CHAPTER I

INTRODUCTION

Introduction to the Nuclear Hormone Receptor Superfamily

Multicellular organisms require the ability to integrate complex external physiological cues into a defined transcriptional response at the cellular level. One of the major genetic networks that have evolved to coordinate this activity is the nuclear hormone receptor (NHR) superfamily (reviewed in (Evans, 1988; Mangesldorf et al., 1995)). NHRs represent the largest known class of metazoan transcription factors and members of the superfamily play essential roles in virtually every physiological process including development, proliferation, differentiation, inflammation, apoptosis, and metabolic homeostasis. Not surprisingly, NHRs have been implicated in a number of disease states and as ligand modulated receptors are particularly amenable to pharmaceutical manipulation. In fact, small molecule agonists or antagonists of various NHRs are currently being used to treat such diverse pathologies as cancer, diabetes, hormone resistance syndromes, and skin disorders.

The traditional definition of NHR action is of a lipophilic hormone (*e.g.* estrogen or progesterone) that is released into the bloodstream and proceeds to elicit diverse effects in multiple organs systems by transversing the lipid bilayer of cellular membranes and binding to a specific intracellular receptor. Ligand binding initiates conformational changes in the receptor that leads to a defined transcriptional response at specific DNA elements residing in the regulatory region of target genes. The first cDNA for a NHR member, the glucocorticoid receptor, was cloned in 1985 and since then the superfamily has grown to now include more than 150 different proteins. While the classic definition of NHR action still predominates, *i.e.* lipophilic hormone modulation of transcription

factor activity, variations on this theme are now emerging as more members of the superfamily are characterized.

NHR DNA Binding Motifs - the Hormone Response Element

NHRs bind as monomers, heterodimers, or homodimers to a specific DNA element termed the hormone response element (HRE) that is found in the 5' regulatory region of target genes (reviewed in (Renaud and Moras, 2000)). In the case of NHRs that bind as homodimers or heterodimers, the HRE consists of two half-site hexad nucleotide repeats spaced by a variable number of nucleotides (Fig. 1A). The half-site repeats can be oriented as a direct repeat (DR), a palindrome (inverted repeat or IR), or as an everted repeat (ER). The perfect HRE for most steroid receptors consists of the core hexad sequence AGAACA while the consensus hexad sequence for the estrogen receptor (ER) and other NHRs is AGGTCA. However, natural HREs can deviate widely from these consensus sequences. Specificity of DNA binding between different NHRs is achieved in part through variation in the nucleotide sequence 5' to the HRE as well as in the number of nucleotides between half-sites.

Classification of NHRs

Mangelsodorf *et al*. have proposed to classify NHRs into four different classes (Fig. 1B) (Mangesldorf et al., 1995):

1. Class I – The Steroid Receptors. This class includes receptors for progestins (PR), estrogens (ER), androgens (AR), glucocorticoids (GR), and mineralocorticoids (MR). Class I receptors are sequestered within the cytosol in complexes with heat shock proteins and thus are not thought to influence gene expression in the absence of ligand. Ligand binding induces conformational changes that lead to release from heat shock proteins and entry into the nucleus. Steroid receptors bind to DNA as homodimers on a hexad repeat which is inverted and spaced by three nucleotides (IR-3).

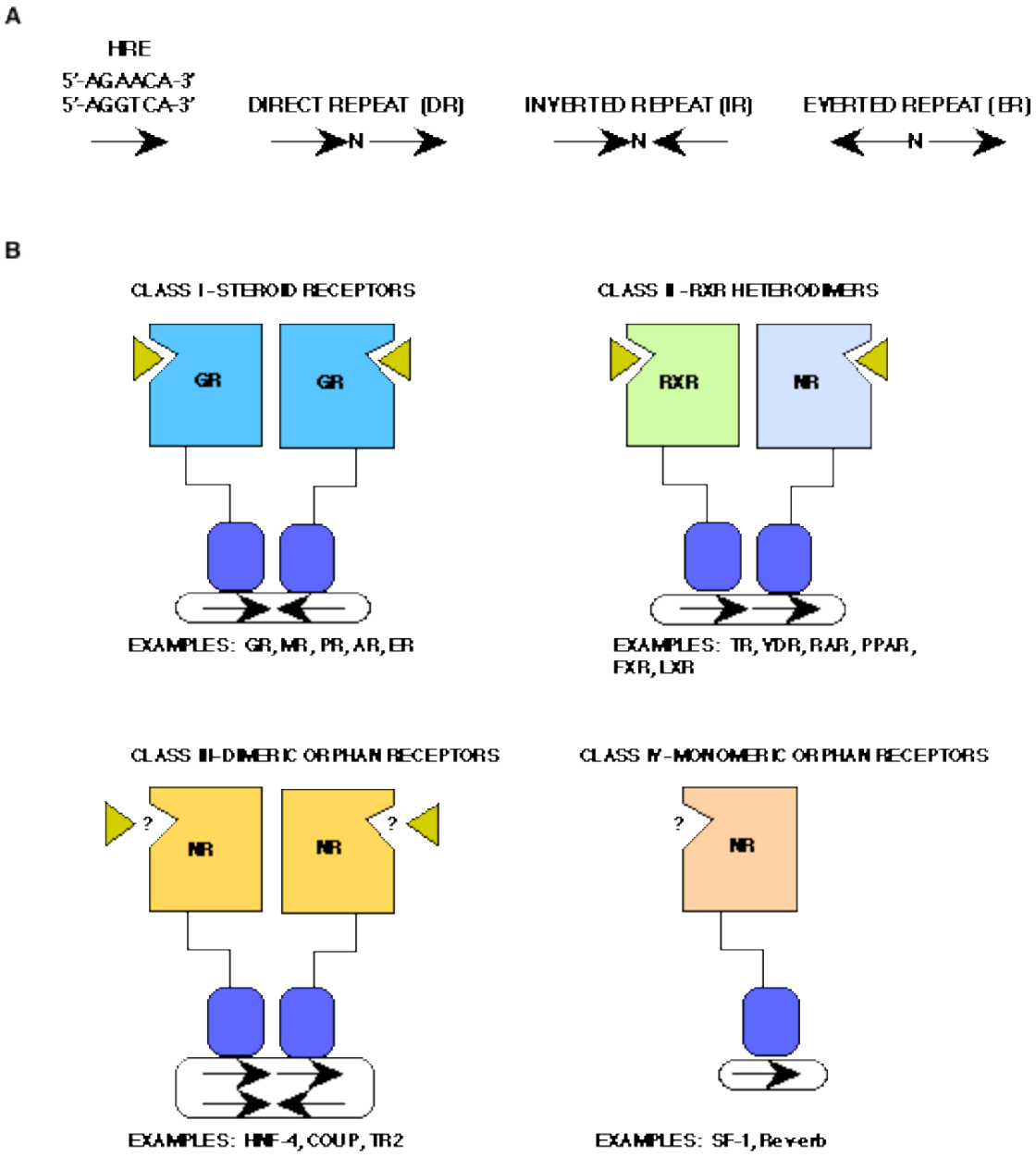

Figure 1. A) The DNA binding motif of NHRs consists of a two core hexad half-sites oriented as palindromes (inverted repeats, IR), everted repeats (ER), or direct repeats (DR). B) NHRs can be classified into four major categories based on their modes of transcriptional regulation. See text for details.

