed as described previously (52). Total RNA was extracted using RNAqueous (Ambion), according to the manufacturer’s protocols. RNA quality and integrity were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies) along with the Agilent RNA 6000 nano kit using Agilent 2100 Expert software (Agilent Technologies) following the manufacturer’s protocols. The RNA integrity number was used as an objective measurement of RNA quality. Any sample with an RNA integrity number of less than 8.5 was discarded from further analysis. Reagents for qPCR analysis of mRNA abundance were purchased from Applied Biosystems. Analysis was carried out using 2 ng eq of cDNA on an Applied Biosystems 7900 thermal cycler, and data were analyzed using the SDS 2.3 software package. All data were normalized to expression of GAPDH, and data are routinely expressed as 2^(-(ΔΔCt)).

Microarray Preparation, Quality Control, Preprocessing of Probe Level Data, and Analysis of Differential Expression—mRNA from technical replicates in the presence or absence of RS5444 at 6-h intervals (0, 6, 12, 18, and 24 h) was isolated as described above. Gene profiling analysis was performed using HG133 version 2 Gene Chip Microarrays (Affymetrix) as described previously (14). Affymetrix CEL files, extracted from Affymetrix GCOS software, were loaded into the R language environment (version 2.2.1) with the Bioconductor (68) libraries (version 1.7.1) and run under the Linux distribution OpenSuse, version 10, 64-bit operating system with 10 gigabytes of available memory. CEL image files were visually inspected. Any image that displayed surface artifacts such as scratches, ghosting, or uneven liquid pooling was excluded from further analysis.

Computational preprocessing was performed using several algorithms to obtain different figures of merit. The first metric calculated was the Bioconductor’s implementation of Affymetrix MAS 5.0 change call using the mas5calls function with default parameters under the affy package (version 1.8.1) (69). The second metric was the Bioconductor implementation of Affymetrix MAS 5.0 summarization values using the mas5 function under the affy package with default parameters (neither normalize nor background correct) (69). The third metric was the Irizarry implementation of robust multichip average (RMA) summarization values (log base 2 output) using the justRMA function call with default parameters under the affy package (70). Finally, the fourth metric utilized the Wu and Irizarry implementation of GCRMA summarization values (log base 2 output) using the justGCRMA function call with default parameters under the package GCRMA (version 2.2.0) (71). The data sets were then exported into Insightful Splus 7 with ArrayAnalyzer 2.0. Both Bland-Altman plots and principal components analysis were calculated to determine more refined analysis of across-replicate and across-time variation of the gene chips (72). Summarization data were then imported into a data base and filtered based on change call information, i.e. only probe sets that were determined by the Bioconductor.
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mas5calls function to be either present “P” or marginally present “M” across all conditions were used for further analysis.

Three different statistical models were considered for scoring significant differential expression, each making different assumptions about the data. The first was a nonparametric model making no assumptions at all about the input data structure or possible underlying biological meaning. The second model was the polynomial regression model from the Bioconductor library maSigPro (68). Here, distances were computed based on a two-step least squares regression fit to find probe sets with significant temporal expression changes and significant differences between experimental groups. The third model is Bioconductor library time course (57, 73). The generalized multivariate t test, known as the HotellingT2 test, was modified by inferring from the data hyper-parameters (tuning parameters). These tuning parameters adjust the co-variance matrix and ultimately reduce both type I and type II statistical error. A detailed mathematical description of the algorithms can be found in the Supplemental Material.

Gene Ontology Analysis—The gene ontology analysis program ErmineJ, version 2.1.12, was used to calculate the over-representation of gene ontology terms (74, 75). Longitudinal microarray data were used to define groups of “GO terms” that consist of functionally related genes. Over-representation analysis uses a cut-off threshold to determine whether there are gene sets within GO classes that are statistically over-represented. We used over-representation analysis instead of the more generalized re-sampling calculation of the functional class score (76) for statistical confidence of functional groupings. ErmineJ evaluates 6127 GO classes for biological function. Only classes with a minimum of 5 and maximum of 100 genes were taken into account, as very small or very large classes are unlikely to be as informative (either too specific or too general) (77). The data input file consisted of the initial 10227 probe sets with a MAS 5 change call of either “P” or “M” across all conditions. A HotellingT2 score cutoff of 281.7 (corresponding to an MB statistic cutoff of $\pm 2.0$) was used as the figure of merit in ErmineJ to calculate p values. Only GO classes with a p value of 0.05 or less were considered for further analysis.

