Hic-5 regulates an epithelial program mediated by PPARγ

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PPARγ is a dominant regulator of fat cell differentiation. However, this nuclear receptor also plays an important role in the differentiation of intestinal and other epithelial cell types. The mechanism by which PPARγ can influence the differentiation of such diverse cell lineages is unknown. We show here that PPARγ interacts with Hic-5, a coactivator protein expressed in gut epithelial cells. Hic-5 and PPARγ colocalize to the villus epithelium of the small intestine, and their expression during embryonic gut development correlates with the transition from endoderm to a specialized epithelium; expression of both these factors is reduced in tumors. Forced expression of Hic-5 in colon cancer cells enhances the PPARγ-mediated induction of several gut epithelial differentiation/maturation markers such as L-FABP, kruppel-like factor 4 (KLF4), and keratin 20. siRNA directed against Hic-5 specifically reduces PPARγ-mediated induction of gut epithelial genes in colon cells and in an ex vivo model of embryonic gut differentiation. Finally, forced expression of Hic-5 during 3T3-L1 preadipocyte differentiation inhibits adipogenesis while inducing inappropriate expression of several mRNAs characteristic of gut epithelium in these mesenchymal cells. These results indicate that Hic5 is an important component in determining an epithelial differentiation program induced by PPARγ.

PPARγ is a member of the nuclear receptor superfamily that functions in a heterodimeric complex with RXR (for review, see Spiegelman 1998). While the identity of the natural, endogenous ligand for this receptor is not clear, PPARγ is the functioning receptor for the antidiabetic thiazolidinedione drugs such as rosiglitazone and pioglitazone (Forman et al. 1995; Lehmann et al. 1995). PPARγ is a dominant regulator of fat cell differentiation, being both necessary and sufficient for this process (for review, see Lowell 1999; Rosen et al. 2000). Expression and ligand activation of PPARγ converts most fibroblastic cells into adipocytes (Tontonoz et al. 1994; Hu et al. 1995). Conversely, cells or animals lacking PPARγ show a complete loss of fat cells and fat tissue development (Barak et al. 1999; Kubota et al. 1999; Rosen et al. 1999).

Although PPARγ is expressed at highest levels in adipose tissues, it is also expressed at significant levels in a number of epithelial cell types, including several that are important in human cancer, such as colon (Mansen et al. 1996; Sarraf et al. 1998; Lefebvre et al. 1999), breast (Mueller et al. 1998), and prostate (Kubota et al. 1998; Mueller et al. 2000). Ligand activation of this receptor in human cancer cells from these tissues resulted in slowing of growth and an increase in cellular maturation (for reviews, see Koeffler 2003; Michalik et al. 2004). For example, PPARγ activation in human colon cancer cells reduces cell growth and induces multiple RNAs associated with a more differentiated phenotype, such as keratins 18, 19, and 20 and carcinoembryonic antigen (CEA) family members (Sarraf et al. 1998; Gupta et al. 2001). There is also an alteration in cell morphology, including an increase in cytoplasmic to nuclear ratio, consistent with a more differentiated phenotype (Sarraf et al. 1998).

The notion that PPARγ can alter the growth and differentiation state of epithelial cells has led to a number of studies of its potential role in the cause and treatment of cancer. Although agonist treatment of animals suggests possible both pro- or anticancer properties in the colon (Lefebvre et al. 1998; Saez et al. 1998; Sarraf et al. 1998), genetic studies unambiguously demonstrate that PPARγ functions as a tumor suppressor in chemically induced colon carcinogenesis in mice (Girnun et al.

2002). Human clinical trials have shown that application of a PPARγ ligand to humans with certain forms of liposarcoma yielded impressive histological evidence of tumor differentiation (Demetri et al. 1999); trials of PPARγ ligands as a monotherapy in several advanced human malignancies have shown no efficacy (Kulke et al. 2002; Burstein et al. 2003). Taken together, these studies suggest that PPARγ may play an important role in the differentiated states of several forms of epithelia, whereas the use of PPARγ agonists in the human cancer clinic may be limited to the treatment of early stage disease, or in combination with other drugs.

The apparent ability of PPARγ to promote the differentiation and maturation of fat cells of mesenchymal origin and cells of epithelial origin raises interesting issues. Because PPARγ and its heterodimeric partner RXR are essentially identical in all of these tissues, this suggests that the execution of radically different genetic programs must occur as a consequence of other molecules that interact, directly or indirectly, with PPARγ. Recent data have indicated that coactivator proteins, such as PGC1α or myocardin, can dramatically influence the activity of nuclear receptors and other transcription factors, thereby influencing cell fate (Puigserver et al. 1998; Wang et al. 2001; Yoon et al. 2001; Yoshida et al. 2003). We show here that intestinal epithelial cells contain a coactivator protein, Hic-5, that stimulates PPARγ transcriptional activity. Moreover, this factor promotes an epithelial-maturation activity when combined with PPARγ in malignantly transformed epithelial cells, and fetal small intestine. Hic-5 can even stimulate expression of PPARγ-dependent epithelial genes when expressed in preadipose cells.

Results

Hic-5 binds and coactivates PPARγ

We considered the possibility that PPARγ may execute a particular program related to gut epithelial biology through the function of a tissue-selective coactivator protein. To identify candidate cofactors of PPARγ, we used a yeast two-hybrid assay to screen a human colon cancer cDNA library, using the ligand-binding domain (LBD) of PPARγ as bait. Two positive clones, each isolated multiple times, were identified as Hic-5, previously described as a TGFβ-inducible gene (Shibanuma et al. 1994) and as a LIM-domain coactivator of the androgen receptor in prostate cells, also known as androgen receptor activator 55 (ARA55) (Fujimoto et al. 1999). Hic-5 is known to be expressed in several cell types of epithelial origin: prostate, lung, small intestine, and colon (Shibanuma et al. 1994; Zhang et al. 2000). This pattern of expression along with the ability of Hic-5 to coactivate various nuclear receptors (Fujimoto et al. 1999; Yang et al. 2000) and to be regulated by TGFβ, an important regulator of the colonic epithelium, prompted our further studies.

The ability of Hic-5 to affect PPARγ transcriptional activity was determined using Bosc cells transiently transfected with a luciferase reporter gene linked to a multiple copies of a PPARγ response element (DR1). Hic-5 potentiated PPARγ activity in a ligand-dependent manner (pioglitazone 1 µM) (Fig. 1A). PGC1α, a bona fide coactivator of PPARγ (Puigserver et al. 1998), was used as a positive control and showed similar stimulation of PPARγ transcriptional activity, although as expected, this activity was largely ligand independent (Fig. 1A).

To determine whether the ability of endogenous Hic-5 to increase PPARγ activity results from a physical interaction, as implied by the yeast two-hybrid results, cells were transfected with either Flag-tagged PPARγ or a control vector. As a positive control for physical interaction, cells were cotransfected with a tagged PPARγ and PGC1α. We could not use endogenous PGC1α in this experiment because this coactivator cannot be detected in Bosc cells. As shown in Figure 1B, endogenous Hic-5 coprecipitated with PPARγ in the presence of rosiglitazone, while PGC1α coprecipitated with PPARγ in a ligand-independent manner. The domains involved in the interaction between Hic-5 and PPARγ were determined using a modified GST pull-down assay. Bosc cells were transiently transfected with plasmid vectors expressing different domains of Hic-5 fused to gfp. The C-terminal and wild-type gfp-Hic-5 have been reported to be localized primarily to focal adhesion sites by fluorescent microscopy (Fujita et al. 1998). In contrast, N-terminal gfp-Hic-5 is localized diffusely throughout the cell. We observed similar localization patterns and did not see changes in localization in the presence of PPARγ ligands (data not shown). Lysates from these cells were then incubated with GST fused to full-length PPARγ. After washing, proteins were eluted and were immunoblotted for gfp. Figure 1C shows that GST-PPARγ binds to full-length Hic-5. However, a Hic-5 ΔLIM protein containing amino acids 1–208 was not coprecipitated with PPARγ, while Hic-5(209–444) was coprecipitated with PPARγ. The domains of Hic-5 responsible for the interaction with PPARγ were further mapped by using fragments of Hic-5 translated in vitro. Figure 1D shows that GST-PPARγ binds to the full-length Hic-5(1–444) but fails to interact with the Hic-5(1–208), ΔLIM, in a similar pattern shown in Figure 1C. Various combinations of LIM motifs (LIM1, LIM1–2, LIM2–4) were sufficient to restore Hic-5 binding to PPARγ (Fig. 1D). These data imply that each LIM motif in Hic-5 might contribute to its interaction with PPARγ. This finding resembles those in a recent study showing that another LIM domain protein, FHL2, binds to the androgen receptor through multiple LIM domains (Muller et al. 2000).