2. Class II – The RXR Heterodimers (also known as the thyroid/retinoid/vitamin D_3 subclass). This class includes the vitamin D_3 receptor (VDR), thyroid hormone receptor (TR), all-*trans* retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), and farnesoid X receptor (FXR). Class II receptors are normally found within the nucleus bound to their cognate HRE in the absence of ligand (for review see (Mangelsdorf and Evans, 1995)). Some members of this subclass are capable of exerting a repressive effect on gene transcription in this state. Binding of ligand initiates conformational changes that cause recruitment of transcriptional co-activators and transcriptional activation. Normally, members of this subclass bind to DNA as heterodimers on a direct hexad repeat (DR). The VDR and TR bind to DRs spaced by 3 or 4 nucleotides (DR-3 and DR-4, respectively). In contrast, PPAR and RAR bind to DR-1 elements, while RAR can also bind to both DR-2 and DR-5 elements.

Remarkably, the sole heterodimerization partner for class II NHRs is the retinoic X receptor (RXR). The natural ligand for RXR is 9-*cis* retinoic acid, and this receptor appears to function as a master regulator that is capable of integrating multiple hormone signaling cascades. The binding of a class II NHR-RXR heterodimer complex on DNA is asymmetric, with RXR occupying the 5' half-site on DR-3, DR-4, and DR-5 elements and the 3' half-site on DR-1 and DR-2 elements. Finally, RXR may either be transcriptionally responsive or silent depending on its heterodimer partner. For example, when RXR is bound to TR, VDR, and RAR, it is repressed and transcriptionally unresponsive to ligand. However, PPAR-RXR and LXR-RXR complexes are "permissive" in the sense that addition of ligand to either receptor in the complex leads to transactivation of a promoter.

3. Class III – Dimeric Orphan Receptors. Before the advent of DNA cloning, the NHR field was focused on the identification of receptors for hormones with known

biological activities. However, beginning in the late 1980's, many receptors were cloned and classified as NHRs based simply on sequence homology. These receptors were termed orphan receptors because the ligand that activated them was not known, although it is now recognized that some members of this category are not likely to be ligand modulated. Class III NHRs represent orphan receptors that bind as homodimers to HREs oriented as IRs or ERs. Members of this family include hepatocyte nuclear factor-4 (HNF-4), chicken ovalbumin upstream promoter (COUP) transcription factors, and testis receptor (TR2).

4. Class IV – Monomeric Orphan Receptors. As the name would suggest, receptors in this subclass are orphan receptors that bind to DNA as monomers, often to a HRE consisting of a single hexad half-site in which the 5' flanking sequence is critical in determining binding specificity. Class IV NHRs are often positive regulators of transcription that display constitutive activity. Members of this family include steroidogenic factor 1 (SF-1) and rev-erb.

Structure of NHRs

NHRs are modular transcription factors and the basic design of the protein is well conserved among members of the superfamily. NHRs can be divided in to five major domains (Fig. 2A).

1. A/B Domain. The A/B domain is also known as the modulator domain. It is located at the amino terminus of the protein and is the least conserved domain within the superfamily. Many NHRs will encode splice variants with unique biological properties that differ solely in the composition of the A/B domain. The A/B domain also contains a ligand independent transactivation function termed Activator Function-1 (AF-1) and often contains amino acids that are targets of post-translational modification (*e.g.* phosphorylation). Recent evidence suggests that cell-specific co-regulators also bind to the A/B domain.

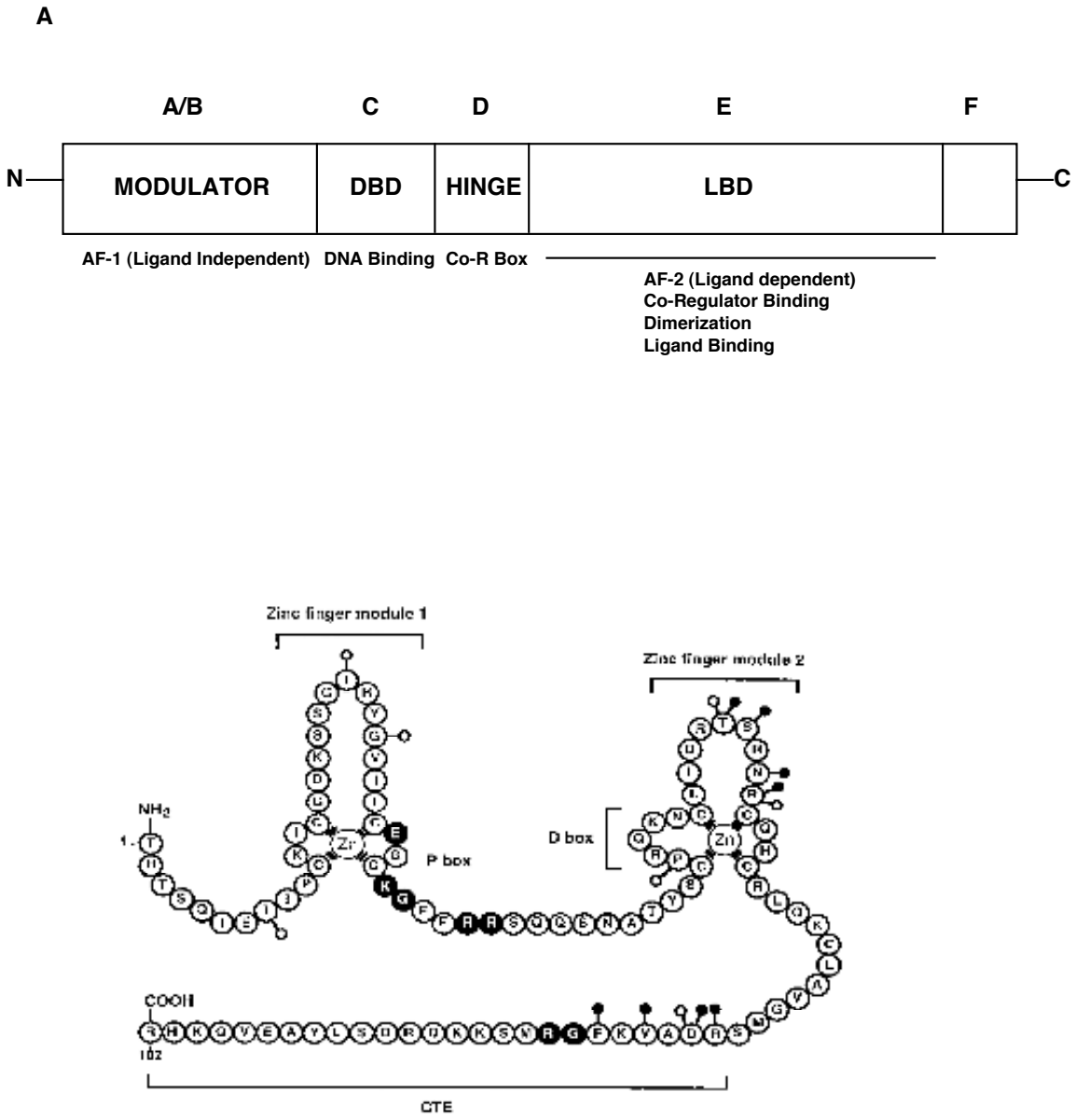

Figure 2. Anatomy of NHRs. A) NHRs can be divided into five major domains (see text for details). B) A NHR DNA binding domain as visualized by Renaud and Moras (Renaud and Moras, 2000). See text for details.