RESULTS

MOSER Cells as a Model for Suppression of Early Events in Colon Carcinogenesis—The MOSER colon carcinoma cell line was established from a spontaneous primary human colon tumor (55). The MOSER S line that we have used in these experiments is sensitive to TGFβ (54, 78). TGFβ sensitivity is generally lost in colorectal cancer cells during the transition from adenoma to adenocarcinoma, suggesting that MOSER cells represent a very early adenocarcinoma or late adenoma stage in colon cancer development. The conventional “Vogelgram” representation of colon carcinogenesis represents cancer progression as a process whereby specific genetic mutations are acquired in a defined sequence (79, 80). Early stage transformation is associated with mutation in critical components of the APC/β-catenin pathway, followed by loss of heterozygosity. Progression from adenoma to adenocarcinoma is associated with acquisition of K-Ras mutations and loss of TGFβ sensitivity. MOSER genomic DNA was isolated, and exon 15 of the APC gene, exon 3 of β-catenin, and codons 12, 13, and 61 of K-Ras were sequenced. A substitution mutation (C to T) was detected at nucleotide position 4069 in exon 15 of APC (supplemental Fig. S1A), resulting in a nonsense mutation. The sequence analysis suggested that the wild-type exon 15 allele was not present in genomic DNA, and Western blot analysis indicated that MOSER cells express only a 150-kDa APC protein, with no evidence of the 310-kDa wild-type APC (supplemental Fig. S1B). These data indicate that MOSER cells have sustained a loss of function APC mutation, followed by loss of heterozygosity at the APC locus. We detected no mutations to the N terminus of β-catenin or to codons 12, 13, or 61 of K-Ras. Because K-Ras mutations and loss of TGFβ sensitivity commonly occur during the transition from adenoma to adenocarcinoma during colon carcinogenesis (80), we conclude that MOSER represents a very early stage colon cancer cell and is therefore an appropriate model to study the effects of PPARγ on early stage colon carcinogenesis.

MOSER cells express PPARα, -δ, and -γ isoforms (56, 78), but we have previously shown that RS5444 activates PPARγ but does not activate PPARα or PPARδ (14). MOSER cells were cultured in the presence of RS5444 for 24 h. RNA was isolated every 2 h after treatment, and protein was isolated every 12 h after treatment. RS5444 rapidly induced the PPARγ target gene PDK4 (Fig. 1A, closed circles). RS5444 did not alter the expression of PPARγ mRNA (Fig. 1A, open circles) or protein (Fig. 1A, inset).

Activation of PPARγ inhibits growth of nontransformed intestinal epithelial cells (14). Growth of MOSER cell cultures was likewise inhibited by the thiazolidinediones RS5444 (Fig. 1B, squares) or rosiglitazone (Fig. 1B, diamonds). No cytological evidence of apoptosis was observed within 3 days after activation of PPARγ, although cells slowly die beginning 4–5 days after addition of the agonist (data not shown). We have also observed that activation of PPARγ in nontransformed intestinal epithelial cells causes irreversible withdrawal from the cell cycle (14). To determine whether MOSER cells exhibit a similar phenotype, the cells were treated with Me2SO or RS5444 for 24 h. Thereafter cells were harvested and counted, and a known number of cells was plated in complete media that did not contain RS5444. The ability to form clonal colonies was estimated thereafter, as described under “Materials and Methods.” Transient activation of PPARγ reduced the clonogenic potential of MOSER cells to less than 60% of that of control cells (Fig. 1C, p < 0.005). These data indicate that transient activation of PPARγ in MOSER cells caused irreversible withdrawal from the cell cycle, similar to the response we observed in nontransformed intestinal epithelial cells (14). The data are consistent with the hypothesis that PPARγ either induces differentiation or promotes senescence in MOSER cells. MOSER cells exhibit anchorage-independent growth in soft agar. Addition of RS5444 to soft agar inhibited colony formation by MOSER cells (Fig. 1D, p < 0.002). These data indicate that PPARγ inhibits both anchorage-dependent and anchorage-independent growth of MOSER cells.

PPARγ regulates motility of nontransformed intestinal epithelial cells (14, 52). To determine the effects of PPARγ on MOSER cell invasion and migration, cells were treated with

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Me\textsubscript{2}SO or RS5444 for 24 h and then plated onto Matrigel-coated Boyden transwell chambers to measure invasion or onto collagen-coated transwell chambers to measure migration. Activation of PPAR\textsubscript{γ} significantly inhibited invasion by MOSER cells (Fig. 1E, \(p < 0.01\)). MOSER cells, in contrast to nontransformed intestinal epithelial cells (14), are highly motile. PPAR\textsubscript{γ} stimulates motility of nontransformed intestinal epithelial cells (14, 52) but had no effect on motility of MOSER cells, assessed by the ability to penetrate collagen-coated membranes (Fig. 1E).