Hic-5 and PPARγ are coexpressed in normal and malignant gut development

Gut endoderm develops into a structured epithelium between embryonic days 13 and 15 (E13 and E15) (Simon and Gordon 1995). We investigated whether Hic-5 and PPARγ were coexpressed during gut development, using a fetal mouse intestine as a model. After microscopic dissection of intestines at E12.5 to E17.5, RNA was ex-

tracted and relative levels of Hic-5, PPARγ mRNA, and several other markers of gut development were determined. As shown in Figure 2A, PPARγ mRNA increases 2.2-fold between E12.5 and E13.5 and a further fourfold between E13.5 and E15.5. Hic-5 mRNA levels also increase twofold between E12.5 and E13.5 (Fig. 2A). This increase in PPARγ and Hic-5 transcripts correlates with the induction of several molecular markers of gut epithelial maturation between E13.5 and E15.5 including keratin 18, keratin 19, and liver fatty-acid-binding protein (L-FABP) (Fig. 2A). To further examine whether PPARγ and Hic-5 colocalize in the intestine, paraffin-embedded sections of intestine from E13.5, E15.5, E17.5, and 2-wk-old mice were analyzed by immunohistochemistry. As shown in Figure 2B, PPARγ protein is localized mainly to the epithelium of embryonic developing gut and in the mucosal lining of the adult villus, with a gradient of increasing expression toward the villus tip (Fig. 2B). Hic-5 shows a similar graded pattern of expression in epithelial cells (Fig. 2A), although its expression is not restricted to the epithelial layer in agreement with previous reports (Fig. 2B; Shibanuma et al. 1994; Yuminamochi et al. 2003). Importantly, these data illustrate that although Hic-5 expression pattern in the gut is broader than the expression of PPARγ, PPARγ and Hic-5 are colocalized only in the gut epithelial layer and are induced during epithelium development.

We also investigated Hic-5 and PPARγ expression in pathological development, particularly in epithelial tumorgenesis. Mice were treated with azoxymethane (AOM), a chemical that induces colon tumors, as previ-

ously described (Burdette Walter 1970; Girnun et al. 2002). Hic-5 and PPARγ mRNA levels were analyzed in colon tumors and compared with those in adjacent normal tissue, using quantitative real-time RT–PCR. Figure 2C shows that PPARγ expression was decreased by almost 70% in the tumor compared with the normal epithelial tissue. Hic-5 levels were decreased by >50%. mRNA of paxillin, a Hic-5 homolog that has been implicated as a positive regulator of cell survival and motility (Schaller 2001; Hanks et al. 2003), was increased 2.5-fold in these tumors. As expected, several molecular markers of colon differentiation, such as keratin 20 (Calnek and Quaroni 1993; Moll et al. 1993) and kruppel-like factor 4 (KLF4), a transcription factor linked to epithelial differentiation (Chen et al. 2003), were decreased in the tumor tissue. Taken together, these data show a correlation between Hic-5 and PPARγ levels and levels of certain gut differentiation markers.

Hic-5 enhances PPARγ-mediated induction of epithelial genes, in colon cancer cells

A gene-array analysis was used to study the program of endogenous gene expression induced by PPARγ in poorly

Figure 1. Hic-5 binds and coactivates PPARγ in a ligand-depended manner (A) Bosc cells were transiently cotransfected with 50 ng of expression vector for PPARγ and 50 ng of a luciferase reporter plasmid with three PPARγ response elements (DR1-sites), in the presence of 250 ng Hic-5 or 100 ng PGC1α expression vectors. Empty vector was used as a control. The following day cells were treated with 1 µM pioglitazone (Pio). Cell lysates were then analyzed for relative transcriptional activity, and data were normalized to β-gal activity, derived from a cotransfected β-gal expressing vector. Values were expressed relative to activation of empty vector and presented as mean ± SE of three different experiments. (B) Coimmunoprecipitation assay. Bosc cells were transfected with either control vector or vector expressing Flag-PPARγ. Cell lysates were then incubated with beads bound to an antibody directed against the Flag epitope. The interaction between Flag-PPARγ and endogenous Hic-5 was tested in the presence and absence of rosiglitazone. Ectopic expression of gfp-PGC1α was used as a positive control for binding to PPARγ. (C) Bosc cells were transiently transfected with various gfp-Hic-5 alleles. The cells were harvested, and extracts were incubated with immobilized GST-PPARγ, which was purified from Sf21 baculovirus cells in the presence of PPARγ ligand troglitazone (Tro). After washing, bound proteins were eluted and separated by SDS-PAGE, followed by Western blotting using an anti-gfp antibody. (D) In vitro interaction assay. Either GST or GST-PPARγ was incubated with [35S]-labeled Hic-5 alleles (shown on the *right* panel). SRC-1 was used as positive control to show in vitro binding to PPARγ.
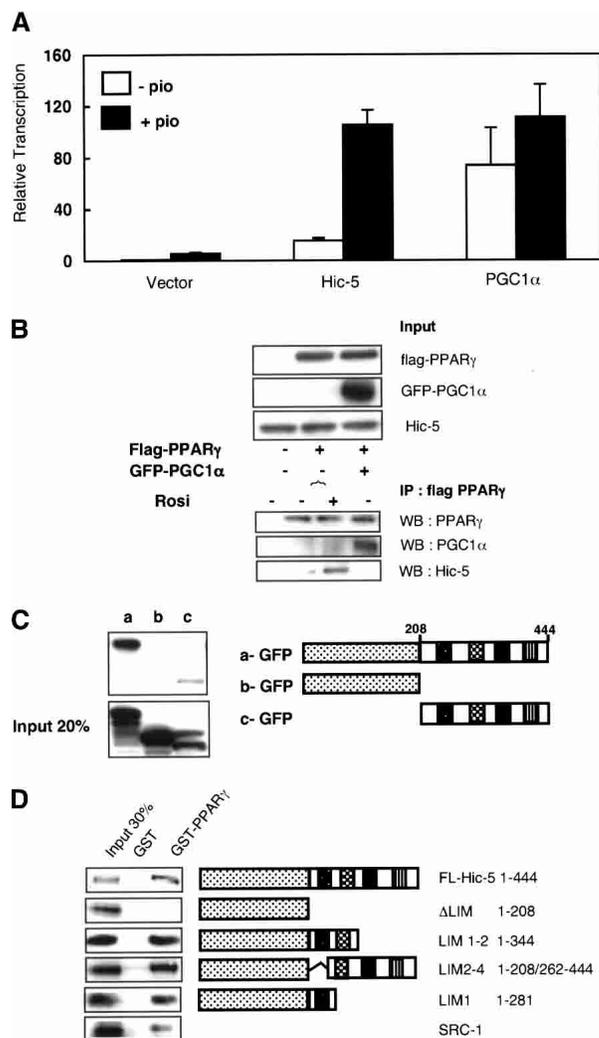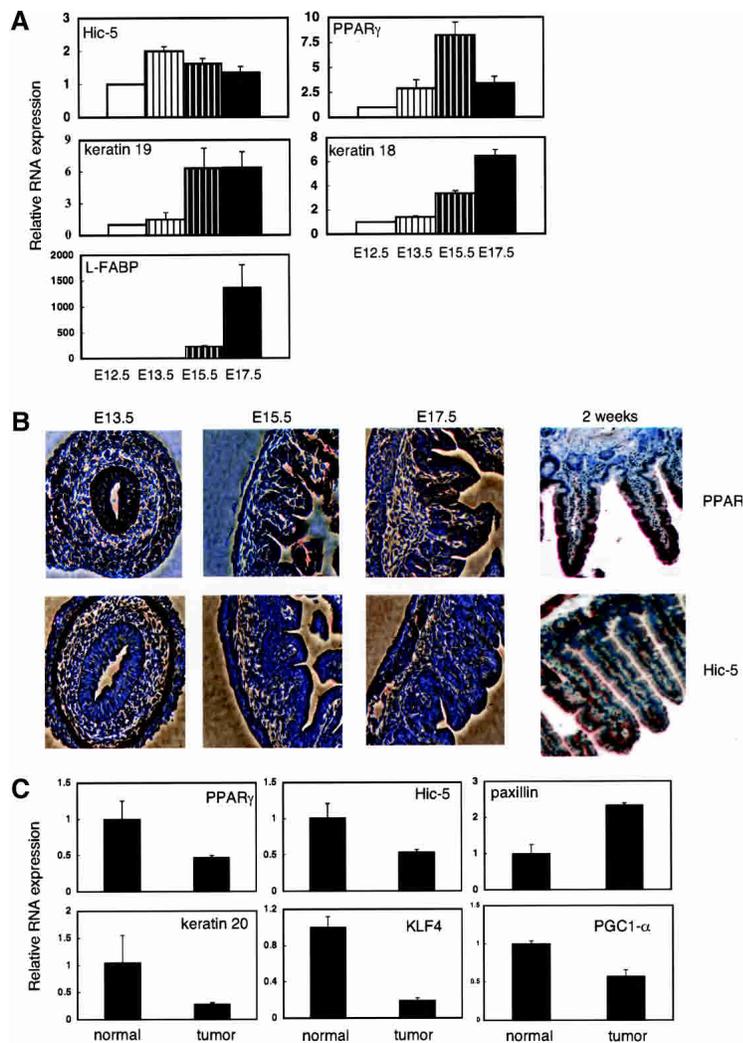
Figure 2. Hic-5 and PPARγ are coexpressed in normal and malignant gut development. (*A*) Small intestines from mouse embryos were dissected at different stages of embryonic development, and total RNA was extracted. The level of expression of PPARγ, Hic-5, keratin 19, keratin 18, and L-FABP was analyzed by using real-time RT–PCR assay. (*B*) Immunohistochemistry of PPARγ and Hic-5 in sections of embryonic and adult mouse small intestines. Brown color indicates positive staining; blue denotes nuclear counter-staining. (*C*) Mice were treated with AOM as described earlier (Girnun et al. 2002). Colonic tumors and adjacent tissue were harvested, and RNA was extracted. Levels of PPARγ, Hic-5, paxillin, keratin 20 KLF4, and PGC1α were analyzed by real-time RT–PCR. The levels of RNA expression were normalized with TBP and are mean ± SD. (*n* = 5). Statistical analysis was done by using Student's *t*-test. (*) $p < 0.01$

