

Published in final edited form as:

*Int J Cancer*. 2009 December 15; 125(12): 2802–2809. doi:10.1002/ijc.24683.

## KLF4-dependent, PPAR $\gamma$ -induced expression of GPA33 in colon cancer cell lines

Julie Rageul<sup>1</sup>, Stéphanie Mottier<sup>1</sup>, Anne Jarry<sup>2</sup>, Yatrik Shah<sup>3</sup>, Sandrine Théoleyre<sup>1</sup>, Damien Masson<sup>4</sup>, Frank J. Gonzalez<sup>3</sup>, Christian L. Laboisse<sup>2</sup>, and Marc G. Denis<sup>1,\*</sup>

<sup>1</sup> Faculté de Médecine, CNRS UMR 6061, Université Rennes 1, IFR140, Rennes, France

<sup>2</sup> Faculté de Médecine, EA Biometadys, Université de Nantes, Nantes, France

<sup>3</sup> Center for Cancer Research, National Cancer Institute, Bethesda, MD

<sup>4</sup> Faculté de Médecine, INSERM U913, Université de Nantes, Nantes, France

### Abstract

The glycoprotein A33 (GPA33) is a colon cancer antigen. Phase I trials with <sup>131</sup>I and <sup>125</sup>I monoclonal antibody A33 in colon carcinoma patients showed excellent localization to colorectal cancer and some evidence of tumor response. Using DNA microarrays, we have identified the *GPA33* gene as a target of PPAR $\gamma$  in HT29-C1.16E colon cancer cells. Treatment of HT29-C1.16E, Caco2, SW1116 and LS174T colon cancer cells with the PPAR $\gamma$  agonist GW7845 induced a 2- to 6-fold increase in GPA33 mRNA as determined by real-time PCR. This induction was also found in HT29-C1.16E cells treated with rosiglitazone and ciglitazone and was prevented by cotreatment with the PPAR $\gamma$  antagonist GW9662, indicating that this regulation was PPAR $\gamma$  dependent. No canonical PPAR responsive element was found in the *GPA33* promoter. We therefore analyzed the expression of transcription factors involved in GPA33 expression. CDX1, CDX2 and KLF5 expression was not modified by PPAR $\gamma$  activation. By contrast, a significant increase in KLF4 was seen, both at mRNA and protein levels. Furthermore, chromatin immunoprecipitation studies demonstrated that an increased amount of KLF4 protein was bound to the *GPA33* promoter in cells treated with rosiglitazone. Finally, downregulation of KLF4 expression by siRNA reduced rosiglitazone-induced GPA33 expression. This indicates that PPAR $\gamma$  activation induces KLF4 expression, which in turn increases GPA33 expression. We also demonstrate that PPAR $\gamma$  activation leads to increased (p21<sup>WAF1/Cip1</sup> and keratin 19) or decreased (cyclin D1) expression of known KLF4 targets, suggesting that KLF4 is a nodal player in a network of PPAR $\gamma$ -regulated genes.

### Keywords

GPA33; PPAR $\gamma$ ; KLF4; regulation; colon cancer

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Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) is a member of the nuclear receptor superfamily of ligand-activated transcriptional factors. PPAR $\gamma$  is expressed throughout the gastrointestinal epithelium from duodenum to rectum and plays a regulator role in differentiated functions of intestinal epithelial cells.<sup>1–3</sup> Furthermore, numerous studies showed that PPAR $\gamma$  is expressed in a variety of malignant tissues including prostate, breast and colon. The implication of PPAR $\gamma$  in colorectal carcinogenesis is still debated. In fact, contrasting with the observation of an increase in the number and burden of naturally occurring

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\*Correspondence to: Faculté de Médecine, CNRS UMR 6061, 2 avenue Pr Léon Bernard, Rennes, 35043 France. Fax: +33-223-234607. marc.denis@univ-rennes1.fr.

intestinal tumors in APC<sup>Min</sup> mice fed with a diet containing a PPAR $\gamma$  agonist,<sup>4,5</sup> several models suggest that PPAR $\gamma$  agonists have colonic anticancer activity. *In vitro*, treatment of colorectal carcinoma cell lines with PPAR $\gamma$  ligands induces cell-cycle blockade resulting in the inhibition of cell proliferation, stimulation of cell differentiation and/or promotion of cell death.<sup>6,7</sup> *In vivo*, thiazolidinediones, synthetic PPAR $\gamma$  agonists, decrease the development of tumors derived from colon cancer cells in xenograft models,<sup>8-10</sup> suppress colon carcinogenesis induced by azoxymethane in mice<sup>11</sup> and are able to reduce the number of chemically induced aberrant crypt foci, which are early precursor lesions of colon cancer.<sup>12</sup> Consistent with these findings, PPAR $\gamma$  heterozygous knockout mice (PPAR $\gamma^{+/-}$ ) have an increased susceptibility to develop tumors, including colon tumors, after administration of a carcinogen.<sup>13</sup> These data, together with the antiproliferative activity of PPAR $\gamma$  ligands observed in many human colorectal cell lines, suggest that these molecules may have promise as anticancer drugs.