2. C Domain. The C domain is the DNA binding domain (DBD) of the receptor and is the most highly conserved region of NHRs. The DBD of a NHR contains two zinc finger motifs consisting of 66-70 amino acids and an amino acid carboxy terminal extension (CTE) of approximately 25 amino acids that mediates both DNA-protein and protein-protein interactions (Fig. 2C). Site-directed mutagenesis and x-ray crystallography studies have identified a subdomain termed the P box that mediates recognition to the core half-site nucleotide sequence of the HRE. The D box is a subdomain involved in receptor dimerization and recognition of spacing between half-sites.

3. D domain. The D domain is also known as the hinge region; its primary purpose is to serve as a hinge between the DBD and the ligand binding domain (LBD) of the receptor. The presence of this hinge is believed to allow for some NHRs to bind to both IR and DRs, (in which case the DBDs have to be able to rotated 180°) while maintaining identical orientation of the LBD. The CoR box, which is a subdomain important for interaction with corepressors, is located in the D domain.

4. E domain. The E domain is also known as the LBD and mediates heat shock protein binding, dimerization, ligand binding, and cofactor binding. At the carboxy terminus of the E domain is an activation function-2 motif (AF-2) that is essential for ligand-dependent transactivation (see below). X-ray crystallography studies suggest that the LBD is made up of 11-13 □-helices. The overall structure has been termed an □-helical sandwich because it consists of three antiparallel layers of □-helices, the core of which serves as a hydrophobic pocket for ligand binding.

5. F domain. The function of the F domain of NHRs is poorly understood and not present in all receptors.

Transcriptional Activities of NHRs

NHRs can regulate the expression of a gene by at least four distinct mechanisms (Fig. 3A) (Glass and Rosenfeld, 2000). The prototypical transcriptional activity of NHR is ligand-induced transcriptional activation when the receptor is bound to its cognate HRE (ligand-dependent transactivation). Some NHRs can also serve as coactivators for other transcription factors; for example, ligand bound GR can serve as a coactivator for certain STAT5 responsive genes (ligand-dependent coactivation). Additionally, some members of the NHR superfamily, notably TR and RAR, are capable of ligand independent repression of a target gene (active repression). This type of repression requires both binding of the NHR to its HRE and the recruitment of corepressors and results in gene silencing in the absence of ligand. Finally, NHRs are also capable of repressing certain target genes in the presence of ligand (ligand-dependent transrepression) in a manner that is independent of the DNA binding activity of the NHR. A classic example of transrepression is the ability of ligand-bound GR to antagonize the activity of AP-1 transcription factors (Kamei et al., 1996). This phenomenon is thought to be due in part to a "squelching" effect in which ligand-bound GR causes sequestration of transcriptional coactivators such as CBP and p300. This sequestration inhibits the ability of CBP/p300 proteins (present within the cell in limiting amounts) to coactivate AP-1.

The Role of Coregulators in NHR function

Simplistically, the function of a NHR is to influence the rate of transcriptional initiation at target gene promoters. Transcriptional initiation is a complex process mediated by RNA polymerase II (RNA Pol II) and a host of general transcription factors (GTF's) (reviewed in (Roeder, 1996)). One way that NHRs could influence transcription initiation rates is through direct contact with either RNA Pol II or GTFs. In fact, several nuclear receptors can directly interact with TATA binding polypeptide (TBP) and several

TBP-associated factors (TAFs), although the relevance of these interactions remains to be determined. In contrast, it has now become clear that a major mechanism by which NHRs regulate target gene promoter initiation events is through the recruitment of a plethora of accessory factors termed cofactor or coregulators. These coregulators serve to bridge the NHR to two crucial elements of gene expression: 1) reorganization of the chromatic template that creates either a permissive or nonpermissive environment for transcriptional initiation and 2) communication with the basal transcriptional machinery. A comprehensive analysis of the complex field of NHR coregulators is beyond the scope of this introduction and can be found elsewhere (McKenna et al., 1999); the following discussion will be limited to a brief overview of NHR coactivators and corepressors.

NHR Coactivators

Complexes that serve as coactivators for NHRs can broadly be divided into three major categories based on their mechanism of action: 1) ATP-dependent chromatin remodeling complexes; 2) complexes with acetyltransferase activity; and 3) mediator complexes that interact with the basal transcriptional machinery. It has long been recognized that DNA is organized into a highly ordered structure, chromatin, which is composed of core nucleosome repeats that consist of DNA wrapped around a complex of histone proteins. This compact structure represents a physical barrier to initiation of gene transcription and a crucial requirement of gene activation is chromatin remodeling. Yeast contain a complex termed SWI/SNF that is able to assist in the binding of sequence specific transcription factors to nucleosomes as well as initiate ATP-dependent local changes in chromatin structure. Human homologs of SWI/SNF proteins include Brg1 and hBrm (Murchadt and Yaniv, 1993); co-transfection of mutant Brg1 or hBrm can reduce the transactivation function of several nuclear receptors. Moreover, the ability of GR to activate the mouse mammary tumor virus (MMTV) promoter is dependent on Brg-1 if the promoter is integrated into chromosomal DNA (Fryer and Archer, 1998).

A second major category of coactivators are proteins that can either directly or indirectly mediate the acetylation of lysine residues in histones. Modification of histones by acetylation has emerged as a significant means by which transcriptional cofactors can regulate gene expression; actively transcribed genes are associated with hyperacetylation while regions of chromatin that are transcriptionally silent consist of histones that are largely hypoacetylated (Chen et al., 2001). One widely studied protein with intrinsic histone acetyltransferase (HAT) activity is CREB binding protein (CBP) and its close homolog p300. CBP was first isolated as a binding protein for the cAMP response element binding protein (CREB) and CBP/p300 can serve as coactivators for a large number of transcription factors including NHRs. For example, microinjection of anti-CBP antibodies into cells can inhibit ligand-induced gene expression of a NHR while *p300*-/- mouse fibroblast cells are severely defective in retinoic acid mediated gene transcription. In addition to histone acetylation, CBP/p300 may also potentiate gene transcription through direct interactions with RNA Pol II. p/CAF is another transcriptional coactivator with intrinsic HAT activity. p/CAF can directly interact with NHRs and the interaction domain has been shown to be distinct from the NHR domain involved in binding to CBP or p300. Finally, the p160/steroid receptor coactivator (SRC) family of coactivators consists of three highly related genes that bind NHRs in a ligand dependent manner. SRC family members have weak intrinsic HAT activity and also bind independently to CBP, suggesting that they may also serve as a "bridging" factor between a NHR and CBP.