The data indicate that MOSER cells are minimally transformed and probably represent a late adenoma or early adenocarcinoma state. Activation of PPAR\textsubscript{γ} in such cells inhibits growth, causes irreversible withdrawal from the cell cycle, and inhibits anchorage-independent growth and invasion. MOSER cells therefore represent an appropriate model to study the tumor-suppressive effects of PPAR\textsubscript{γ}. We reasoned that because PPAR\textsubscript{γ} is a transcription factor, the tumor suppressive-mechanism(s) might be inferred from a functional genomic analysis of PPAR\textsubscript{γ} target genes in MOSER cells.

**Functional Genomics Analysis of PPAR\textsubscript{γ} Effects upon MOSER Cells**—The data shown in Fig. 1 give a clear, albeit high level, picture of the cellular processes that are affected by activated PPAR\textsubscript{γ}. To gain mechanistic insight into these responses, it was important to obtain the best possible functional genomic data set during the initial 24 h after addition of the PPAR\textsubscript{γ}-mediated pathway regulation. We utilized a two-class longitudinal array experimental design in which technical biological replicates (replicate samples collected simultaneously from parallel cultures treated in the same manner) were isolated at intervals after addition of the PPAR\textsubscript{γ} agonist or vehicle (Me\textsubscript{2}SO).

Three samples were analyzed from control and treated cells, harvested at 6-h intervals from 0 to 24 h. Three different algo-
rithms were evaluated for preprocessing, normalization, and background correction using all 30 chips: MAS5 from Affymetrix, RMA from Bioconductor, and GCRMA from Bioconductor. Bland-Altman (MvA) plots, generated using the array analyzer Splus, were used to compare the performance of each algorithm versus raw unprocessed data, as described in the supplemental Fig. S2A. Bland-Altman analysis of these three different algorithms indicated that GCRMA outperformed the other summarization protocols for this two-class time course data set (supplemental Table SII), and all subsequent analyses employed GCRMA for normalization, background correction, and summarization. Principal components analysis indicated that the first and second principal components of the control chips clustered in a manner that was independent of the time variable, whereas the first and second principal components of the treated chips diverged from the controls as a function of time, as would be predicted for a time course experiment in which changes in gene expression were initiated at 0 h (supplemental Fig. S2B).

A statistical bootstrapping method was used to compare an empirical Bayesian machine learning algorithm, a polynomial regression algorithm, and a nonparametric algorithm and to calculate the statistical power for each error model. The empirical Bayesian machine learning algorithm clearly outperformed the other two error models, as demonstrated by statistical power to identify true positive and true negative values (supplemental Table SIII), and the statistical machine learning algorithm employing an empirical Bayesian implementation of the multivariate HotellingT2 score was chosen to define differentially expressed genes over time.

Within the full list of 10,227 probe sets eligible for differential expression analysis by the empirical Bayes model, values for the HotellingT2 scores ranged from 5590.9 to 0.02, with higher scores reflecting a higher degree of confidence that one has identified a target gene. The corresponding MB statistic scores ranged from −0.11 to −7.29. Higher HotellingT2 and MB statistic scores represent higher confidence that the profile of a particular probe set is different in control and treated samples. A full list of probe sets, their HotellingT2 score, and their MB statistic is provided in the supplemental Table SIII. An MB statistic cutoff score of −2.0 was chosen to define the subset of differentially expressed probe sets. This cutoff is rather conservative, within the context of Bayesian statistics, and virtually guarantees the resultant probe set list as representing true differential expression. Therefore, any probe set with an MB statistic of −2.0 or greater was included for further analysis. The resulting subset contained 1975 probe sets from probe set 222912 at “Arrestin Beta 1” with a HotellingT2 score of 5590.9, to 218354 at “hematopoietic stem/progenitor cells 176” with a HotellingT2 score of 281.7.