In an effort to identify PPAR $\gamma$  gene targets in colon cancer cells, we used microarray technology. The differentiated cell line HT29-Cl.16E, a clonal derivative from the HT29 cell line,<sup>14</sup> was grown on filters and cultured for 24 hr in the presence or in the absence of a PPAR $\gamma$  agonist. RNA was then extracted, amplified and hybridized to pan-genomic DNA microarrays. This allowed us to identify the *GPA33* gene as a potential PPAR $\gamma$  target. The *GPA33* gene encodes a 43-kDa transmembrane glycoprotein<sup>15</sup> of the junctional adhesion molecule family,<sup>16</sup> with homology to the immunoglobulin superfamily.<sup>15,17,18</sup> *GPA33* consists of two extracellular immunoglobulin domains, a single transmembrane domain and a short intracellular tail containing four acylation sites.<sup>15,18</sup> Extensive immunohistochemical analysis has shown that the antigen is present on the basolateral surfaces of pyloric stomach, small intestine and colon epithelial cells,<sup>19</sup> and that it is homogeneously expressed by >95% of colon cancers.<sup>19,20</sup>

The *GPA33* structure is consistent with a putative role as a cell adhesion molecule or a novel cell surface receptor, but no function has been assigned to date. However, the restricted pattern of expression in normal tissue makes this antigen a possible target for immunotherapy of colorectal carcinomas. Phase I and II trials with <sup>131</sup>I and <sup>125</sup>I humanized murine monoclonal antibody A33 in patients with colon carcinoma showed excellent localization to colorectal cancer and some evidence of tumor response.<sup>21-23</sup>

Here, we demonstrate that the *GPA33* gene is regulated by PPAR $\gamma$  activation. This regulation is mediated by PPAR $\gamma$ , but is indirect, and involves *Krüppel-like factor 4* (KLF4), also known as gut-enriched Krüppel-like factor (GKLF). KLF4 is a member of the KLF family of zinc-finger-containing transcription factors.<sup>24,25</sup> It is expressed in epithelial cells of the gastrointestinal tract,<sup>26</sup> where it plays important roles in differentiation and cell maturation.<sup>27,28</sup> PPAR $\gamma$  activation regulates the expression of known KLF4 targets, suggesting that KLF4 is a nodal player in a network of PPAR $\gamma$ -regulated genes.

## Material and methods

### Human colonic cancer cell lines

Several human colonic cancer cell lines were used. The differentiated cell lines, HT29-Cl.16E and Caco2, were grown on trans-well filters (12-well Transwell Clear, 0.45  $\mu$ m porosity, Corning-Costar, Cambridge, MA). The nondifferentiated cell lines, SW1116 and LS174T cells, were grown on plastic. All these cell lines were cultured in DMEM (InVitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (FBS, InVitrogen) and used at postconfluency (SW1116 and LS174T) or until full differentiation (HT29-Cl.16E and Caco2).

## Receptor ligands

The following synthetic ligands were used in our studies to determine the specificity and selectivity of GPA33 induction: rosiglitazone (PPAR $\gamma$  agonist; 10  $\mu$ M), GW7845 (PPAR $\gamma$  agonist; 5  $\mu$ M), ciglitazone (PPAR $\gamma$  agonist; 20  $\mu$ M), GW9662 (irreversible PPAR $\gamma$  antagonist; 10  $\mu$ M), WY14643 (PPAR $\alpha$  agonist; 20  $\mu$ M), 9-*cis* retinoic acid (RXR agonist; 10  $\mu$ M).

Rosiglitazone and GW7845 were generously provided by GlaxoSmithKline (Research Triangle Park, NC), and the other ligands were obtained from Sigma-Aldrich (Lyon, France). All synthetic ligands were dissolved in DMSO. The final DMSO concentration in culture medium of all experiments was kept constant at 0.05%. Unless otherwise stated, cells were exposed to these ligands for 24 hr.

## cDNA synthesis and real-time PCR

Total RNA was extracted from cells and colonic tissues using TRIzol<sup>®</sup> protocol (<http://www.invitrogen.com/search.cfm>). cDNA were synthesized as previously detailed.<sup>29</sup>

Primers were designed from the sequence of the human cDNAs using the Universal ProbeLibrary Assay Design Center (<https://www.roche-applied-science.com/sis/rtpcr/upl>). They were selected for binding to separate exons to avoid false positive results arising from the amplification of contaminating genomic DNA. We verified that all amplifications did not yield any product when reverse transcriptase was omitted in the cDNA synthesis reaction. The sequences of these primers are available upon request. PCR amplifications were performed using the LC480 SYBR Green I Master mix (Roche Diagnostics, Meylan, France) in a Rotorgene 3000 instrument (Corbett Research, BioLabo, Archamps, France). The reaction mixture contained 10  $\mu$ l of the supplied 2 $\times$  mix, 0.5  $\mu$ l of each primer (final concentration 0.25  $\mu$ M each), 9  $\mu$ l of the template (cDNA diluted 1/40). The cycling conditions were as follows: denaturation for 5 min at 95 $^{\circ}$ C; amplification for 35 cycles, with denaturation for 5 s at 95 $^{\circ}$ C, annealing for 5 s and extension for 5 s at 72 $^{\circ}$ C. To exclude primer-dimer artifacts, fluorescence was not measured at the end of the extension step, but a separate detection step was added (10 s) at a temperature above the melting point of primer-dimers and below the melting point of the specific PCR product. A standard curve was generated with serial dilutions of pooled cDNAs samples. The amount of transcripts was calculated from these standard curves using the RotorGene software. For each sample, the ratio between the relative amount of each specific transcript and GAPDH was then calculated to compensate for variations in quantity or quality of starting mRNA as well as for differences in reverse transcriptase efficiency. Initial DNA micro-array experiments demonstrated that PPAR $\gamma$  activation did not alter GAPDH expression level. Expression of  $\beta$ -actin and  $\beta$ 2-microglobulin was also unaffected, and similar results were obtained when  $\beta$ -actin was used to normalize expression in quantitative RT-PCR experiments (not shown). Each amplification was performed in triplicate.

## Western blot analysis

Total proteins were extracted from cultured cells using standard RIPA buffer. Nuclear proteins were prepared using the Qproteome cell compartment kit (QIAGEN, Courtaboeuf, France). Proteins were separated by SDS-PAGE and transferred to PVDF membranes as described.<sup>29</sup> Membranes were probed with the A33 monoclonal antibody (gift from Joan Heath) and the H-180 anti-KLF4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Equal protein sample loading was monitored by incubating the same membrane filter with an anti- $\alpha$ / $\beta$  tubulin (2148, Cell Signalling, Danvers, MA) or an anti-lamin A/C antibody (sc7293, Santa Cruz Biotechnology). The probed proteins were detected by using IRD700 and IRD800 conjugated secondary antibodies and the membranes were scanned using a LICOR Odyssey infrared imager (Sciencetec, Les Ulis, France).