The last major category of NHR coactivators is the mediator complexes. Yeast contain a complex termed mediator that is associated with RNA Pol II and appears to be essential for transcriptional activation. A large family of proteins in humans has been identified that represent homologs of the yeast mediator complex and individual members of this group of proteins have been shown to bind multiple transcription factors including NHRs (*e.g.* the TR associated protein (TRAP) and Vitamin D interacting protein (DRIP)

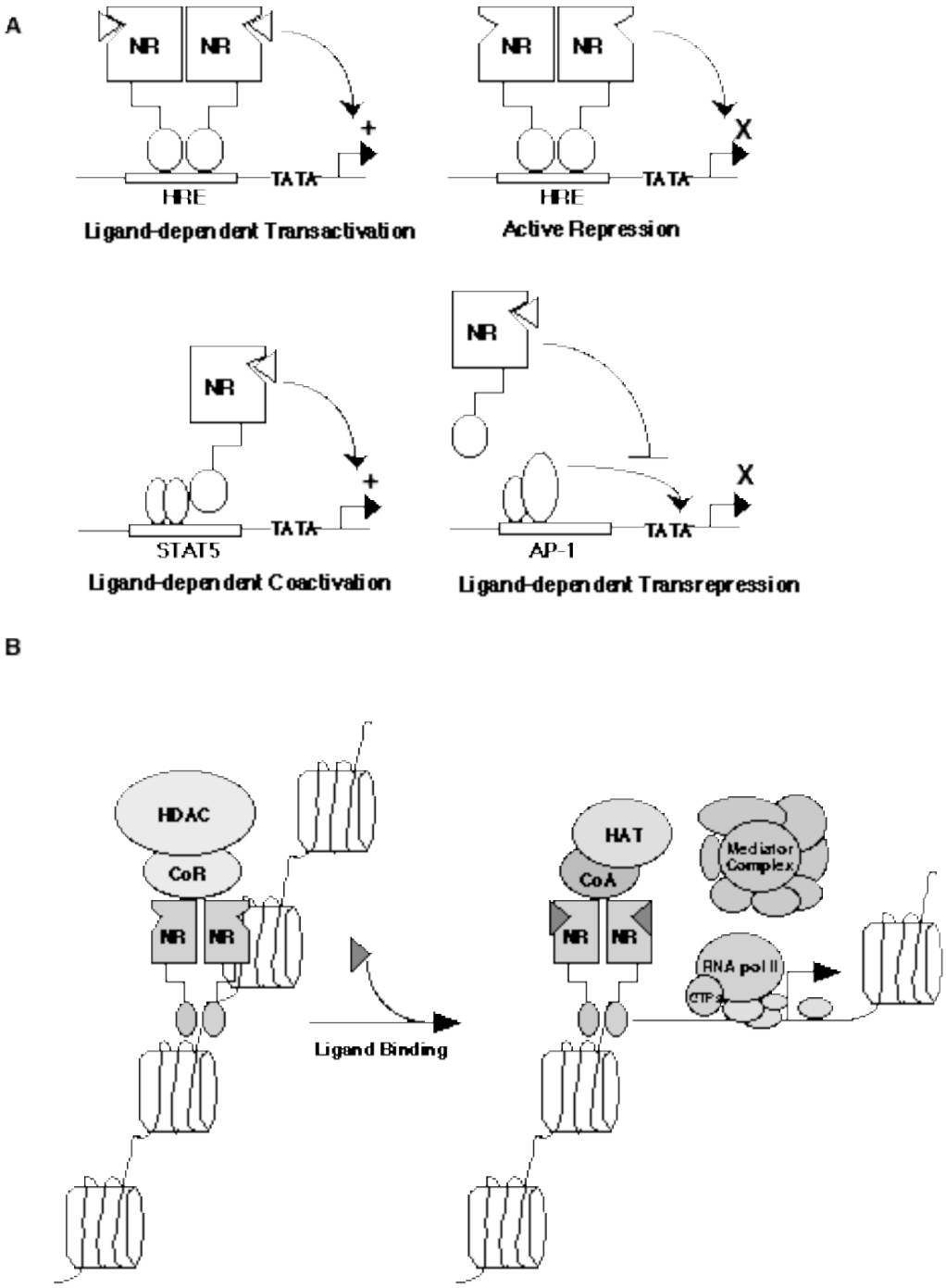

Figure 3. A) The different transcriptional activities of NHRs classified by Glass and Rosenfeld(Glass and Rosenfeld, 2000). B) A schematic of the coregulator exchange, in which ligand binding to a NHR causes release of corepressors and the recruitment of coactivators. See text for details.

complexes) (Rachez and Freedman, 2001). These complexes do not contain intrinsic HAT activity but can bind and stabilize both RNA Pol II and GTFs. Thus, NHRs appear to initiate and retain RNA Pol and GTFs at the site of transcriptional initiation in part through direct interactions with individual components of the mediator complex.

NHR Corepressors

A number of NHRs, including TR and RAR, can silence gene expression in the absence of ligand (Hu and Lazar, 2000). This activity is dependent on DNA binding and is referred to as active repression (as opposed to the transrepression function of some NHRs that does not require the DBD). Two highly related proteins that bind to NHRs in the absence of ligand have been purified and identified as nuclear receptor corepressor (NcoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT). Both of these proteins contain transferable repressor domains and bind to TR or RAR in the absence, but not presence, of ligand. The mechanisms by which NcoR or SMRT mediate transcriptional repression became more clear with the discovery that they could interact with mammalian homologs of the yeast Sin3 proteins, although it does not appear that NcoR and SMRT are stable components of the Sin3 repressor complex. In yeast, transcriptional repression complexes are composed of Sin3 proteins as well as histone deacetylases (HDACs). Thus, it appears that NHRs can silence gene expression by associating with factors (NcoR and SMRT) that then serve as a bridge to a core complex capable of inducing hypoacetylation of histones at a transcriptional locus.

Molecular Mechanisms of Coactivator and Corepressor Binding to NHRs

Almost all coactivators that bind to NHRs in a ligand dependent fashion contain at least one copy of the signature motif, LXXLL (where L is leucine and X is any amino acid) and mutagenesis experiments have confirmed that the LXXLL motif is essential for

NHR-coactivator binding. X-ray crystallographic studies have suggested a common mechanism by which NHRs can bind to coactivators. Essentially, in the absence of ligand the carboxy terminus AF-2 helix of the NHR is oriented away from the surface of the LBD. Binding of ligand causes a conformation change such that the AF-2 helix is repositioned and becomes tightly associated with the LBD and also makes direct contacts with ligand. This repositioning of the AF-2 helix results in a highly receptive hydrophobic cleft on the LBD that binds to the short LXXLL □-helix of a coactivator. The LXXLL helix is positioned by a "charge clamp" that at one end consists of the carboxy end of the LXXLL helix making direct contact with a highly conserved lysine residue found in H3 of NHRs and at the other end involves direct association between the amino terminus of the LXXLL helix and a highly conserved glutamate residue in the AF-2 helix.