differentiated epithelial cells. Moser human colon cancer cells were treated in the presence and absence of rosiglitazone for 14 h. Genes that showed statistically significant differences in expression between rosiglitazone-treated and -untreated cells were then classified according to their function: epithelial differentiation, cell adhesion, tumor suppressors, oncogenes, lipid metabolisms, and insulin signaling (Supplementary Fig. 1). Since these studies focused on expression of a gut epithelial program, genes classified as related to epithelial-selective functions or cell adhesion were further studied. To determine the role of Hic-5 in the expression of these epithelial cell-selective genes, Moser human colon cancer cells were then retrovirally infected with either a vector expressing Hic-5 or an empty vector, and treated with rosiglitazone; as shown earlier, these cells express relatively high amounts of endogenous PPARγ protein (Sarraf et al. 1998). Figure 3A shows that in cells containing only the control vector, rosiglitazone induced several mRNAs characteristic of gut epithelium identified in our gene array analysis, such as keratin 20 (Moll et al. 1993; Gupta et al. 2001) L-FABP (Roth et al. 1990; Gupta et al. 2001), and KLF4 (Chen et al. 2003).

Without the addition of a PPARγ ligand, expression of Hic-5 had no significant effect on these epithelial transcripts, and cells underwent no obvious morphological changes. However, the combination of Hic-5 expression and addition of rosiglitazone resulted in an increased induction of each of these epithelial genes (Fig. 3A). The increase in KLF4 and keratin 20 was twofold compared with control cells treated with rosiglitazone, while Hic-5-mediated increase in L-FABP mRNA was ~40-fold. In contrast, Hic-5 made no detectable contribution to the induction of adipophilin (ADRP) (Fig. 3A), a PPARγ target gene known to be induced in adipogenesis that is also expressed in colon cancer cells (Gupta et al. 2001). Taken together, these data suggest that certain aspects of a specific program of gut epithelial maturation are executed through Hic-5/PPARγ cooperation. However, induction of certain other PPARγ target genes implicated in cell adhesion such as BPG (CEACAM1), CEA (CEACAM5) (Sarraf et al. 1998; Gupta et al. 2001), and annexin A1 (Karasik et al. 1988) was not affected by cooperation between ectopic Hic-5 and the PPARγ ligand (Fig. 3B).

We next asked whether the molecular phenotypes observed were specific for Hic-5 or occurred with the el-
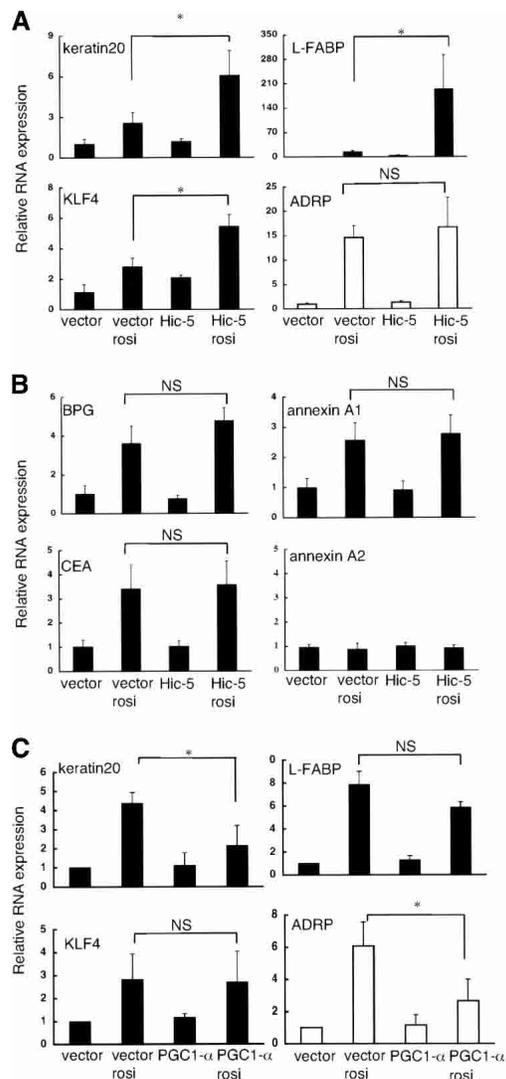
Figure 3. Ectopic expression of Hic-5 enhances PPARγ-mediated induction of several epithelial markers in colon cancer cells. (*A*) Human colon cancer cells (Moser) were infected with a control retrovirus or a retrovirus expressing Hic-5. Cells were treated with or without 1 μM rosiglitazone and were then harvested, and RNA was extracted. Levels of mRNA for keratin 20, L-FABP, KLF4, and ADRP were quantified by real-time RT–PCR. Statistical analysis was done using Student's *t*-test. (*) $p < 0.05$; (NS) not significant. (*B*) Cells were treated as described in *A*. Levels of mRNA for BPG, CEA, annexin A1, and annexin A2 were determined by real-time RT–PCR analysis. Statistical analysis was done using Student's *t*-test. (NS) Not significant. (*C*) Moser cells were infected with either gfp or PGC1α expressing adenovirus. Cells were treated with or without 1 μM rosiglitazone and were then harvested, and RNA was extracted. Levels of mRNA for keratin 20, L-FABP, KLF4, and ADRP were quantified by real-time RT–PCR. Statistical analysis was done using Student's *t*-test. (*) $p < 0.05$; (NS) not significant.

evation of any PPARγ coactivator. We therefore expressed another PPARγ coactivator, PGC1α and examined its effects on epithelial gene expression. Interestingly, PGC1α was recently described to be down-regulated

in human colon tumors (Feilchenfeldt et al. 2004). Figure 3C shows that PGC1α did not increase the PPARγ-mediated induction of epithelial target genes. In fact, PGC1α expression decreased PPARγ-mediated induction of keratin 20, and ADRP (Fig. 3C). These data indicate some selectivity for Hic-5 in mediating PPARγ induction of epithelial-selective genes.