## Chromatin immunoprecipitation assay

HT29-Cl.16E cells ( $2 \times 10^7$ ) were treated with DMSO or rosiglitazone (10  $\mu$ M) for 24 hr and were subjected to chromatin immunoprecipitation with the EZ ChIP Assay kit (Upstate Cell Signaling Solutions). Briefly, formaldehyde was used to crosslink proteins with DNA, and cells were lysed in SDS lysis buffer. The cell lysate was sonicated to shear the DNA. Chromatin samples were precleared with protein G agarose for 2 hr at 4°C and immunoprecipitated overnight at 4°C with normal mouse IgG or an anti-KLF4 antibody (H-180) bound to protein G agarose. Formaldehyde crosslinking was reversed by incubation at 65°C followed by proteinase K digestion and DNA purification. Quantitative PCR were then performed as described earlier, using specific primers. The region between -212 and -50 nucleotides of the GPA33 promoter (163 bp), which contains a KLF4 binding site,<sup>30</sup> was amplified with the following primers: 5'-GGG GGA GAC TTC CTG TTT TC-3'(sense) and 5'-TCA GAG GTA GGG TTC CAA GC-3'(antisense). A 241-bp fragment of the *FABP1* promoter was amplified as negative control with the following primers: 5'-AAG GCT TCC TGC TTG ACT GA-3' (sense) and 5'-GCT CCC TCT TCA CGC ATG CA-3'(antisense).

## Downregulation of KLF4 expression using siRNA

RNA interference was achieved using a validated human KLF4 (NM\_004235) siRNA (Qiagen) or the AllStars negative control siRNA (Qiagen). This validated negative control siRNA has no homology to any known mammalian gene and it has been shown to ensure minimal nonspecific effects on gene expression. HT29 cells were seeded to ~50% confluence in 24-well plates in triplicate. On the following day, transfections were performed with the use of INTERFERin (Ozyme, Saint-Quentin-Yvelines, France), according to the manufacturer's recommended protocol. Briefly, siRNA were diluted in serum-free medium. INTERFERin was added (2  $\mu$ l for 100  $\mu$ l of diluted siRNA). After incubation at room temperature for 10 min, the mixture was added to culture wells containing 0.5 ml of complete cell culture medium. The final concentration of siRNA was 10 nM. After 24 hr, cells were treated with DMSO (control) or rosiglitazone (10  $\mu$ M). Cells were lysed and RNA was extracted 24 hr later.

## Statistical analysis

Results are expressed as means  $\pm$  SEM. Differences between group means are analyzed by Student's *t*-test and are considered significant at  $p < 0.05$ .

## Results

### GPA33 is increased in colon cancer cell lines treated with GW7845

We first analyzed the regulation of the *GPA33* gene in the following human colon cancer cell lines: 2 differentiated cell lines cultured on porous filters (HT29-Cl.16E and Caco2) and 2 nondifferentiated cell lines cultured on plastic (SW1116 and LS174T). These cell lines which all expressed PPAR $\gamma$ <sup>31</sup> were treated with a synthetic PPAR $\gamma$  agonist, GW7845.<sup>32</sup> As a control of induction, we measured the expression level of the *FABP1* (fatty acid binding protein 1) gene, a *bona fide* PPAR $\gamma$  target gene. An increase in FABP1 level (1.6- to 33.4-fold) was found in the different cell lines (Fig. 1). In all these cell lines, an increased expression of GPA33 was seen upon GW7845 treatment, ranging from 1.6-fold in LS174T cells to 5.6-fold in HT29-Cl.16E cells (Fig. 1). A more detailed analysis of regulation mechanisms involved was performed using HT29-Cl.16E cells as a model.

### GPA33 regulation by PPAR $\gamma$ in HT29-Cl.16E

We next examined the effect of different ligands on GPA33 expression. All PPAR $\gamma$  agonists tested (rosiglitazone, GW7845 and ciglitazone) increased GPA33 transcripts (Fig. 2a). In contrast, GW9662, an irreversible PPAR $\gamma$  antagonist,<sup>33</sup> did not affect GPA33 expression. More

importantly, it prevented the increase induced by rosiglitazone (Fig. 2a) and by the other agonists (not shown). This indicated that the PPAR $\gamma$  receptor was involved in *GPA33* regulation. In contrast, WY14643 (PPAR $\alpha$  ligand) and retinoic acid (RXR ligand) did not affect *GPA33* expression (Fig. 2a).

This induction observed at the mRNA level was confirmed at the protein level. Total proteins were extracted from untreated or treated cells and analyzed by immunoblotting. A 4.5- to 6.2-fold increase of *GPA33* was seen with all the PPAR $\gamma$  agonists, whereas the PPAR $\gamma$  antagonist had no effect (Fig. 2b).

By quantitative RT-PCR, *GPA33* expression levels were shown to be increased in a concentration-dependent manner. A significant increase was observed with the lowest dose tested (100 nM), with a maximal effect reached at 300 nM (Fig. 2c). *GPA33* also increased in a time-dependent manner, by 10  $\mu$ M rosiglitazone (Fig. 2d). The *GPA33* mRNA amount was significantly increased after a 3-h exposure to rosiglitazone, and the induction increased for up to 72 hr.

We also analyzed the mRNA levels in HT29-CI.16E cells after rosiglitazone treatment in either presence or absence of the protein synthesis inhibitor cycloheximide (CHX). HT29-CI.16E cells grown in the presence of CHX alone were used as a control. *FABP1* induction was not blocked by CHX, in agreement with the direct effect of PPAR $\gamma$  on transcription of the *FABP1* gene (Fig. 3). In contrast, the CHX presence abolished the rosiglitazone effect on *GPA33* expression (Fig. 3). This suggested the involvement of *de novo* protein synthesis in the mechanism of *GPA33* regulation by PPAR $\gamma$ .