A signature motif termed the CoRNR box has also been identified that is critical in the ability of corepressors to bind NHRs. This motif is an □-helix that consists of the consensus sequence LXXI/HIXXXI/L (where L is leucine, X is any amino acid, and I is isoleucine). The CoRNR box occupies the same hydrophobic cleft on the LBD of a NHR as the LXXLL helix of coactivators. Ligand binding, and subsequent repositioning of the AF-2 helix of the NHR, orients the surface of the LBD such that it can no longer bind corepressor but can facilitate binding of coactivators.

The Coregulator Exchange in NHR Function

The observation that ligand occupied NHRs bind to coactivators associated with HAT activity while apo-NHR mediated active repression was due to corepressors associated with HDACs has led to a generalized model for NHR function in which ligand binding initiates conformational changes that results in the exchange of corepressors for coactivators (Fig. 3B) (Glass and Rosenfeld, 2000). This exchange causes significant changes in the chromatin structure of the transcriptional locus such that the

transcriptional initiation site of the gene now becomes accessible to RNA Pol II and GTFs. NHRs appear to stabilize the binding of these initiation factors in part by the recruitment of mediator complexes. The net result is elevated transcription of a gene which was previously silent. Finally, it should be emphasized that this highly simplistic schematic is limited to only certain NHRs (particularly events depicted in the absence of ligand) and experimental evidence for the "exchange" model has largely been limited to studies with TR and RAR.

Introduction to Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome proliferators are a group of diverse compounds that include fibrates, herbicides, leukotriene antagonists, and plasticizers. In rats and mice, chronic administration of peroxisome proliferators induces extensive biogenesis of hepatocyte peroxisomes and eventually leads to the development of hepatocellular carcinoma (reviewed in (Reddy and Mannaerts, 1994)). These compounds are not mutagenic and thus represent non-genotoxic carcinogens. In 1990, Green and colleagues identified a novel member of the nuclear hormone receptor superfamily that was ligand activated by peroxisome proliferators and hence named the receptor peroxisome proliferator activated receptor (PPAR) (Issemann and Green, 1990). This first cDNA was subsequently designated PPAR□ and two other receptor subtypes in this family were soon cloned and identified as PPAR□and -□(for reviews see (Desvergne and Wahli, 1999; Kersten et al., 2000; Willson et al., 2000)). The three PPAR subtypes are highly homologous in both the DBD and the LBD (Fig. 4A). However, there is significant variation in the amino acids of each subtype that line the ligand binding pocket (Nolte et al., 1998; Xu et al., 1999) and each receptor has a distinct pharmacological profile (Kliewer et al., 1994). In addition, there is considerable variation in the amino acid sequence of the A/B domain of each receptor subtype; this domain may be critical for subtype specific protein-protein

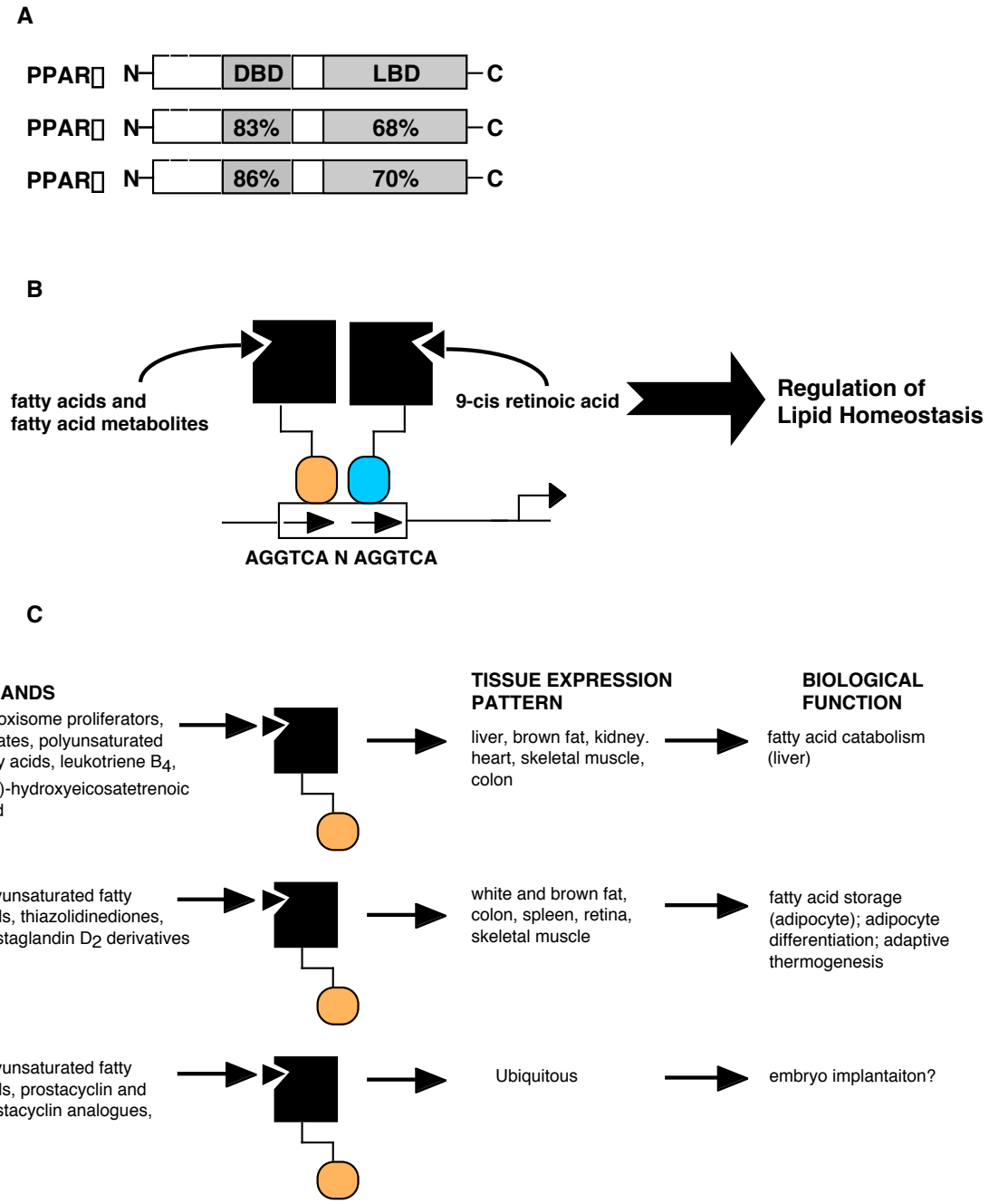

Figure 4. Overview of PPARs. A) Homology between different PPAR subtypes. B and C) Schematics of mechanisms of PPAR transcription and the different biological activities of PPAR subtypes.

interactions. Collectively, PPARs have been shown to play important roles in a number of physiological processes including lipid homeostasis, adaptive thermogenesis, and inflammation. They also represent important drug targets for a range of common diseases including diabetes and atherosclerosis.