### Table 1

The top 20 positively regulated and negatively regulated probe sets

| Probe set | Gene title | Gene symbol | HotellingT2 score | MB statistic | Max FC |
|-----------|------------|-------------|-------------------|--------------|--------|
| 205757_s_at | Carcinoembryonic antigen-related cell adhesion molecule 6 | CEACAM6 | 3958.13 | −0.190 | 4.48 |
| 211893_s_at | Carcinoembryonic antigen-related cell adhesion molecule 1 | CEACAM1 | 2674.21 | 0.219 | 3.80 |
| 209498_at | Carcinoembryonic antigen-related cell adhesion molecule 1 | CEACAM1 | 4892.80 | −0.135 | 3.68 |
| 212099_at | ras homolog gene family, member B | RHOB | 3225.16 | 0.253 | 3.67 |
| 245560_at | Trophoblast-derived noncoding RNA | TnRNA | 1230.80 | −0.709 | 3.63 |
| 249407_at | Abhydrolase domain containing 3 | ABHD3 | 1433.04 | −0.468 | 3.22 |
| 213816_s_at | Met proto-oncogene (hepatocyte growth factor receptor) | MET | 1773.12 | 0.502 | 3.06 |
| 211657_at | Carcinoembryonic antigen-related cell adhesion molecule 6 | CEACAM6 | 2038.04 | 0.491 | 3.93 |
| 201650_at | Keratin 19 | KRT19 | 1590.98 | 0.558 | 2.91 |
| 209122_at | Adipose differentiation-related protein 5 | ADFP | 2825.39 | 0.399 | 2.88 |
| 212531_at | Lipocardin 2 (oncogene 24p3) | LCN2 | 1150.28 | −0.747 | 2.80 |
| 214581_s_at | Tumor necrosis factor receptor superfamily, member 21 | TNFRSF21 | 2159.26 | 0.408 | 2.77 |
| 202672_s_at | Activating transcription factor 3 | ATF3 | 1516.05 | 0.585 | 2.74 |
| 222529_at | Down syndrome critical region gene 1 | DSCR1 | 2256.07 | 0.503 | 2.71 |
| 209365_s_at | Extracellular matrix protein 1 | ECM1 | 1792.35 | 0.496 | 2.62 |
| 212800_at | KIAA0367 | KIAA0367 | 1586.62 | −0.560 | 2.58 |
| 217173_s_at | Low density lipoprotein receptor | LDLR | 1140.01 | −0.758 | 2.51 |
| 202842_s_at | Dnal (Hsp40) homolog, subfamily B, member 9 | DNAJB9 | 997.23 | 0.849 | 2.44 |
| 212570_at | KIAA0830 protein | KIAA0830 | 1086.05 | −0.790 | −3.93 |
| 234987_at | Chromosome 20 open reading frame 118 | C2orf118 | 3697.08 | −0.210 | −3.93 |
| 212860_at | Sterile α-motif and leucine zipper containing kinase AZK | AZK | 717.36 | −1.100 | −3.96 |
| 210292_at | EST | EST | 2621.56 | −0.327 | −3.99 |
| 228250_at | KIAA1961 gene | KIAA1961 | 5132.36 | 0.124 | −4.00 |
| 225773_at | KIAA1972 protein | KIAA1972 | 2579.89 | 0.025 | −4.00 |
| 230143_at | Frequent homolog (Drosophila) | FREQ | 2097.36 | −0.421 | −4.03 |
| 222912_at | Arrestin Beta 1 | ARRB1 | 5590.89 | 0.105 | −4.53 |
| 203255_at | F-box protein 11 | FBXO11 | 4072.81 | 0.614 | −4.39 |
| 223984_s_at | Nucleoporin like 1 | NUP11 | 2822.08 | −0.300 | −4.42 |
| 222912_at | Arrestin, β1 | ARRB1 | 5590.89 | 0.105 | −4.53 |
| 1555772_a_at | Cell division cycle 25A | CDC25A | 4091.39 | −0.181 | −4.61 |
| 212386_at | Transcription factor 4 | TCF4 | 2164.22 | −0.407 | −4.72 |
| 205471_s_at | Dachshund homolog 1 (Drosophila) | DACH1 | 2362.74 | −0.369 | −5.01 |
| 221276_s_at | Syncoilin, intermediate filament 1 | SYN1 | 4188.57 | −0.175 | −5.24 |
The majority of the PPARγ target genes displays down-regulation compared with control. If one uses the 24-h time point as a reference point for measuring fold changes between the control and RS5444-treated replicates, 33 probe sets had a positive fold change of 2 or greater, and 356 had a negative fold change of 2 or less. If one considers all time points and takes the largest difference of median values at any time point, either negative or positive (defined as the maximum difference of medians), then 133 probe sets had a positive fold change of 2 or greater, and 1807 probe sets had a negative fold change of 2 or less, suggesting that the empirical Bayes algorithm models many more genes as repressed than induced by PPARγ. The polynomial regression calculated 4,693 probe sets as having a Benjamini-Hochberg adjusted p value of 0.05 or less, and the nonparametric model calculated 6,546 probe sets as having an adjusted p value of 0.05 or less. Both models calculated about the same percentage of up-regulated and down-regulated genes as did the empirical Bayes model (data not shown). These results are consistent with the bootstrapping analysis and suggest that analysis of longitudinal time course data using statistical models that lack moderation results in moderate to large type I error.