#### **KLF4 is induced by PPAR $\gamma$ in HT29-CI.16E cells**

PPAR $\gamma$  functions by regulating gene transcription *via* binding to DNA sequences known as peroxisome proliferator response elements (PPREs) located in the promoter regions of target genes. We performed an extensive analysis of the *GPA33* promoter, but we did not find any PPRE in this sequence. We thus hypothesized that this regulation might be indirect, involving other factors. A comprehensive analysis of the 5'-regulatory region of the human *GPA33* gene has been performed. This revealed positive *cis*-regulatory elements incorporating consensus Krüppel-like factor and caudal-related homeobox (CDX)-binding sites, located just upstream from the human *GPA33* antigen transcription start site.<sup>34</sup> Further analysis provided evidence that *GPA33* is a target gene for the intestine-specific homeobox transcription factor, CDX1,<sup>34</sup> and that the gut-enriched Krüppel-like factor (GKLF-KLF4) binds to the promoter region of the *GPA33* gene in colonic carcinoma cells.<sup>30,34</sup>

We therefore analyzed the expression of these genes, CDX1 and KLF4, in HT29-CI.16E cells treated with rosiglitazone. We also included 2 related genes in our series: KLF5 (IKLF, intestinal KLF) and CDX2. The quantitative RT-PCR performed clearly showed that rosiglitazone induced expression of KLF4, but the other transcription factors were not modified (Fig. 4a). Furthermore, this induction was prevented by cotreatment with the PPAR $\gamma$  antagonist GW9662, indicating that this induction was also PPAR $\gamma$ -dependent. This increase was confirmed at the protein level by immunoblotting experiments (Fig. 4b). A 2.7-fold increase was seen after 24 hr and reached 4.6-fold after 48 hr of treatment.

An increase in KLF4 expression was also found at the RNA level in the other cell lines tested (1.5-, 2.3- and 2.5-fold increase in Caco2, LS174T and SW1116 cells, respectively; data not shown).

### KLF4 is involved in PPAR $\gamma$ induction of GPA33 expression

Chromatin immunoprecipitation experiments were first performed to determine whether PPAR $\gamma$  activation led to increased binding of KLF4 to the *GPA33* promoter. HT29-C1.16E cells were treated with DMSO or rosiglitazone for 24 hr and then subjected to chromatin immunoprecipitation. Following DNA purification, quantitative PCR was performed. No differences were seen when a fragment of the *FABP1* gene, which does not contain KLF4 binding sites, was amplified (Fig. 5). In contrast, Figure 5 clearly shows that upon rosiglitazone treatment, the amount of *GPA33* promoter DNA immunoprecipitated with an anti-KLF4 antibody significantly increased.

To determine whether or not GPA33 induction by PPAR $\gamma$  activation was due to this KLF4 increase and binding to the GPA33 promoter, we undertook a siRNA approach. KLF4 expression was significantly decreased (~2-fold) in cells transfected with a specific KLF4 siRNA as compared to cells treated with a control siRNA, both in DMSO- and in rosiglitazone-treated cells (Fig. 6). Similarly, GPA33 expression was significantly lower in cells treated with the specific KLF4 siRNA than in cells treated with the control siRNA, both in control conditions and following rosiglitazone treatment (Fig. 6). Inhibition of KLF4 expression did not affect induction of FABP1 by rosiglitazone (Fig. 6). Altogether, these data demonstrated that GPA33 induction was, at least in part, due to KLF4 increased expression.

### PPAR $\gamma$ activation regulates additional KLF4 targets

In colon cancer cells, constitutive overexpression of KLF4 induces cell cycle arrest at the G<sub>1</sub> phase and inhibits cell proliferation.<sup>35,36</sup> These effects appear to be mediated through transcriptional upregulation of the negative cell-cycle-regulatory cyclin-dependent kinase inhibitor *p21<sup>WAF1/Cip1</sup>* gene.<sup>37,38</sup> On the other hand, KLF4 represses several positive cell-cycle-regulatory gene promoters, such as *cyclin D1*.<sup>35,39</sup>

To determine whether the PPAR $\gamma$ -induced KLF4 increase would also modify the expression level of other genes, we measured the expression level of known KLF4 target genes: cyclin D1, *p21<sup>WAF1/Cip1</sup>* and keratin 19. Real-time PCR analysis revealed that PPAR $\gamma$  activation leads to an increase in p21 and Ker19 expression and a decrease in cyclin D1 expression (Fig. 7). This suggests that some of the responses affected by PPAR $\gamma$  are the result of changes in the activity of the KLF4 signaling network.

### Discussion

The biological activities of PPAR $\gamma$  are very broad. It is generally accepted as a master transcriptional regulator of lipid and glucose metabolism, but it has also been implicated in tumorigenesis. Indeed, PPAR $\gamma$  agonists, such as thiazolidinediones (rosiglitazone, troglitazone and pioglitazone), prevent tumorigenesis in animal models, inhibit growth of malignant human cells and cause cell cycle arrest and apoptosis in a broad spectrum of epithelial-derived tumor cell lines.<sup>6</sup> Several potential downstream targets of PPAR $\gamma$  mediating its antitumor effects have been identified in colorectal cancer cell types. The activation of PPAR $\gamma$  negatively regulates cell cycle progression by modulating a number of cell cycle regulators such as cyclin D1 expression in Ras-transformed intestinal epithelial cells.<sup>40</sup> The activation of PPAR $\gamma$  has also been reported to inhibit tumor cell growth by upregulation of the transcriptional repressor TSC22 in colon cancer cells<sup>41</sup> and to suppress tumor cell invasion by downregulation of matrix metalloproteinase-7.<sup>42</sup>

To provide further insights into the role of PPAR $\gamma$  in colorectal carcinogenesis, we sought to identify novel PPAR $\gamma$ -regulated genes. Using microarray technology, we identified *GPA33* as a PPAR $\gamma$ -regulated gene in HT29-C1.16E cells. A similar approach has been used by Gupta *et*

*al.*,<sup>10</sup> using a different cell line (MOSER S cells). They selected genes induced or repressed 2.5 times or greater after treatment with the PPAR $\gamma$  ligand GW7845. They identified several PPAR $\gamma$ -selective targets: genes linked to growth regulatory pathways (regenerating gene IA), colon epithelial cell maturation (*GOB-4* and keratin 20), immune modulation (neutrophil-gelatinase-associated lipocalin) and members of the carcinoembryonic antigen family. They did not identify *GPA33* as a PPAR $\gamma$  target. We do not know whether the *GPA33* gene is expressed in this cell line or whether it is induced less than 2.5-fold.