N terminus of Hic-5 functions as a dominant-negative allele and alters epithelial gene expression

Both the C and N termini of Hic-5 were previously suggested to function as dominant-negative (DN) alleles of Hic-5 (Fujimoto et al. 1999; Kasai et al. 2003). We therefore analyzed the ability of these fragments to alter PPARγ-mediated transcription. As shown in Figure 4A, the C-terminal domain (209–444) retains some ability to coactivate PPARγ, although the magnitude of this activity is much lower than that seen with full-length Hic-5. In contrast, the N terminus of Hic-5(1–208) lacks the ability to coactivate PPARγ (Fig. 4A). To evaluate whether either of these Hic-5-derived proteins might have a suppressive effect on wild-type Hic-5 activity, the full-length protein was expressed with increasing concentration of either the N-terminal or C-terminal halves (Fig. 4B). Coexpression of Hic-5 and its N-terminal fragment resulted in a dose-dependent inhibition of PPARγ coactivation (Fig. 4B, lanes 5–10), while this fragment had no significant effect on the ligand activation of PPARγ in the absence of added wild-type Hic-5 (Fig. 4A). In contrast, coexpression of full-length Hic-5 with increasing amounts of the C-terminal fragment of Hic-5 resulted in a minor increase in PPARγ coactivation (Fig. 4B, lanes 11–16). These results suggest that the N-terminal fragment of Hic-5 can function as a dominant-negative allele and can interfere with Hic-5 coactivation of PPARγ. Importantly, this fragment does not appear to interfere significantly with PPARγ activity in the absence of wild-type Hic-5.

We examined the effect of this dominant-negative allele of Hic-5 on expression of keratin 20, a specific marker of enterocyte maturation (Moll et al. 1993; Gupta et al. 2001), in Moser human colon cancer cells. Moser cells were retrovirally infected with a vector expressing Hic-5, the domonant-negative allele of Hic-5 or a control gfp protein (Fig. 4C), and cells were then treated with or without the PPARγ agonist pioglitazone. After 2 d of treatment, the induction of keratin 20 mRNA and protein was analyzed using either real-time RT–PCR (Fig. 4C, upper panel) or by Western blot analysis (Fig. 4C, lower panel). As shown in Figure 3A, expression of wild-type Hic-5 enhanced the PPARγ-mediated induction of keratin 20. Expression of the dominant-negative allele of Hic-5 completely abolished PPARγ-mediated induction of keratin 20 at the mRNA and protein levels (Fig. 4C). Interestingly, TGFβ, a well-known inducer of the Hic-5 protein (Shibanuma et al. 1994) also enhanced PPARγ-mediated induction of keratin 20, an effect also suppressed by expression of the dominant-negative version of Hic-5 (Fig. 4C). However, addition of TGFβ could not
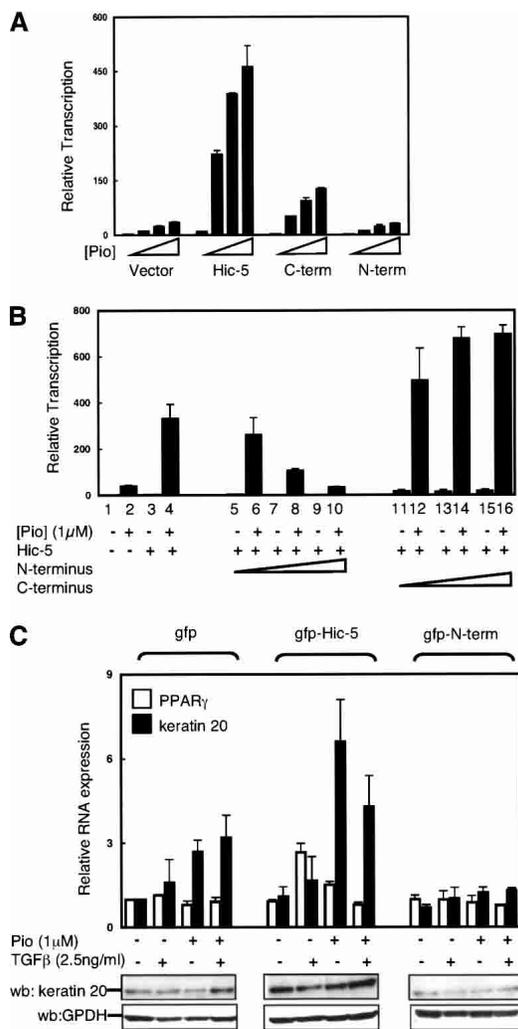
Figure 4. TGFβ enhances PPARγ induction of keratin 20, while dominant-negative Hic-5 blocks it. (*A*) Cos-1 cells were transiently cotransfected with 50 ng expression vector for PPARγ, 50 ng luciferase reporter plasmid with three PPARγ response elements (DR1-luc) in the presence of 250 ng gfp-Hic-5, 400 ng gfp-C terminus Hic-5(209–444), gfp-N terminus Hic-5(1–208) expression vectors, or gfp expressing vector as a control. Cells were treated with increasing concentrations of pioglitazone (Pio) as shown. Cell lysate were then analyzed for relative transcriptional activity, as described in Figure 1A. (*B*) Cos-1 cells were transiently tranfected and treated as described in *A*. To determine potential dominant-negative activities, Hic-5 expressing vector (100 ng) was cotransfected with increasing amount of either gfp-N terminus Hic-5 or gfp-C terminus Hic-5(100–400 ng). (*C*) Moser cells transduced with retroviral expressing Hic-5, dominant-negative Hic-5 (gfp-N terminus), or gfp were incubated with or without TGFβ or PPARγ ligand (pioglitazone). After 2 d, total RNA and protein were extracted. (*Top*) Relative keratin 20 and PPARγ mRNA levels were analyzed by real-time RT–PCR. Values of keratin 20 and PPARγ were normalized to expression level without treatment in control cells, corrected to β-actin and were mean ± SE of three different experiments. (*Bottom*) Protein lysates were extracted and separated by SDS-PAGE gels. Keratin 20 and GPDH levels were analyzed by Western blot using keratin 20 and GPDH antibodies respectively. GPDH levels are shown as a loading control.

further enhance PPARγ-mediated induction of keratin 20 in the presence of exogenous expressed Hic-5, consistent with the notion that the induction of Hic-5 by TGFβ might be a major link between the TGFβ and PPARγ pathways in colon cancer.
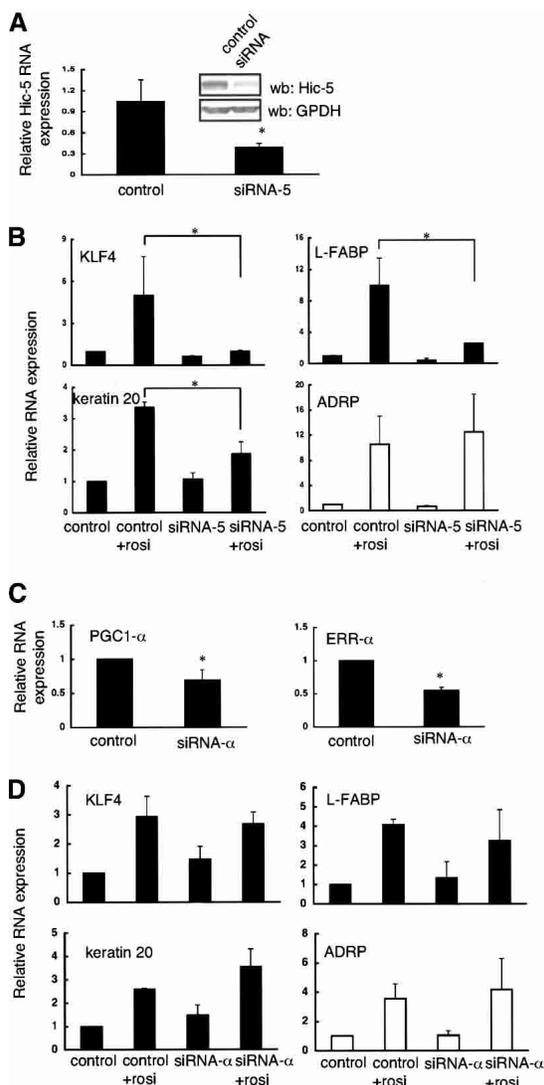
siRNA directed against Hic-5 inhibits PPARγ induction of epithelial-selective genes