The top 20 positively regulated and negatively regulated probe sets are listed in Table 1. CEACAM6 probe set “203757_s_at” (carcinoembryonic antigen-related cell adhesion molecule 6), which is a known PPARγ target (56), had the greatest positive regulation with a fold change of 2^4.8. SYNC1 probe set “221276_s_at” (syncoilin, intermediate filament 1) had the greatest negative regulation with a fold change of 2^-5.24.

Genomic Ontology of PPARγ Target Genes—ErmineJ was used to analyze gene ontology (GO) of the 1975 differentially expressed probe sets identified by the empirical Bayes HotellingT2 model. Of the 6127 GO classes considered, 112 GO classes were found to have a p value of 0.05 or less by over-representation analysis. A full list of GO classes represented in the data set can be found in the supplemental Table SIV. The list of GO terms calculated by ErmineJ grouped into three superfamilies of biological function, as illustrated in Fig. 2. The most significant GO class includes genes that are involved in metabolism, and among these lipid, energy, and carbohydrate metabolism are notably represented. It is known that PPARγ regulates metabolism in adipocytes (9, 13, 16, 17) and intestinal epithelial cells (14), and our data indicate that metabolic control is likely to be a major function of PPARγ in colon cancer cells. Heat maps corresponding to these metabolic target genes are shown in Fig. 3A. As can be seen from this representation, some of the metabolic genes, such as LDLR, were induced after activation of PPARγ, whereas others, such as TMEM23, were repressed. These results indicate that the metabolic response to activation of PPARγ in colon cancer cells is both dramatic and complex.

Among the highest scoring GO classes are genes that are involved in proliferation and replication (Fig. 2). A heat map representation of PPARγ target genes that are involved in pro...
liferation/DNA replication (Fig. 3) reveals that all of the genes in this proliferative cohort were repressed after activation of PPARγ, consistent with the observation that PPARγ inhibits MOSER cell proliferation. A third major GO class includes genes that are involved in migration, motility, and cell structure (Fig. 2), which exhibit both positive and negative regulation (Fig. 3). We have previously shown that PPARγ regulates a cohort of genes that are involved in cellular motility, cell-cell interaction, and interaction with the extracellular matrix of intestinal epithelial cells (14), and that PPARγ regulates motility of such cells (52). Our analysis of genomic response of MOSER cells is consistent with the observation that PPARγ affects invasive properties of such cells.

Genomic ontology analysis indicates that PPARγ regulates functional cohorts of genes that are involved in metabolism, proliferation, or motility, consistent with the observed phenotype of RS5444-treated MOSER cells and/or with published observations concerning cellular responses to PPARγ. However, the power of genomic ontology analysis rests in the ability to infer regulation of signaling pathways that might not be intuitively obvious. ErmineJ inferred a statistically significant cohort of PPARγ target genes that are involved in calcium signaling (Fig. 3D). This outcome was unanticipated because, to our knowledge, no one has shown a relationship between PPARγ and calcium signaling. However, calcium is a major regulator of cellular proliferation and motility, and it is plausible that PPARγ suppression of MOSER cell transformed properties might be linked to regulation of calcium signaling.

**Ingenuity Pathways Analysis Infers a DSCR1-Calcineurin-NFATc Signaling Axis**—The PPARγ target genes with their associated HotellingT2 scores and difference of median distances were used as figures of merit to interrogate IPA functions. Significant cross-talk between PPARγ and calcium signaling was inferred by the observation that RS5444 inhibits expression of a number of critical calcium signaling components, summarized in Fig. 4 (see supplemental Fig. S3 for the detailed IPA pathway.) These PPARγ-regulated calcium signaling genes included the p85 regulatory subunit of phosphatidylinositol 3-kinase (p85), the endoplasmic reticulum inositol 1,4,5-trisphosphate receptor, calmodulin, the PPP3CB catalytic subunit of calcineurin, calmodulin kinase kinase, and cAMP-response element-binding protein 1. PPARγ also induced the endogenous calcineurin inhibitor Down syndrome critical region 1 (DSCR1, also known as MCIIP1, Adapt78, and calcipres-
Ingenuity pathway analysis inferred that PPARγ affects signal transduction between DSCR1, the serine/threonine phosphatase calcineurin (PPP3), and the cytoplasmic component of the transcription factor nuclear factor of activated T-cells (NFATc), whose activity is regulated by calcineurin-mediated dephosphorylation (81). Similar results were obtained using Metacore pathway analysis tools (not shown). The gene candidates were interrogated using IPA functions, as shown in Fig. 4B. (The detailed IPA functional assignment map is shown in supplemental Fig. S3B.) The functional matches for this pathway included growth of eukaryotic cells (66, 82–84), colony formation of eukaryotic cells (85, 86), and proliferation of cells (66, 87–89). The inferred PPARγ/calcium signaling axis was also associated with functions related to invasiveness as represented by ingenuity knowledge base. IP3, inositol 1,4,5-trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; CREB, cAMP-response element-binding protein; CAMKK, calmodulin kinase kinase; ER, endoplasmic reticulum.