The human and mouse *GPA33* antigens are Type I transmembrane glycoproteins of the immunoglobulin superfamily.<sup>15,16</sup> Immunohistochemical studies of normal tissues identified the large and small intestinal mucosa as the principal site of *GPA33* expression.<sup>20</sup> The expression pattern of *GPA33* is characterized by robust and uniform expression by epithelial cells throughout the intestine. Tests in tumor samples demonstrated that only tumors of the gastrointestinal tract are consistently A33 antigen positive.<sup>20</sup> The biological function of *GPA33* is not yet understood. It has been suggested that it may be a novel cell surface receptor or cell adhesion molecule in the immunoglobulin superfamily.<sup>15</sup>

Our results demonstrate for the first time that, in a time- and concentration-dependent manner, *GPA33* expression levels were upregulated by PPAR $\gamma$  ligands both at mRNA and protein levels. The induction of *GPA33* expression by PPAR $\gamma$  agonists was observed in different human colon cancer cell lines. We also demonstrated a significant, although much lower (40% increase), induction in colon of mice receiving rosiglitazone (not shown).

This induction was PPAR $\gamma$ -dependent as demonstrated by the inhibitory effect of GW9662. A similar conclusion was drawn from experiments performed with mice with targeted disruption of the PPAR $\gamma$  gene in intestinal epithelial cells generated using a villin-Cre transgene and floxed PPAR $\gamma$  allele.<sup>43</sup> We did not find an increase in *GPA33* expression in animals that did not express PPAR $\gamma$  in their intestinal epithelial cells.

However, this regulation is indirect since the PPAR $\gamma$ -induced regulation of *GPA33* was abolished by coincubation with cycloheximide suggesting that this regulation required *de novo* protein synthesis. This is in agreement with the kinetic of induction, which shows a rather slow increase of *GPA33* transcripts upon rosiglitazone treatment. Among the cell lines used, the effects on *GPA33* were in general more modest and different than the pattern seen for *FABP1*, suggesting that the regulation of *GPA33* is more complex and multidimensional. In addition, we found no correlation between *KLF4* and *GPA33* expression levels determined by quantitative real-time PCR in cell lines and in a series of human colorectal tumors (data not shown).

The promoter region alignment revealed several conserved binding sites for transcription factors previously implicated in the regulation of gene expression in intestinal epithelial cells. A CDX-binding sequence was identified, and the binding of the intestine-specific homeobox transcription factor CDX1 was demonstrated in this site.<sup>34</sup> In addition, potential binding sites for *KLF4* and *KLF5* were identified by Mao *et al.*<sup>30</sup>

In our model, PPAR $\gamma$  activation did not significantly modify *CDX1*, *CDX2* and *KLF5* expression. In contrast, it specifically increased *KLF4* mRNA and protein levels. We examined the effect of cycloheximide on the expression of *KLF4* mRNA in the presence of rosiglitazone. This combination resulted in an 18-fold increase in *KLF4* mRNA level (data not shown). These results are in agreement with those obtained using 15-deoxy- $\Delta^{12,14}$  prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), a natural PPAR $\gamma$  ligand, and indicate that early *de novo* protein synthesis may not be required for induced *KLF4* upregulation. Using specific inhibitors, Chen and Tseng<sup>44</sup> demonstrated that the MEK/ERK pathway is engaged in the induction of *KLF4* mRNA by 15d-PGJ<sub>2</sub>.

*KLF4* does not seem to contain a potential conserved consensus of PPRE in its promoter. Chen and Tseng<sup>44</sup> conclude that the PPAR $\gamma$ -induced *KLF4* up-regulation is most likely to be mediated through a PPAR $\gamma$ -independent mechanism. They also based this conclusion on results suggesting that endogenous PPAR $\gamma$  in HT29 cells may not be functional or is transcriptionally inactive. We strongly disagree with these conclusions and clearly show here that PPAR $\gamma$  agonists always strongly induced expression of the *FABP1* gene, a well-known PPAR $\gamma$  target. How is *KLF4* upregulated upon PPAR $\gamma$  activation remains to be elucidated. The transcription factors, SOX9 and TCF4, recently demonstrated by Flandez *et al.*<sup>27</sup> for having an effect on *KLF4* expression might be good candidates.

Sodium butyrate has been shown to increase *KLF4* mRNA and protein levels.<sup>27,45</sup> We found that the treatment of HT29 cells with sodium butyrate also increased *KLF4* expression. Interestingly, this treatment also induced GPA33 expression (data not shown).

*KLF4* expression is altered in various models of intestinal tumorigenesis. The level of *KLF4* transcript is significantly decreased in intestinal adenomas of multiple intestinal neoplasia (APC<sup>Min/+</sup>) mice and in colonic adenomas of familial adenomatous polyposis (FAP) patients.<sup>37,46</sup> Similarly, *KLF4* mRNA and protein levels are reduced in sporadic colorectal carcinomas of patients when compared with normal colonic tissues.<sup>36,47</sup> Bearing in mind the negative effect of *KLF4* on cell proliferation, it might be interesting to increase *KLF4* expression in these cancer cells by activating PPAR $\gamma$ .