siRNA directed against Hic-5 were designed and cloned into a retroviral vector. Scrambled oligonucleotides cloned into the same vector were used as a control. Moser cells were infected with both control and siRNA retroviruses. siRNA against Hic-5 was able to downregulate Hic-5 mRNA levels by at least 60% (Fig. 5A) and had an even greater effect on the level of Hic-5 protein (Fig. 5A, inset). Infected cells were treated with or without rosiglitazone. As expected, rosiglitazone was again able to induce L-FABP, KLF4, keratin 20, and ADRP in control cells (Fig. 5B). The expression of the siRNA against Hic-5 did not significantly change the level of these genes in the absence of PPARγ agonist (Fig. 5B). However, the ability of the PPARγ ligand-mediated induction of each transcript was dramatically decreased in cells expressing the siRNA against Hic-5, while the induction of ADRP was not affected (Fig. 5B). Together, these loss-of function data, using both a dominant-negative allele of Hic-5 and Hic-5-specific siRNA, indicate that Hic-5 and PPARγ cooperate specifically to induce multiple genes characteristic of gut epithelial differentiation.

To again evaluate the specificity of these effects, Moser cells were infected with either adenovirus expressing siRNA targeted against PGC1-α (siRNA-α) (Koo et al. 2004), or a scrambled oligonucleotide, and treated with or without rosiglitazone. The level of PGC1α mRNA was reduced by 30% (Fig. 5C, left panel), and the mRNA for a well-known PGC1α target gene ERRα (Schreiber et al. 2003) was reduced by >50% in cells infected with siRNA directed against PGC1α (siRNA-α) (Fig. 5C, right panel). Figure 5D shows that the induction of PPARγ epithelial target genes KLF4, L-FABP, and keratin 20 is not affected in cells expressing siRNA directed against PGC1α. Taken together, these data indicate that Hic-5 but not PGC1α is part of the transcriptional pathway inducing the set of epithelial target genes mediated by PPARγ.

Modulation of PPARγ and Hic-5 levels in gut development

Mice with total knock out of PPARγ are not viable, but heterozygous mice have normal viability (Barak et al. 1999; Kubota et al. 1999). Therefore we used small intestines from PPARγ heterozygous and wild-type mice (Akiyama et al. 2002) to examine the role of PPARγ in the normal process of gut epithelial differentiation. Figure 6A shows that small intestines harvested from

Figure 5. siRNA against Hic-5 inhibits PPARγ induction of certain epithelial genes. (*A*) Moser cells infected with siRNA expressing vector (pSuper-neo) directed against Hic-5 (siRNA-5) mRNA or scrambled oligonucleotides as a control. After antibiotic selection cells were treated with and without 1 μM rosiglitazone. (*Inset*) RNA and proteins were isolated, and Hic-5 mRNA levels were determined by real-time RT–PCR and Western blot analysis. The levels of RNA expression were normalized with TBP and are mean ± SD of three different experiments. Statistical analysis was done using Student's *t*-test. (*) *p* < 0.01. (*B*) Cells were treated as described in *A*. RNA was isolated, and levels of mRNA for, KLF4, L-FABP, keratin 20, and ADRP were analyzed by real-time RT–PCR. Statistical analysis was done using Student's *t*-test. (*) *p* < 0.05. (*C*) Moser cells infected with adenovirus expressing either siRNA directed against PGC1α mRNA (siRNA-α) (Koo et al. 2004), or scrambled oligonucleotide as a control. After 48 h cells were harvested, and levels of PGC1α and its target gene ERRα were measured by real-time PCR. Analysis was done as described in *A*. (*D*) Moser cells were infected as described in *C*. After infection cells were treated with or without rosiglitazone (1 μM). Levels of KLF4, keratin 20, L-FABP, and ADRP were analyzed as described in *B*.

PPARγ heterozygous (PPARγ$^{+/-}$) mice express 50% less mRNA for PPARγ compared with small intestines from *Pparγ* wild-type (PPARγ$^{+/-}$) mice (Fig. 6A, upper left panel). Importantly, small intestines from PPARγ$^{+/-}$ mice also showed 50% reduction in the expression of several mRNA markers of gut epithelium (Fig. 6A): KLF4, keratin 20, and keratin 19 L-FABP, and keratin 18 mRNA levels showed the same trend, albeit, not statistically significant. These results further demonstrate a physiological role for PPARγ in gut epithelial differentiation.

We used an ex vivo embryonic model to study Hic-5 role during embryonic gut development, which has recently been described (Tou et al. 2004). Primitive guts were dissected from murine embryos and placed in a chemically defined media. When cultured over several days, these explants can undergo growth, peristalsis, and eventually cytodifferentiation as evidence by villus morphogenesis and changes in gene expression reminiscent of gut differentiation. This system allows the manipulation of specific genes, by injections of siRNA expression plasmids or mammalian expression plasmids into the lumen followed by electroporation. Importantly, the expression plasmids in this system have been shown to be targeted mainly to the evolving epithelium layer (Tou et al. 2004). The manipulation of Hic-5 levels is therefore expected to occur mainly in the epithelial layer despite the fact that Hic-5 is also present in the mesenchymal layer of the gut. We first injected embryonic guts with either plasmids expressing siRNA directed against Hic-5 (Fig. 6B, siRNA-5-I, siRNA-5-II) or plasmids expressing scrambled oligonucleotides as control (Fig. 6B, control). After electroporation, these embryonic small intestines were kept under in vitro differentiation conditions (Tou et al. 2004). After 2 d, protein lysates were harvested, and the levels of Hic-5 protein and epithelial markers were determined by Western blots analysis. Figure 6B shows that in total guts treated with siRNA directed against Hic-5, expressed 22% and 62% (siRNA-I and siRNA-II) less Hic-5 protein compared with fetal intestine electroporated with control plasmid. Since Hic-5 in the epithelium represents only a portion of the total Hic-5 in the gut, the percentage reduction in the expression level is presumably somewhat larger than this value. These reductions in Hic-5 levels resulted in decreases in several epithelial markers; 42% and 86% reduction in keratin 19 and 54% reduction in L-FABP (siRNA-5-II) expression levels. These data indicate that normal Hic-5 expression is required for the epithelial gene expression in the intact embryonic gut.