**FIGURE 4.** Ingenuity pathways analysis infers novel PPARγ regulation in calcium-mediated signaling. A, Ingenuity pathways knowledge base canonical pathway of calcium-mediated signaling with PPARγ represented by maximum fold change. Colored genes indicate probe sets that were significantly regulated, by HotellingT2 and MB statistic; green indicates inhibited and red indicates induced. B, inferred calcium-mediated signaling axis of genes overlaid with functions (fx) of both proliferation and invasiveness as represented by ingenuity knowledge base. IP3, inositol 1,4,5-trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; CREB, cAMP-response element-binding protein; CAMKK, calmodulin kinase kinase; ER, endoplasmic reticulum.

**FIGURE 5.** DSCR1 is regulated by PPARγ in MOSER cells. A, DSCR1 expression in the presence (filled symbols) or absence (open symbols) of RS5444 as modeled by the empirical Bayes HotellingT2 algorithm with GCRMA preprocessed microarray data as input. Ordinate denotes log base 2 expression. B, RNA was extracted from RS5444-treated cells at 6-h intervals, and qPCR was used to measure DSCR1 mRNA, relative to endogenous GAPDH. Data points represent the mean ± S.D. of three technical replicates. C, total RNA from MOSER cells treated with or without RS5444 for 24 h was reverse-transcribed into cDNA, and qPCR was performed using primer sets that were designed to amplify DSCR1 isoforms 1, 2, and 4. Bars represent mean ± S.D. of DSCR1/GAPDH (n = 3).

sequently, our studies focused on defining the role of the DSCR1 in PPARγ suppression of transformed properties of MOSER cells.
**PPARγ Regulation of DSCR1 Expression**—The HotellingT2 model (Fig. 5A) identified DSCR1 as a highly significant target (HotellingT2 rank 44 out of 1975), and the model predicted that DSCR1 mRNA expression increased about 4-fold within 12 h after activation of PPARγ. This prediction was confirmed by qPCR analysis of DSCR1 mRNA expression, which was induced about 3-fold and reached steady state 12 h after addition of RS5444 (Fig. 5B). The DSCR1 gene encodes three functional transcripts: DSCR1-1 (also known as DSCR1A), DSCR1-2 (DSCR1B), and DSCR1-4 (DSCR1C), which arise from alternative promoter utilization and splicing to common exons 5–7 (96). Of these isoforms, only DSCR1-1 and DSCR1-4 are known to be expressed in adult tissues (96, 97). qPCR primers were obtained to measure the abundance of transcripts across the splice junctions between exons 1 and 5 (DSCR1-1), exons 2 and 5 (DSCR1-2) and exons 4 and 5 (DSCR1-4). MOSER cells express both DSCR1-1 and DSCR1-4 isoforms, as shown in Fig. 5C, and DSCR1-2 mRNA was at or near the limits of detection by qPCR. No significant change in DSCR1-1 (p = 0.227) or DSCR1-2 (p = 0.069) expression was observed within 24 h after addition of RS5444, whereas the abundance of DSCR1-4 mRNA increased about 3-fold (p = 0.013, Fig. 5C), indicating that induction of DSCR1 by PPARγ is because of activation of the exon 4 promoter.

The induction of DSCR1 by thiazolidinediones was dose-dependent. The estimated EC_{50} for RS5444 was about 0.3 nM (Fig. 6A), whereas that for rosiglitazone was about 10 nM (Fig. 6B). These data conform to the EC_{50} values that we have measured for induction of PDK4 by PPARγ (14) and suggest that induction of DSCR1 by thiazolidinediones is dependent upon the PPARγ receptor. This prediction was confirmed by lentiviral shRNA-mediated knockdown of PPARγ. Lentiviral populations were screened to identify an shRNA knockdown virus that yields about 85% knockdown of PPARγ at the level of mRNA and protein (supplemental Fig. S4). MOSER cells were transiently infected with either the NT control virus or the HD-32 PPARγ knockdown virus, and RS5444 or Me2SO was added 48 h later.
The NT control virus had no effect on induction of DSCR1 or PDK4 by RS5444 (Fig. 6, C and D). However the PPARγ knockdown virus completely blocked induction of both DSCR1 and PDK4, indicating that induction of DSCR1 is PPARγ-dependent and not because of PPARγ-independent effects of thiazolidinediones (98–100). Cycloheximide was used to determine whether induction of DSCR1 is a primary consequence of activation of PPARγ. As shown in Fig. 6E, simultaneous addition of cycloheximide and RS5444 blocked induction of DSCR1, but had no effect on induction of PDK4 (Fig. 6F). These data are consistent with the conclusion that PPARγ does not directly transactivate the DSCR1 promoter but that de novo protein synthesis is required upstream of induction of DSCR1.