The highly tissue-specific expression of the A33 antigen presents a great opportunity for specific tumor targeting in patients with gastrointestinal cancer. Studies using nude mouse models with xenografts of the human colorectal carcinoma cell lines, SW1222, indicated that the A33 monoclonal antibody was capable of inducing tumor regression.<sup>48</sup> In phase I and II trials, <sup>131</sup>I- and <sup>125</sup>I-labeled murine A33 monoclonal antibodies were shown to have antitumor effects without bowel toxicity.<sup>49,50</sup> A genetically humanized monoclonal antibody A33 (huA33) has been generated, and phase I trials have been undertaken.<sup>21–23,51</sup> Scott *et al.*<sup>21</sup> demonstrated that huA33 localized rapidly and selectively to colorectal carcinoma *in vivo*, and the colon elimination half-life of huA33 was equivalent to basal colonocyte turnover.

The antiproliferative activity of PPAR $\gamma$  ligands observed in many human colorectal cell lines suggests that these molecules may have promise as anticancer drugs. Rosiglitazone is approved for the treatment of insulin-resistant diabetes. No significant effect could be observed when troglitazone alone was administered in patients with metastatic colorectal cancer included in a phase-II clinical trial.<sup>52</sup> However, recent reports demonstrated a synergy between PPAR $\gamma$  ligands and current anticancer therapies such as fluorouracil,<sup>53</sup> carboplatin<sup>54,55</sup> or tamoxifen.<sup>56</sup> Thus, PPAR $\gamma$ -induced GPA33 expression might increase the efficiency of anti-GPA33 monoclonal antibodies. A combination of both approaches might be effective.

In colon cancer cells, constitutive overexpression of *KLF4* induces cell cycle arrest at the G<sub>1</sub> phase and inhibits DNA synthesis and cell proliferation.<sup>35,36</sup> These effects appear to be mediated through the regulation of genes such as *p21<sup>WAF1/Cip1</sup>*<sup>37,38</sup> and *cyclin D1*.<sup>35,39</sup> We also demonstrated that, in addition to GPA33, PPAR $\gamma$  activation leads to increased *p21<sup>WAF1/Cip1</sup>* and decreased cyclin D1 expression, suggesting that *KLF4* is a nodal player in a network of PPAR $\gamma$ -regulated genes. Further work will be necessary to identify additional genes involved in this network.

Collectively, our results provide potential clinical applications and will help to define the mechanisms by which PPAR $\gamma$  and *KLF4* are involved in colorectal cancer cell biology.



## Acknowledgments

Ligue Départementale Contre le Cancer; Grant numbers: 35, 44, 56; Grant sponsor: Association Pour la Recherche Contre le Cancer; Grant number: ARC-3732; Grant sponsor: Région Bretagne; Grant number: ACOMB 2627.

The A33 monoclonal antibody was kindly provided by Dr. Joan Heath (Ludwig Institute for Cancer Research, Victoria, Australia). DNA microarray experiments that allowed us to identify the *GPA33* gene as a potential PPAR $\gamma$ -target were performed on the “plateforme Ouest-Génopole” (Dr. Marie-Dominique Anne-Galibert, Pr. Jean Mosser).