After the initial cloning of PPAR, it was soon realized that PPARs did not readily form homodimers but rather were members of the class II family of NHRs and hence formed heterodimers with RXR (Kliewer et al., 1990) (Fig. 4B). The consensus PPAR response element (PPRE) is a DR-1 composed of the sequence AGGTCA A/T AGGTCA. In addition, studies *in vitro* using recombinant PPARs and a range of naturally occurring PPREs have failed to identify any key differences that would suggest that particular PPREs specify binding of a specific PPAR subtype (Juge-Aubry et al., 1997). However, these studies did suggest that the 5' sequencing flanking a DR-1 can serve to help distinguish the binding of PPAR versus PPAR The PPAR-RXR complex is defined as permissive because both RXR and PPAR ligands will induce transactivation of a PPAR-RXR driven promoter (Bardot et al., 1993). In some instances, combination addition of PPAR and RXR ligands results in transcriptional synergy (Schulman et al., 1998), a phenomenon that may be due to the ability of PPARs and RXRs to recruit different transcriptional coactivators.

The fact that peroxisomes are important in the metabolism of long chain fatty acids provided the first clue that the natural ligand for PPARs might be a fatty acid or fatty acid metabolite. Unexpectedly, a broad range of polyunsaturated fatty acids (PUFAs) were found to activate PPARs in the low micromolar range (Gottlicher et al., 1992; Schmidt et al., 1992). This raised the possibility that fatty acids could serve as hormones that regulate gene transcription via modulation of PPARs. To date, all known PPAR target genes with functional PPREs play roles in lipid or glucose metabolism (Table I). Thus, a central theme in PPAR signaling that has developed over the last decade is the role of these receptors as fatty acid "sensors" that integrate dietary fat intake

Table I

PPAR target genes with identified PPREs

Target Gene	Gene Function	PPAR[2]
Acyl-CoA synthase	Fatty acid oxidation	PAR
cyl-CoA oxidase	Peroxisomal -oxidation	PPAR
Apolipoprotein A-I	Blood transport of fatty acid	PPAR
Apolipoprotein A-II	Blood transport of fatty acid	PPAR
Apolipoprotein C-III	Bood transport of fatty acid	PPAR
aP2 adipocyte lipid binding protein	Inttracellular fatty acid binding	PPAR
Bifunctional enzyme[1]	Peroxisomal -oxidation	PPAR
CPTI carnitine palmitoyl transferase I	Entry of fatty acyl into mitochondria	PPAR
Cyp4A1/P420 IV family	Microsomal -oxidation	PPAR
Cyp4A6/P450 IV family	Microsomal -oxidation	PPAR
Fatty acid transport protein	Fatty acid transport across cell membranes	PPAR/
Lipoprotein lipase	Fatty acid release from lipoprotein-bound triglycerides	PPAR/
Liver fatty acid binding protein	Intracellular fatty acid binding protein	PPAR
Liver-specific type I sugar transporter	Sugar Transport	PPAR
Malic enzyme	Fatty acid synthesis/NADPH production	PPAR
Medium chain acyl-CoA dehydrogenase	Mitochondrial -oxidation	PPAR
Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	Ketone body synthesis	PPAR
Phophoenolpyruvate carboxykinase	Glycerogenesis (adipose tissue)	PPAR
Scavenger receptor CD36	Uptake of modified LDL in macrophages	PPAR
Stearoyl-CoA desaturase I	Desaturation of fatty acyl-CoA	PPAR
Uncoupling protein I (brown adipocytes)	Nonshivering thermogenesis	PPAR

[1](enoyl-CoA hydratase/3-Hydroxy-acyl-CoA dehydrogenase)

[2]The PPAR subtype that was used in studying the indicated promoter is listed; however, other PPAR subtypes may also regulate this gene

with the transcriptional control of genes involved in glucose and lipid metabolism (Fig. 4B and C). The discussion below will begin with a brief overview of the PPAR and - subtypes followed by a more comprehensive discussion of the PPAR subtype.

Overview of PPAR

PPAR is primarily expressed in liver, brown fat, kidney, colon, skeletal muscle, and heart (Mukherjee et al., 1997). It serves as a central regulator of fatty acid catabolism in these tissues by regulating the transcription rates of multiple genes involved in lipid metabolism (Dreyer et al., 1992) (see Table I), presumably in response to fluxes in dietary fat intake. During the fasting state, PPAR (thought to be activated by fatty acids released by adipose depots) stimulates the transcription of multiple genes (*e.g.* acyl-CoA oxidase and keto-acyl-CoA thiolase) involved in the oxidation of fatty acids to ketone bodies (Kersten et al., 1999; Leone et al., 1999). These studies also used mice null for PPAR to confirm the essential role of PPAR in mediating the fasting response; PPAR knockout mice exhibit dramatic defects in fatty acid oxidation and ketogenesis when placed on a fasting diet and suffer from elevated serum levels of free fatty acids along with hypoketonemia and hypoglycemia.

The administration of synthetic ligands for PPAR (*e.g.* fibrates) causes favorable changes in lipid profiles including a reduction in triglycerides(Staels et al., 1998)(due to a PPAR-stimulated increase in fatty acid oxidation) as well an increase in high density lipoprotein (HDL) that is likely due to the ability of PPAR to induce apolipoprotein-A1 and –AII (Vu-Dac et al., 1995; Vu-Dac et al., 1994). There is also evidence indicating that PPAR may have a direct anti-atherosclerotic effect, potentially due to the ability of the receptor to negatively regulate inflammation in vascular smooth muscle cells(Staels et

al., 1998). Finally, a consensus endogenous ligand for PPAR has not been discovered, but the receptor can be activated by a range of PUFAs (Forman et al., 1997; Kliewer et al., 1997) as well as some oxidized fatty acids. In addition, the lipoxygenase products leukotriene B_4 (LTB_4) (Devchand et al., 1996) and 8(S) hydroxyeicosatetrenoic acid (8S-HETE) (Yu et al., 1995) can activate PPAR but it remains to be proven that these molecules are relevant activators of the receptor *in vivo*.

Overview of PPAR

PPAR is ubiquitously expressed in almost all tissues in the body and a major biological role for PPAR has not yet been established (Auboeuf et al., 1997). Mice null for PPAR show negligible phenotypes, although they appear to exhibit focal areas of demyelination in their central nervous system and have a slight increase in the level of inflammation in the skin following topological exposure to TPA (Peters et al., 2000). Other studies have suggested that the cyclooxygenase (COX) metabolite prostacyclin (PGI_2) can transactivate PPAR (Gupta et al., 2000) and that this signaling cascade may be operative during COX-2 mediated regulation of embryo implantation(Lim et al., 1999). PPAR was recently identified as a downstream target gene of the tumor suppressor adenomatous polyposis coli (APC) (He et al., 1999) and a colorectal cancer cell line with both alleles of PPAR removed exhibits reduced tumorigenicity, suggesting a role for PPAR in colorectal carcinogenesis (Park et al., 2001).