The DSCR1-4 promoter contains 15 tandem NFATc-binding sites (97), and DSCR1 is known to be induced by calcineurin-dependent dephosphorylation of NFATc (58, 60). Cyclosporin A binds to calcineurin, inhibits activity of calcineurin, and prevents nuclear uptake of NFATc (101). Cyclosporin A blocked the induction of DSCR1 by RS5444 (Fig. 6G) but had no effect on induction of PDK4 (Fig. 6H). This observation indicates that calcineurin is required for DSCR1 induction, and suggests that PPARγ induces DSCR1 by a mechanism that involves activation of NFATc. To test this hypothesis, we initially screened for NFATc isoform expression in MOSER cells. These cells express NFATc3 almost exclusively (supplemental Fig. S5A). Lentiviral shRNA knockdown vectors were screened to identify a virus that yielded >85% transient knockdown at the level of NFATc3 mRNA (supplemental Fig. S5B). As shown in Fig. 6I, transient knockdown of NFATc3 reduced basal expression of DSCR1 and attenuated induction of DSCR1 in RS5444-treated MOSER cells, with no effect on induction of PDK4 (Fig. 6J). These data indicate that PPARγ induces DSCR1 via a calcineurin/NFATc-dependent mechanism.

DSCR1 Effects on Transformed Properties of MOSER Cells—Lentiviral shRNA knockdown was used to determine whether induction of DSCR1 plays a role in PPARγ-mediated inhibition of cell proliferation and/or invasion. We screened a series of lentiviral shRNA knockdown vectors and identified one that inhibits basal DSCR1 expression by about 85% (supplemental Fig. S6A) and completely blocked induction of DSCR1 by PPARγ (Fig. 7A). Transient DSCR1 knockdown efficiency was maintained at >75% for >5 days in these cells (supplemental Fig. S6B). Knockdown of DSCR1 blocked RS5444-dependent inhibition of MOSER cell proliferation (Fig. 7B). Furthermore, DSCR1 knockdown blocked PPARγ inhibition of MOSER cell invasion (Fig. 7C). These data indicate that DSCR1 is required for PPARγ suppression of the transformed properties of early stage colorectal cancer cells.

DISCUSSION

Both pharmacological and naturally occurring PPARγ agonists inhibit experimental colon carcinogenesis in rodents, and a number of human colon cancer lines undergo growth arrest, differentiation, or apoptosis when treated with PPARγ agonists. In contrast, a weak PPARγ agonist had no significant effect on advanced, metastatic colon cancer in a small phase II clinical trial. These data suggest that PPARγ blocks some early event in transformation, and we have focused on defining the effects of PPARγ agonists on a well characterized model of early stage colon cancer, the MOSER cell line.

We posited that a detailed genomic profile was required to understand the response of early stage colon cancer cells to PPARγ agonists. We reasoned that a relatively dense time course experiment would facilitate discrimination between early, potentially causal, and late events, which may be indirect. A time course experiment can identify genes that may be regulated in a transitory fashion; and in theory, kinetic modeling might be used to infer regulatory links between pathways that are activated at different times. Therefore, much richer detail of the response may be derived from a longitudinal two-class microarray analysis of a single cell line, as opposed to a one-class microarray analysis of multiple cell lines. The decision to undertake a two-class analysis presented a number of interesting statistical problems. What is the best algorithm for preprocessing, background correction, normalization, and summarization of the large number of chips that are required for this sort of analysis? What is the best statistical model for measuring significant changes in gene expression in a time course data set of this sort? We used our data set to compare different methods for preprocessing and summarization of a two-class study in which samples were collected in parallel from control and treated cells over a number of time points. Our results are consistent with those reported for one-class studies (102), and validate the use of GCRMA for two-class studies. Our bootstrap analysis demonstrated that the empirical Bayes statistical machine learning model is much more robust than polynomial or nonparametric statistical models for this longitudinal two-class data set.