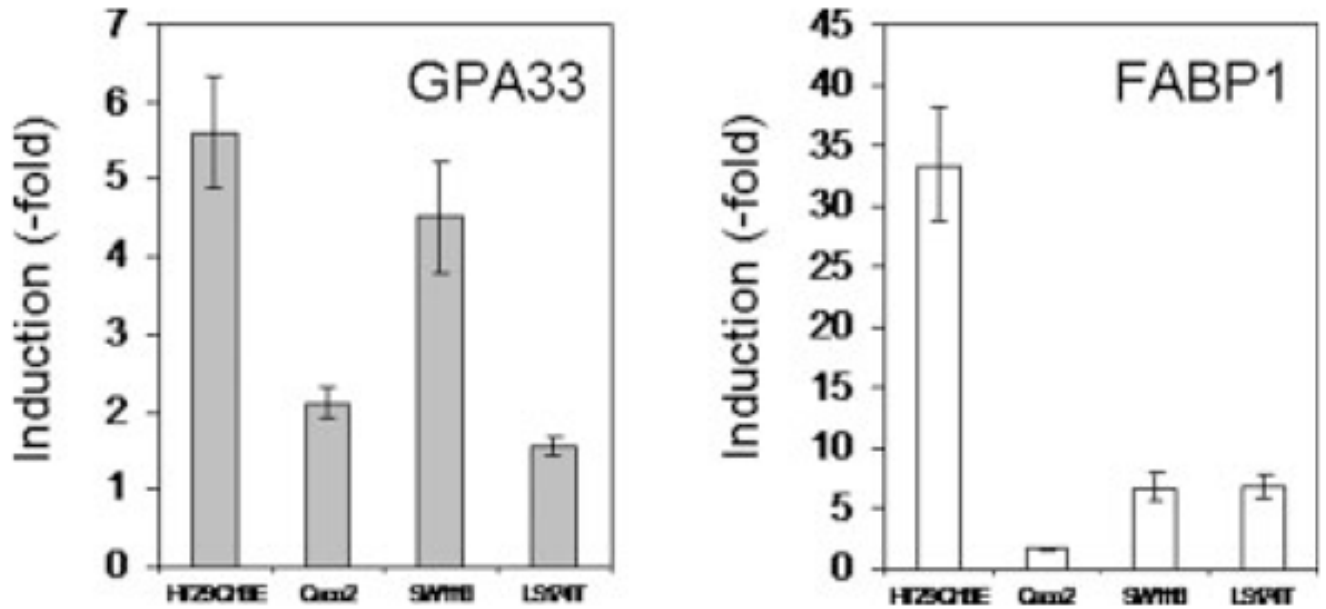
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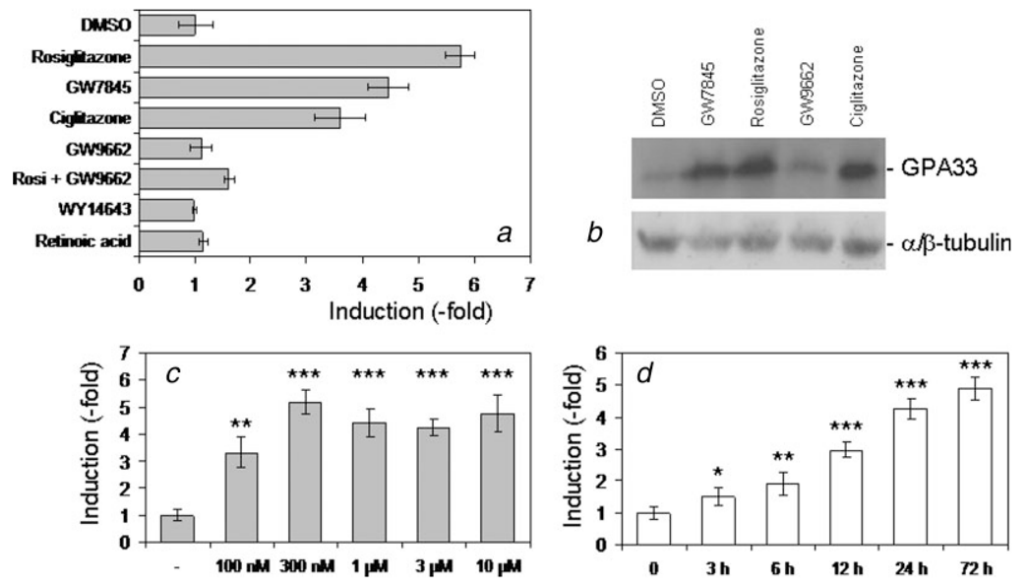
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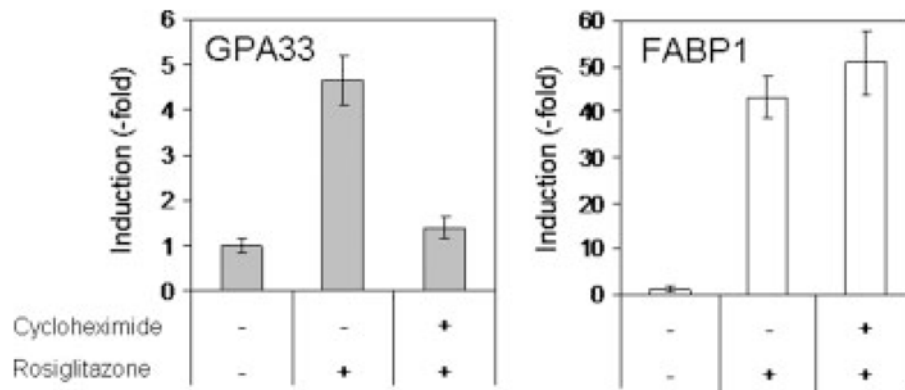
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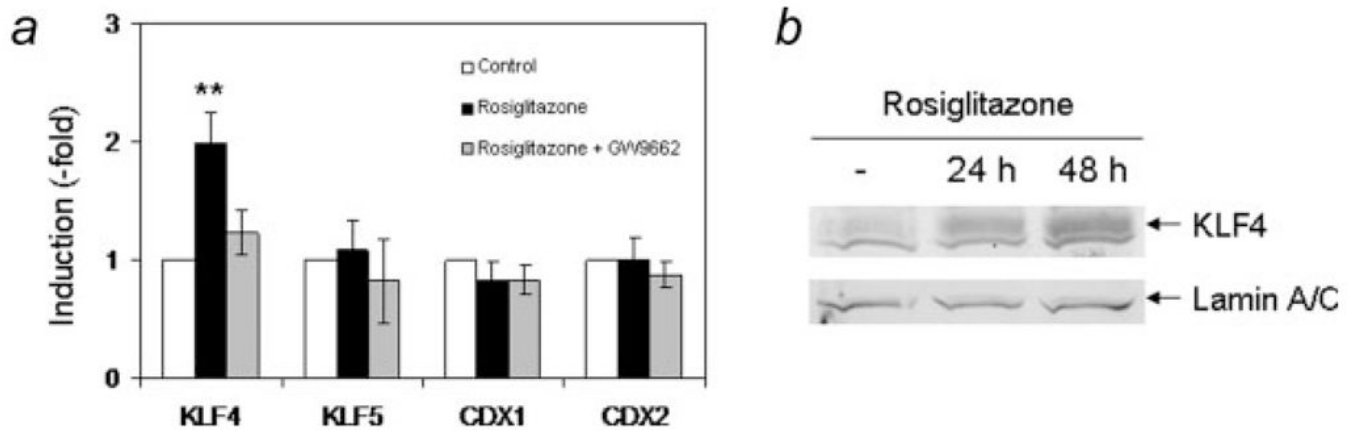
**Figure 1.** PPAR $\gamma$  activation induces GPA33 expression in colon cancer cells. RNA was extracted from colon cancer cell lines treated for 24 hr with DMSO or GW7845 (10  $\mu$ M). GPA33 and FABP1 expression was measured by real-time PCR and normalized to GAPDH. Results presented are mean  $\pm$  SD of 3 different experiments.

**Figure 2.**

Characterization of GPA33 induction in HT29-C1.16E. HT29-C1.16E colon cancer cells were treated with different ligands for 24 hr. GPA33 expression was measured by real-time PCR and normalized to GAPDH (a). Proteins extracted from treated cells were analyzed by Western blotting using the A33 antibody. The  $\alpha/\beta$  tubulin antibody was used as a loading control (b). HT29-C1.16E colon cancer cells were treated with rosiglitazone at the indicated concentration for 24 hr (c), or with 10  $\mu$ M for the indicated time (d). RNA was extracted and analyzed by quantitative RT-PCR. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Results presented are mean  $\pm$  SD of 3 different experiments.