Introduction to PPAR

PPAR was originally identified in studies focused on the cloning of PPAR related cDNAs (Zhu et al., 1993) as well as experiments focused on the identification of nuclear proteins that bound to the fat-specific enhancer element of the aP2 gene (an intracellular fatty acid binding protein) (Tontonoz et al., 1994). PPAR is most highly expressed in white and brown adipose tissue, colon, spleen and retina with lower levels in

skeletal muscle and many other tissues (Fajas et al., 1997; Mukherjee et al., 1997). The location of the gene has been mapped to 3p25 (a region that often displays loss of heterozygosity in human cancers) (Greene et al., 1995). Three different splice variants of PPAR that arise through alternative promoter usage have been identified (Fajas et al., 1997; Fajas et al., 1998): PPAR 1, - 2, and - 3. There is no difference in the coding sequence of PPAR 1 and PPAR 3; however, PPAR 2 contains an additional 30 amino acids at its amino terminus and is exclusively expressed in adipose tissue. To date, no functional differences between PPAR 1 and PPAR 2 have been identified. The A/B domain of PPAR contains a serine residue at codon 112 that serves as a substrate for mitogen activated protein kinase (MAPK) (Hu et al., 1996). Phosphorylation of PPAR at Ser^{112} by MAPK reduces PPAR transcriptional responsiveness, an effect that is likely due to a decrease in affinity for ligand (Shao et al., 1998).

The identification of PPAR as a regulator of the aP2 gene, along with its strong expression in adipose tissue, suggested an important role for PPAR in adipocyte biology. In fact, soon after the discovery of PPAR, experiments by Tontonoz *et al.* established PPAR as a central regulator of adipocyte differentiation. Forced expression of PPAR in cultured fibroblasts induced their differentiation into mature adipocytes that could accumulate lipid and express a number of markers of adipocyte differentiation (Tontonoz et al., 1994). Since these initial discoveries, PPAR has been shown to be a global regulator of glucose and lipid homeostasis that also appears to play an important regulatory role in cholesterol trafficking in macrophages. It may also play a role in the control of the inflammatory response in different cell types. The discussion below will first focus on PPAR ligands and cofactor interactions followed by a detailed discussion of the known biological activities of the receptor.

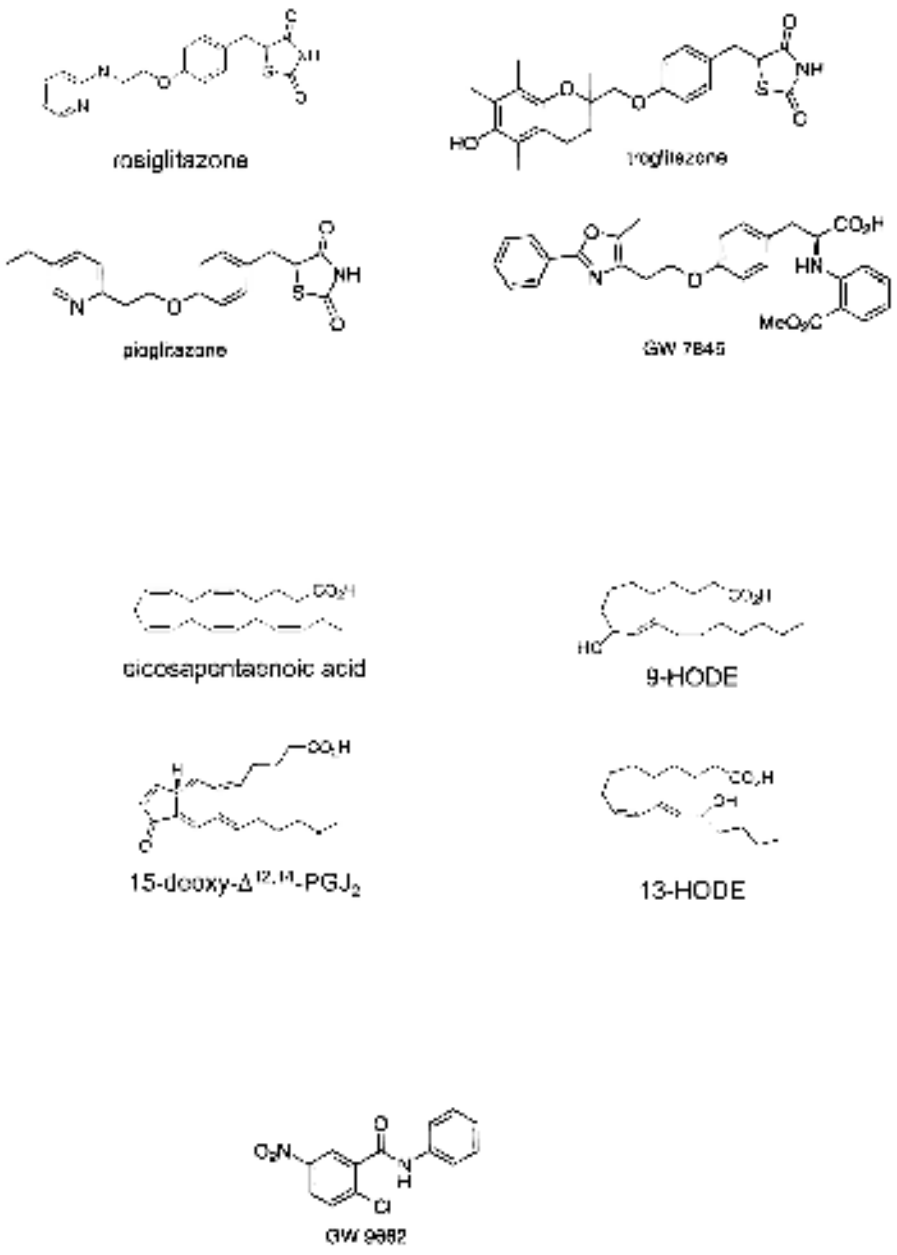

Figure 5. PPAR ligands – see text for details.

PPAR Ligands

PPAR is activated by a diverse array of natural and synthetic ligands (Fig. 5). As is the case with PPAR and -, PUFAs (*e.g.* eicosapentaenoic acid) can activate PPAR in the low micromolar range (Kliewer et al., 1997). Other potential natural ligands for PPAR include the prostanoid 15-deoxy^{12,14} PGJ_2 (Forman et al., 1995; Kliewer et al., 1995) and two oxygenated derivatives of linoleic acid, 9- and 13-hydroxyoctadecadienoic acid (HODE), that are components of oxidized LDL (oxLDL) particles (Nagy et al., 1998). 15-deoxy^{12,14} PGJ_2 is a downstream metabolite of prostaglandin D_2 (PGD_2), which itself is formed from arachadonic acid by the catalytic activities of COX and PGD synthase. The discovery that a prostanoid was a high affinity agonist of a NHR raised the intriguing possibility that prostaglandins might directly modulate gene transcription via modulation of a NHR. However, it is still not clear if 15-deoxy^{12,14} PGJ_2 activates PPAR *in vivo*, and many biological effects of the molecule are clearly independent of PPAR For example, the biological effects of 15-deoxy^{12,14} PGJ_2 are identical in wild type and PPAR-/- macrophage cell lines (Chawla et al., 2001).