We also used the same ex vivo system to study the effects of ectopic Hic-5 expression. Figure 6C shows that Hic-5 mRNA levels were increased threefold in embryonic small intestines injected with Hic-5 expression vector compared with the control injected embryos. This increase caused a threefold increase in keratin 20 levels and a similar trend in KLF4 mRNA levels (Fig. 6C). Taken together, manipulation of Hic-5 expression levels in the fetal mouse intestine resulted in distinct modulation of gut epithelial gene expression. These data
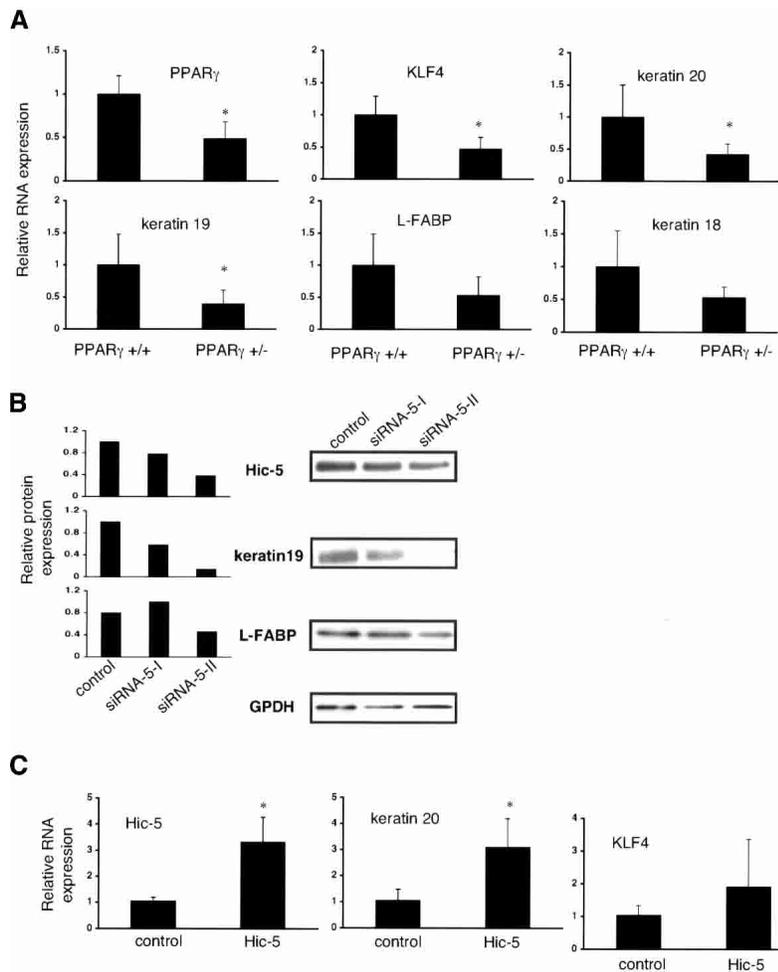
Figure 6. Modulation of PPARγ and Hic-5 expression in epithelial gut development. (*A*) Small intestines were isolated from PPARγ$^{+/+}$ and PPARγ$^{+/-}$ mice (Akiyama et al. 2002). After RNA isolation levels of PPARγ keratin 20, keratin 19, keratin 18, KLF4, and L-FABP were determined using real-time RT–PCR analysis, as described in Figure 5 (*n* = 6). (*B*) Small-intestines were isolated from mice embryos between E12 and E13 and were injected with either siRNA directed against Hic-5 (siRNA-5-I, siRNA-5-II) or scrambled oligonucleotide expressing vector, followed by electroporation to express the plasmids in the epithelial layer. Embryonic small-intestines were then grown for 2 d ex vivo under differentiation condition, and protein lysates were harvested as described in Tou et al. (2004). Hic-5, keratin 19, and L-FABP protein levels were analyzed by Western blot analysis, using GPDH as loading control. Relative protein expression was determined by using densitometer analysis of the Western blot corrected for GPDH expression levels. This is a representative experiment (*n* = 5). (*C*) Embryonic small intestines were isolated and treated as described in *B*, only Hic-5 expressing vector and an empty vector as a control were used. RNA was isolated, and Hic-5, keratin 20, and KLF4 mRNA levels were analyzed using real-time RT–PCR as described in Figure 5.

strongly suggest a physiological role for Hic-5 in gut epithelial development.

Expression of Hic-5 alters the program of adipocyte differentiation.

Preadipocytes are mesenchymal cells that are ontologically far removed from epithelial cell lineages and are not known to express bona fide markers of epithelial cells. We therefore, examined the effects of forced Hic-5 expression during adipogenesis, a pathway where PPARγ plays a dominant role. 3T3-L1 preadipocytes were infected with either an empty retroviral vector or a Hic-5 expressing vector, and the resulting cells were subjected to a standard protocol of differentiation, with or without the addition of a PPARγ ligand for 4 d. Figure 7A shows that while endogenous Hic-5 is expressed at detectable levels in 3T3-L1 preadipocytes, these levels decrease during differentiation. Interestingly, forced expression of Hic-5 inhibited morphological aspects of adipogenesis, as shown by phase contrast microscopy and by the accumulation of lipids, as illustrated by Oil-Red-O staining (Fig. 7B). Furthermore, several mRNAs characteristic of adipocyte differentiation, such as aP2, adipsin, and

acrp30, were all reduced with forced Hic-5 expression. This effect was even more dramatic when PPARγ ligand was added to the differentiation mixture (Fig. 7C). Notably, the cells infected with empty vector do not express several mRNAs characteristic of gut epithelial differentiation, such as keratin 20, L-FABP, and KLF4. Expression of Hic-5 alone mildly increased the levels of these genes. However, when cells expressing Hic-5 were treated with rosiglitazone, robust expression of these epithelial genes was observed (Fig. 7C). These data indicate that Hic-5 and PPARγ can collaborate to induce inappropriate expression of genes characteristic of epithelial cells, even in a classical model of adipose differentiation.

Discussion

Although PPARγ is best known as a dominant regulator of fat cell differentiation, it also participates in the maturation and growth regulation of various epithelial cell lineages. Genes that constitute the fat cell differentiation program, such as adipsin, aP2, and acrp30, are not part of the PPARγ-induced program in colon cancer cells; instead, genes characteristic of gut epithelium are in-
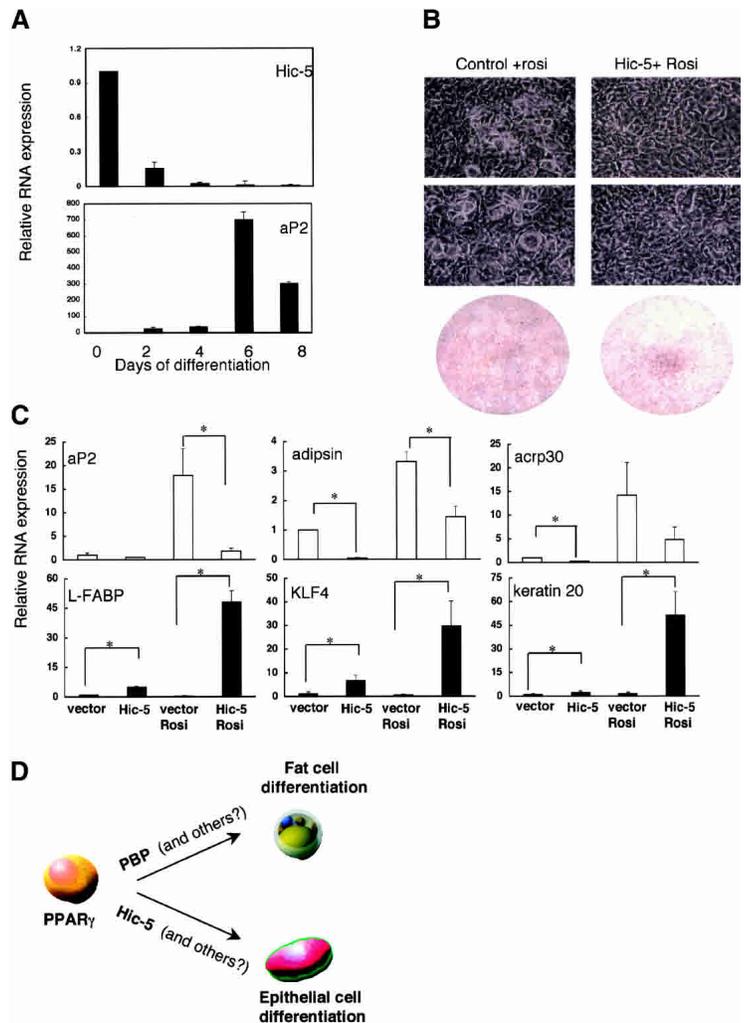
Figure 7. Ectopic expression of Hic-5 in 3T3-L1 pre-adipocytes inhibits adipocyte differentiation while inducing markers of epithelial gut differentiation. (*A*) Hic-5 mRNA levels during 3T3-L1 adipogenesis were analyzed using real-time RT–PCR. (*B*) 3T3-L1 preadipocytes were infected with either retrovirus expressing Hic-5 or an empty retrovirus. Cells were then differentiated using a standard protocol with addition of rosiglitazone. After 4 d cells were fixed, and phase-contrast pictures were taken (*top* panel) or stained for lipid droplet formation by Oil-Red-O (*bottom* panel). (*C*) 3T3-L1 preadipocytes were treated as described in *B*. After 4 d of differentiation with or without rosiglitazone, cells were harvested and RNA was extracted. Levels of mRNA for adipocyte markers adipsin, acrp30, and aP2 and gut epithelial markers KLF4 L-FABP and keratin 20 were determined by real-time quantitative RT–PCR. Statistical analysis was done using Student's *t*-test. (⋆) $p < 0.05$. (*D*) Illustration of Hic-5 and PPARγ combination in epithelial differentiation.