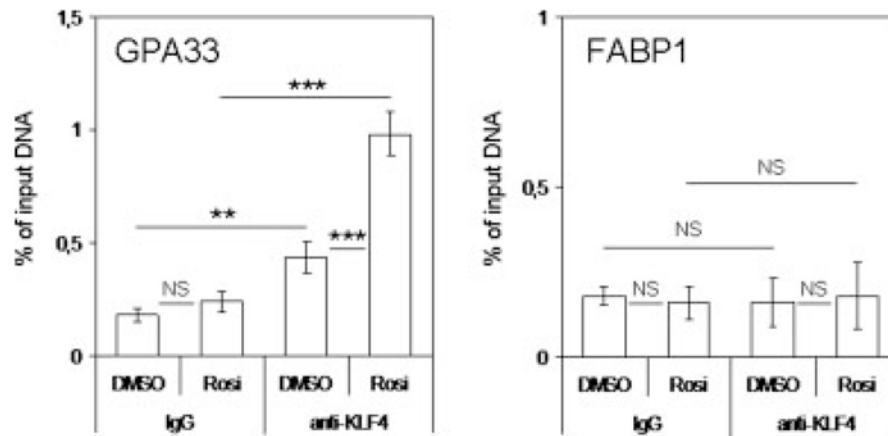
**Figure 3.** GPA33 induction requires protein synthesis. HT29-C1.16E were treated for 24 hr with rosiglitazone (10  $\mu$ M) in the absence or presence of cycloheximide (10  $\mu$ g/ml). RNA was extracted and analyzed by RT-PCR. Results presented are mean  $\pm$  SD of 3 different experiments.



**Figure 4.**

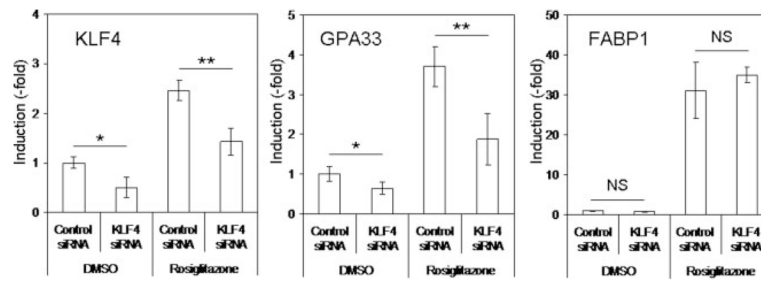
PPAR $\gamma$  activation specifically induces KLF4 expression. (a) HT29-Cl.16E cells were treated for 24 hr with rosiglitazone (10  $\mu$ M) in the absence or presence of GW9662 (10  $\mu$ M). RNA was extracted and expression of KLF4, KLF5, CDX1 and CDX2 was analyzed by RT-PCR and normalized to GAPDH (\*\* $p < 0.01$ ). Results presented are mean  $\pm$  SD of 3 different cultures. (b) Proteins extracted from untreated cells or cells treated with rosiglitazone for 24 or 48 hr were analyzed by Western blotting using an anti-KLF4 antibody. The lamin A/C antibody was used as a loading control.





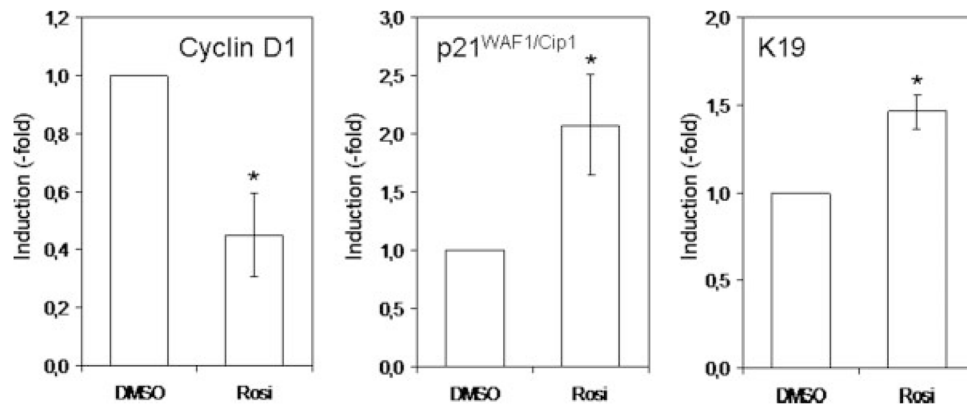
**Figure 5.**

PPAR $\gamma$ -induced KLF4 binding to the *GPA33* promoter. Chromatin immunoprecipitation analysis of KLF4 binding to the target region on the *GPA33* promoter but not on the *FABP1* promoter was performed as described in “Material and Methods” section. HT29-C1.16E cells were treated for 24 hr with DMSO or rosiglitazone (10  $\mu$ M) for 24 hr. Chromatin samples were subjected to immunoprecipitation using a KLF4 antibody or normal mouse IgG as a control. Quantitative PCR were then performed using specific primers. The region between -212 and -50 nucleotides of the *GPA33* promoter, which contains a KLF4 binding site, was amplified. As a control, a fragment of the *FABP1* gene, which does not contain KLF4 binding sites, was used. The amount of precipitated DNA was determined by real-time PCR and expressed as percentage of input DNA (NS, not significant; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).



**Figure 6.**

PPAR $\gamma$ -induced KLF4 expression is involved in GPA33 regulation. RNA interference was performed as described in “Material and Methods” section by using a validated human KLF4 siRNA or a negative control siRNA. After 24 hr, cells were treated with DMSO (control) or rosiglitazone (10  $\mu$ M). RNA was extracted 24 hr later. KLF4, GPA33 and FABP1 expression was determined by real-time PCR. Data were normalized to GAPDH expression (NS, not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ ).



**Figure 7.**

PPAR $\gamma$  activation induces KLF4 target genes. RNA was extracted from colon cancer cell lines treated for 24 hr with DMSO or rosiglitazone (10  $\mu$ M). Cyclin D1, p21/Cip1 and keratin 19 expression was measured by real-time PCR and normalized to GAPDH. Results presented are mean  $\pm$ SD of 3 different experiments (\* $p < 0.05$ ).