The HotellingT2 scores, MB statistics, and median differences in expression were used as figures of merit in bioinformatics analysis of the PPARγ target genes that we have identified. In a sense, the genomic ontology analysis served as a “reality check” on the data set, and the fact that the most significant cohorts of target genes segregate into ontological classes associated with metabolism, proliferation, and motility is reassuring in light of the cellular phenotype that attends PPARγ activation, published data on metabolic effects of PPARγ in...
The genomic ontology inferred an entirely unanticipated connection between PPAR and the nontransformed gastrointestinal epithelial cells (14). However, DSCR1 is a tumor suppressor (66), and it has been speculated that overexpression of DSCR1, because of trisomy 21, may account for the apparent resistance of Down syndrome individuals to certain kinds of cancer (66, 95). The prevailing hypothesis is that DSCR1 is an inhibitor of angiogenesis (66). However, DSCR1 is an NFATc inhibitor, and NFATc has been implicated in invasion and proliferation (91–93, 106) of tumor cells. It has also been reported that silencing of DSCR1 is associated with metastatic behavior of tumor cells (94).

Our data indicate that PPARγ induces DSCR1. It has been calculated that complete inhibition of calcineurin/NFATc signaling may be achieved by as little as 50% increase in DSCR1 expression (107), and our data indicate that DSCR1 abundance increases by 2–4-fold within 6–12 h after activation of PPARγ. Induction of DSCR1 by RS5444 requires PPARγ, indicating that the response is secondary, requires de novo protein synthesis, and is therefore not because of direct interaction between PPARγ and the DSCR1 promoter. The DSCR1-1 promoter has been reported to be regulated by nuclear receptors (97, 108); however, this isoform is not induced by PPARγ in MOSER cells. Induction of DSCR1 requires calcineurin activity, suggesting that PPARγ initially activates calcium/calcineurin/NFATc signaling, which leads to transactivation of the DSCR1-4 promoter. This promoter contains 15 tandem NFATc-binding sites (96, 97) and is therefore likely to be sensitive to very modest increases in NFATc activity. According to our current understanding of DSCR1 function, this protein should then feedback to inhibit calcineurin activity, thereby attenuating NFATc activity.

Our knockdown data indicate that induction of DSCR1 is required for PPARγ-mediated inhibition of MOSER cell proliferation and invasion. Based upon these observations we advance the working hypothesis shown in Fig. 8. We propose that PPARγ initially activates calcineurin, which activates NFATc, which then induces a number of genes that control proliferation and invasion (91–93,106). However, NFATc also induces DSCR1, which feeds back to attenuate the pro-invasive and proliferative effects of NFATc. There are a number of obvious questions concerning the mechanism whereby PPARγ activates calcineurin. Although we have proposed that mobilization of intracellular calcium is involved, this hypothesis has not been tested. Nevertheless, the observation that PPARγ downregulates arrestinβ1 suggests that G-protein coupled receptor-mediated calcium transients may be involved. Experiments to test this hypothesis are currently in progress. The general impression from the genomic analysis is that PPARγ inhibits rather than stimulates calcium signaling. We are inclined to believe that this response reflects feedback inhibition by DSCR1, likely mediated through NFATc, but this hypothesis remains to be tested.

An equally provocative hypothesis is that loss of DSCR1 might convert PPARγ from a colon cancer suppressor to a colon cancer promoter. It is known that DSCR1 is silenced in some tumor cells (94). Loss of DSCR1 in such cells is associated with metastasis. Our model predicts that activation of PPARγ other cell types (103–105), and our analysis of PPARγ effects in nontransformed gastrointestinal epithelial cells (14). However, genomic ontology inferred an entirely unanticipated connection between PPARγ and calcium signaling. Our attention was focused upon this potential cross-talk because of the nodal relationship of DSCR1, which is induced by NFATc and serves as a feedback inhibitor of calcineurin-mediated NFATc dephosphorylation and activation (58, 60). DSCR1 has been identified as a tumor suppressor (66), and it has been speculated that...
in cells that lack DSCR1 might result in unrestrained activation of calcineurin/NFATc signaling. This hypothesis remains to be tested, but the concept may have considerable clinical significance, given the current interest in developing PPARγ agonists as chemopreventive and/or chemotherapeutic agents. Finally, our data indicate that DSCR1 may function as a tumor suppressor in early stage transformation. It has been suggested that the tumor-suppressive effects of DSCR1 are attributable to the role of this entity in controlling calcineurin/NFATc activity and angiogenesis in endothelial cells (66, 67). Our data indicate that DSCR1 may play a significant role in controlling calcineurin activity in tumor cells, and that the tumor suppressive effects of DSCR1 may be, at least in part, directly due to inhibition of tumor cell proliferation and/or invasive potential.

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