duced when PPARγ is activated in this cell type. Since PPARγ itself is apparently identical in both differentiation processes, as far as is known, it is likely that another level of regulation is involved. There are now several well-documented examples of tissue-specific coactivators that are major regulators of different metabolic and cell differentiation programs, such as OcaB (Luo and Roeder 1995; Kim et al. 1996), myocardin (Wang et al. 2001), and PGC-1α (Puigserver et al. 1998; Yoon et al. 2001). We show here that Hic-5 collaborates with PPARγ to induce a program of epithelial-specific gene expression. Of particular interest, the combination of Hic-5 and a PPARγ agonist facilitates the induction of certain epithelial markers and inhibits fat differentiation, even in determined preadipocytes, (this is shown schematically in Fig. 7D). We also show here that Hic-5 and PPARγ are coexpressed in the gut during embryonic development and that this expression coincides with differentiation/emergence of definitive epithelial cells (Fig. 2A,B). Furthermore, the gene expression pattern in small intestine epithelium of PPARγ$^{+/-}$ mice showed a decrease in epithelial markers. Similar results are obtained here by using siRNA against Hic-5 in an ex vivo model of fetal gut

development. Conversely, introduction of a vector expressing Hic-5 resulted in an increase in epithelial marker mRNAs (Fig. 6). These data, taken together, strongly suggest that the molecular partnership between PPARγ and Hic-5 has physiological significance in gut development.

Hic-5, a member of the LIM domain family with striking similarity to paxillin (Shibanuma et al. 1994; Yuminamochi et al. 2003), can shuttle between focal adhesions and the nucleus, suggesting a possible role in relaying cytoplasmic signals into the nucleus (Shibanuma et al. 2003). LIM domain are implicated in various biological processes, including cell lineage determination and organ development (for reviews, see Bach 2000; Gill 2003). Hic-5, in particular, was previously shown to be expressed in epithelial cells (Zhang et al. 2000) and correlated with differentiation processes induced by retinoic acid (Shibanuma and Nose 1998). Indeed, all the genes we examined here that are induced through the combination of Hic-5 and PPARγ are related to gut epithelial differentiation. Keratin 20 belongs to the large family of cytokeratins that are expressed almost exclusively in epithelial cells, in this case mainly in mature

gastrointestinal epithelium, urothelium, and Merkel cells (Moll et al. 1990); keratin 20 expression is also used to distinguish colon from other cancers in clinical pathology (Nishizuka et al. 2003). L-FABP, a fatty-acid-binding protein, is highly expressed in hepatocytes and enterocytes and is an established marker of intestinal epithelial differentiation and development (Roth et al. 1990). KLF4 is a zinc-finger transcription factor that is enriched in postmitotic gut epithelial cells (Dang et al. 2003; Hinnebusch et al. 2004; Zhao et al. 2004) and required for goblet cell differentiation in the colon (Katz et al. 2002). Importantly, robust induction of these genes required the combined activity of PPARγ and Hic-5; expression of Hic-5 alone was not sufficient to activate this program.

Hic-5 has also been implicated in tumorogenesis, although the published literature is ambivalent about its precise role. Hic-5 (ARA55) activates the androgen receptor in prostate cancer and was therefore initially suggested to promote prostate cancer cell growth (Fujimoto et al. 1999; Rahman et al. 2003). However, it was reported more recently that expression of ARA55 (Hic-5), but not that of other androgen receptor coactivators such as ARA54 or ARA70, is decreased in prostate tumors (Mestayer et al. 2003). Consistent with these data, we show in this study that Hic-5 expression is also decreased in AOM-induced colon tumors, further suggesting that Hic-5 is unlikely to be a positive effector of carcinoma progression. The fact that Hic-5 and PPARγ collaborate to induce expression of L-FABP, keratin 20, and KLF4, markers of gut maturation, is more consistent with a role for Hic-5 as a tumor suppressor.

Finally, our data suggest a molecular mechanism by which the tumor suppressor activities of TGFβ and PPARγ may be integrated. Hic-5 was originally isolated as a TGFβ-inducible gene (Shibanuma et al. 1994), and TGFβ regulates a well-established tumor-suppressor signaling pathway in colon cancer that enhances epithelial differentiation and inhibits cell growth. In advanced stages of colon cancer, aberrant TGFβ signaling can enhance malignancy, epithelial to mesenchymal transition (EMT), and metastasis (for reviews, see Akhurst and Derynck 2001; Derynck et al. 2001). Genetic studies, including in human colon cancer patients (Sarraf et al. 1999), implicate PPARγ as a tumor suppressor. PPARγ also regulates cancer cell growth and differentiation in vitro (Sarraf et al. 1998; Gupta et al. 2003). Much like TGFβ, PPARγ has also been shown to be an effective tumor suppressor in the colon early in the process of carcinogenesis. In the presence of damage to the APC gene, PPARγ lost its protective function (Girnun et al. 2002) or even enhanced cancer progression (Lefebvre et al. 1998; Saez et al. 1998). It therefore seems likely that induction of Hic-5 by TGFβ enhances the ability of PPARγ to modulate the program of epithelial differentiation in vivo.

Mechanism-based strategies to promote tumor differentiation represent a promising new area in cancer research. The best example of differentiation therapy to date is the use of all-trans-retinoic acid (ATRA) to treat acute promyelocytic leukemia (APL) (Zelent et al. 2001; Fang et al. 2002; Tallman 2004). The ability of ATRA to induce terminal differentiation of malignant myeloid cells to mature neutrophils depends on the expression of a chimeric PML-RAR α fusion protein resulting from a t(15;17) chromosomal translocation (Zelent et al. 2001; Fang et al. 2002; Tallman 2004). By analogy, the use of PPARγ agonists in differentiation therapy has thus far shown promising results in certain liposarcoma patients, where rosiglitazone induced terminal adipocytic differentiation and tumor regression (Demetri et al. 1999). While clinical trails of PPARγ agonists as the sole therapy in advanced epithelial tumors to date have failed to show significant clinical effects (Kulke et al. 2002), new trials will likely combine PPARγ ligands with other chemotherapy agents. This study suggests that agonists that enhance interaction of PPARγ with Hic-5 could be useful in differentiation therapy of colon and other epithelial cancers.

Materials and methods

Yeast two-hybrid screening

A cDNA encoding amino acids 183–505 of the murine PPARγ was cloned in-frame into the GAL4 DNA-binding domain of pGBKT7 (pGBKT7-PPARγ, Clontech). An SW480 colon cancer cell cDNA expression library was constructed into the pGAD10 plasmid expressing the GAL4 activation domain (custom generation, Clontech). A yeast two-hybrid screening was performed as described in the Clontech Matchmaker two-hybrid system protocol. Briefly, pGBKT7-PPARγ was transformed into Y190 yeast cells by the lithium acetate method and maintained by selection (Y190-PPARγ). The pGAD10 plasmid containing the colon cancer cell cDNA library was transformed into Y190-PPARγ yeast cells, and positive clones were assayed for β-galactosidase activity in a filter assay as described in the Clontech protocol.

Cell culture and transient transfection/reporter assays

293T, Cos-1, Bosc, 3T3-L1, and Moser colon cancer cell lines were cultured in DMEM containing 10% FBS supplemented with penicillin/streptomycin. Sf21 insect cells were cultured in Grace's insect media containing 10% FBS. Bosc or Cos-1 cells were transiently transfected with a PPARγ expression vector; a PPARγ response element fused to a luciferase construct (DR1-luc); and either a control vector, vectors expressing Hic-5, and/or Hic-5 deletion constructs or PGC-1α expression constructs using Superfect transfection reagent (Qiagen). Cells were treated with various ligand concentrations, and luciferase levels were assayed after 18–24 h of ligand exposure.

Protein interaction analysis

Bosc cells were transfected with a control vector, vectors expressing Flag-PPARγ, or gfp-PGC1α as a positive control. Cell lysates were incubated with agarose beads bound to an anti-Flag antibody (Sigma) in the presence or absence of rosiglitazone. Beads were washed and proteins separated on an 8% gel by PAGE and transferred to PVDF. Immunoblotting was performed using antibodies against Hic-5 (BD Transduction Labs), PPARγ (Santa Cruz Biotech), or gfp (a kind gift from Dr. Pam Silver,

Dana-Farber Cancer Institute, Boston, MA). GST-PPARγ was produced using a baculovirus expression system. A cDNA expressing full-length PPARγ was cloned into a pAcGHLT baculovirus expression plasmid (Pharmingen). Sf21 cells were infected with the baculovirus expressing GST-PPARγ for 2 d and then harvested into lysis buffer. GST-PPARγ protein was isolated by incubation with GSH-Sepharose beads and protein quantified by Commassie blue staining. Affinity purification was performed using 1 μg GST-PPARγ protein with cell lysate from Bosc cells transfected full-length gfp-Hic-5 or an N-terminal or C-terminal gfp-Hic-5 deletion constructs (gfp-Nterm and gfp-Cterm, respectively) as shown in Figure 1C (a kind gift from Drs. Nose and Shibanuma, Showa University School of Pharmaceutical Sciences, Tokyo, Japan) (Fujita et al. 1998). Briefly, GST-PPARγ bound to GSH beads was incubated for 2 h with cell lysates at 25°C. Beads were washed and proteins resolved on an 8% SDS gel. Proteins were transferred to PVDF and immunoblotted using an antibody against gfp (a kind gift from Dr. Pam Silver). Mapping of Hic-5 domains that interact with PPARγ was performed by using full-length Hic-5 or Hic-5 deletion constructs, as shown in Figure 1D (a kind gift from Drs. Nose and Shibanuma), or SRC-1 as a positive control. Proteins were translated in vitro in the presence of ^{35}S-methionine according to manufacturer protocol (Promega). GST-PPARγ bound to GSH beads was incubated with in vitro translated protein in binding buffer at room temperature for 1 h. Following washing, proteins were eluted and resolved on a 4%–12% gel (Invitrogen) by PAGE. Gels were dried down and exposed to film.

Retroviral preparation and infections

A full-length Hic-5 cDNA was generated by RT–PCR from mouse spleen RNA using Invitrogens TOPO cloning kit. The resulting cDNA was sequenced and subsequently cloned into a pMSCV retroviral vector (Clontech). siRNA against Hic-5 was designed using Oligoengines online design program. The 293T packaging cell line was transfected using Lipofectamine 2000 (Invitrogen). Cells were transfected with expression vectors for vsv and gag-pol and with the following retroviral vectors: pBabe expressing gfp, gfp-Hic-5, or gfp-Nterm, pmscv-puro, pmscv-puro expressing Hic-5, pSuperRetro-neo control, or pSuperRetro-neo siRNA against Hic-5. After 6–12 h, media was replaced, and cells were transferred to 33°C for 24–72 h. Viral supernatants were collected and filtered. Moser or 3T3-L1 cells were plated and infected on the following day with the viral supernatants and 8 μg/mL polybrene. 3T3-L1 cells were subjected to a differentiation protocol when confluent (see below). Moser cells were split 1:3 and selected with either puromycin or neomycin for 1 wk, or sorted for gfp-expressing cells.

Adipocyte differentiation and Oil-Red-O staining

For differentiation assays, confluent 3T3-L1 cells infected with control or Hic-5 expressing retrovirus were treated with or without 1 μM rosiglitazone plus 0.5 mM 3-isobutyl-1-methylxanthine, 5 μg/mL insulin, and 1 μM dexamethasone for 2 d. Cells were then kept in maintenance medium (with or without rosiglitazone) consisting of DMEM with 10% FBS supplemented with penicillin/streptomycin and 5 μg/mL insulin. RNA was harvested and cDNA prepared as described below. Differentiated 3T3-L1 cells were also washed in PBS and then fixed in 10% buffered formalin (Formaldefresh; Fisher). A stock solution of 0.5% Oil-Red-O (Sigma) in isopropanol (w/v) was diluted 60:40 in water, filtered, and added to fixed cells. Cells were then washed in water and photographed.

Preparation of tissue for RNA and immunohistochemistry

Small intestines were isolated from either E12.5–E17.5 mouse embryos as previously described (Tou et al. 2004), or from adult mice. For immunohistochemistry analysis, isolated small intestine was rinsed with PBS and fixed in 4% paraformaldehyde (EM sciences). Paraffin embedding, sectioning, and immunohistochemistry were performed by the Pathology Core at Brigham and Women's Hospital. A mouse-on-mouse kit was used for Hic-5 antibody staining (Vector labs). Immunohistochemistry was performed using an anti-mouse monoclonal against Hic-5 (1:100) and an anti-rabbit polyclonal antibody against PPARγ (1:200). For gene expression analysis, RNA was isolated, cDNA was synthesized, and mRNA levels were analyzed using real-time PCR analysis. For tumor analysis, 8-wk-old C57Bl/6J mice were treated with the colon-specific carcinogen AOM as previously described (Burdette Walter 1970; Girnun et al. 2002). Tumor and adjacent uninvolved tissue were excised and snap frozen in liquid nitrogen. RNA isolation, cDNA synthesis, and real-time PCR analysis of tumor, normal adjacent and embryonic tissues were performed as described below.

Ex vivo embryonic gut development

Day 12.5 fetal mouse intestines were isolated under a dissecting microscope as previously described (Tou et al. 2004). Briefly, to introduce expression vector into explants, 2 μg of control plasmid or plasmid encoding either Hic-5 or siRNA directed against Hic-5 was injected into the intestinal lumen by using a capillary pipette and Nanoject injection device. Explants were bathed in DMEM, placed between platinum electrodes and exposed to three 10-msec pulses of 60 V each by using the BTX830 square-wave pulse electroporator, washed in DMEM, and cultured. After 2 d in culture, explants were harvested in RIPA buffer for protein analysis or subjected to RNA isolation using Trizol.

Transcriptional profiling

Moser human colon cancer cells were treated for 14 h with vehicle control or 1 μM rosiglitazone. Total RNA was prepared using an RNeasy Mini Kit (Qiagen Inc.) and used for global expression analysis. Affymetrix array hybridization and scanning were performed by the Core Facility at Dana-Farber Cancer Institute using human genome U133A chip (Affymetrix). Array data were analyzed with d-CHIP array analysis program (Li and Wong 2001).

Real-time PCR

Total RNA was prepared from cells or tissues using Trizol reagent (Invitrogen) following the manufacturer's instructions. Briefly, chloroform was added to homogenized samples, and the aqueous layer was isolated following centrifugation. RNA was precipitated with isopropanol and washed in 70% ethanol. One microgram of total RNA was DNase treated, and cDNA was synthesized using iScript reverse transcriptase reagent (Bio-Rad). PCR reactions were performed using SYBR Green PCR Master Mix (Bio-Rad). Real-time PCR reactions were carried out using the following conditions: for 2 min at 50°C and for 10 min at 95°C, followed by 40 cycles of switching between 95°C for 15 sec and 60°C for 1 min (iCycler, Bio-Rad). The cDNA synthesis step included a control reaction without the reverse transcriptase, and the PCR step included a control reaction without the template to rule out contamination and/or genomic amplification. In addition, the TATA-box-binding protein (TBP) gene was used as a control to account for possible variations in initial

RNA quantity and efficiency of the cDNA synthesis reaction. Primers for target genes were designed using the PRIMEREX-PRESS software (Applied Biosystems). Sequences for real-time PCR primers are available upon request.

Acknowledgments

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