A number of high affinity, PPAR subtype selective agonists have been synthesized. The thiazolidinediones (TZDs or glitazones) are a family of compounds that were originally identified through empirical screens as potent sensitizers of insulin in mouse models of diabetes (Hulin et al., 1996). Examples of TZDs include rosiglitazone, troglitazone, and pioglitazone. Both rosiglitazone and pioglitazone are currently being used in the treatment of insulin resistant diabetes mellitus. A major breakthrough that linked PPAR signaling with insulin sensitization and glucose homeostasis came with the discovery that TZDs are potent and selective PPAR ligands (Lehmann et al., 1995). Evidence that TZDs mediate their insulin sensitizing effects via PPAR has come from studies that show a strong correlation between the binding affinity of a TZD to PPAR and its glucose lowering properties *in vivo* (Willson et al., 1996). Recently, a novel class of synthetic PPAR agonists has been developed based on the amino acid L-tyrosine;

these compounds (*e.g.* GW7845) bind to PPARγ at concentrations in the low nanomolar range (Henke et al., 1998) and again, their potency in reducing blood glucose levels *in vivo* correlates with their binding affinity to PPARγ *in vitro* (Brown et al., 1999).

Finally, a potent and irreversible synthetic antagonist of PPARγ, GW9662, was recently developed (Huang et al., 1999). GW9662 alkylates codon C286 in helix 3 (H3) of PPARγ and is selective for PPARγ in the 1-10 micromolar range. The compound inhibits PPARγ activity in cellular transactivation assays and can also inhibit TZD-induced adipocyte differentiation.

PPARγ Coregulators

A number of the coregulator molecules discussed earlier, including SRC-1 and CBP/p300, have been shown to bind PPARγ (DiRenzo et al., 1997; Zhu et al., 1996). An *in vitro* study using fluorescence resonance energy transfer (FRET) suggested that PPARγ has a higher intrinsic affinity for CBP over SRC-1, but the physiological relevance of this is not clear (Zhou et al., 1998). PPARγ also interacts with a protein named PPAR binding protein (PBP) that is identical to TRAP220 and DRIP230 (Zhu et al., 1997). As discussed earlier, TRAP and DRIP complexes fall under the mediator class of coregulators and the TRAP220/DRIP230 subunit contains an LXXLL motif that apparently serves to bridge TR and VDR to the larger mediator complex. Based on the finding that PBP/TRAP220/DRIP230 binds strongly to PPARγ, similar mechanisms are likely to be involved in bridging PPARγ to mediator complexes.

The PPARγ coactivator 1 and -2 (PCG1 and –2) proteins were originally identified in yeast two-hybrid screens using PPARγ as bait (Castillo et al., 1999; Puigserver et al., 1998). These cofactors are unique in that they do not contain LXXLL motifs, have little intrinsic HAT activity, and bind to PPARγ in the absence of ligand. Interestingly, they still participate in ligand-dependent coactivation via cooperative interactions with SRC-1 and CBP (Puigserver et al., 1999). Both PCG-1 and –2 are

examples of NHR coregulators that only appear to function in specific NHR- mediated activities. For example, PCG-1 is exclusively expressed in brown fat and skeletal muscle and is strongly induced under conditions of adapative thermogenesis. Mice that are exposed to low environmental temperatures contain significant elevations in PCG-1 levels within brown fat tissue; under these circumstances PCG-1 serves as an essential coactivator for PPARγ mediated up-regulation of uncoupling protein 1 (UCP1) that then functions to dissipate the mitochondrial proton gradient and generate heat. PCG-2 interacts with the A/B domain of PPARγ and does not bind to PPARα or -δ. Castillo *et al*. have proposed that this interaction may be responsible, in part, for the ability of PPARγ to uniquely induced adipocyte differentiation.

The corepressors N-CoR and SMRT can bind to PPARγ in solution but it is not clear if PPARγ is capable of silencing gene transcription when bound to a typical PPRE. Unlike the case with the TR, transfection of PPARγ does not repress transcription of a reporter gene driven by its cognate DNA response element. Consistent with this, Zamir *et al*. demonstrated that while both PPARγ and TR could bind to these two corepressors in solution, only TR could do so when bound to DNA (Zamir et al., 1997).

PPARγ as a Regulator of Adipocyte Differentiation

As discussed above, one of the earliest biological activities of PPARγ to be discovered was its ability to induce adipocyte differentiation *in vitro*. This has now been confirmed in gene knockout experiments. PPARγ-/- mice are embryonic lethal due to a defect in formation of the placenta; however, this defect can be rescued using aggregation chimeras consisting of PPARγ-/- diploid embryos and wild type tetraploid embryos. Barak *et al*. used this strategy to generate a single PPARγ-/- mouse that was completely devoid of both white and brown adipose tissue (Barak et al., 1999). In contrast, Rosen *et al*. developed chimeric mice in which wild type blastocysts were mixed with PPARγ-/- embryonic stem cells (Rosen et al., 1999). Most tissues developed from both wild type

and PPAR null cells; however, white and brown adipose tissue developed exclusively from wild type cells, suggesting an essential role for PPAR in adipogenesis.

The Role of PPAR in Insulin Signaling

The fact that TZDs are potent activators of PPAR mediated transcription, coupled with pharmacological studies that established a tight correlation between the affinity of a TZD for PPAR and its potency as an insulin sensitizer *in vivo*, suggests that PPAR plays a central role in insulin mediated glucose utilization. There is also genetic evidence implicating a role for PPAR in regulating insulin sensitivity. Two different germline loss of function mutations were recently identified in humans; both of these mutations severely disrupt normal ligand dependent activation of PPAR (Barroso et al., 1999). In fact, coexpression of either mutant receptor and wild type PPAR causes repression of the wild type receptor through a dominant-negative mechanism. Three different patients that were heterozygous for one of these mutations all exhibited severe insulin resistance and hyperglycemia providing a direct link between PPAR transcription and insulin sensitivity.

PPAR agonists such as the TZDs normalize blood glucose levels by increasing glucose utilization in skeletal muscle and decreasing gluconeogenesis in the liver. How exactly does ligand-activated PPAR promote these events, especially when it is highly enriched only in adipocytes? One mechanism could be through the regulation of factors secreted from adipocytes. For example, adipocytes secrete tumor necrosis factor (TNF-) and leptin and both of these molecules can lead to increased insulin resistance. PPAR negatively regulates both TNF- and leptin, suggesting that TZDs and other PPAR agonists can enhance insulin sensitivity in part through the negative or positive regulation of adipocyte secreted factors (De Vos et al., 1996; Kallen and Lazar, 1996; Valverde et al., 1998). A second mechanism has been proposed by Willson *et al.* and involves the glucose-fatty acid (Randle) cycle (Fig. 6) (Willson et al., 2001). In this

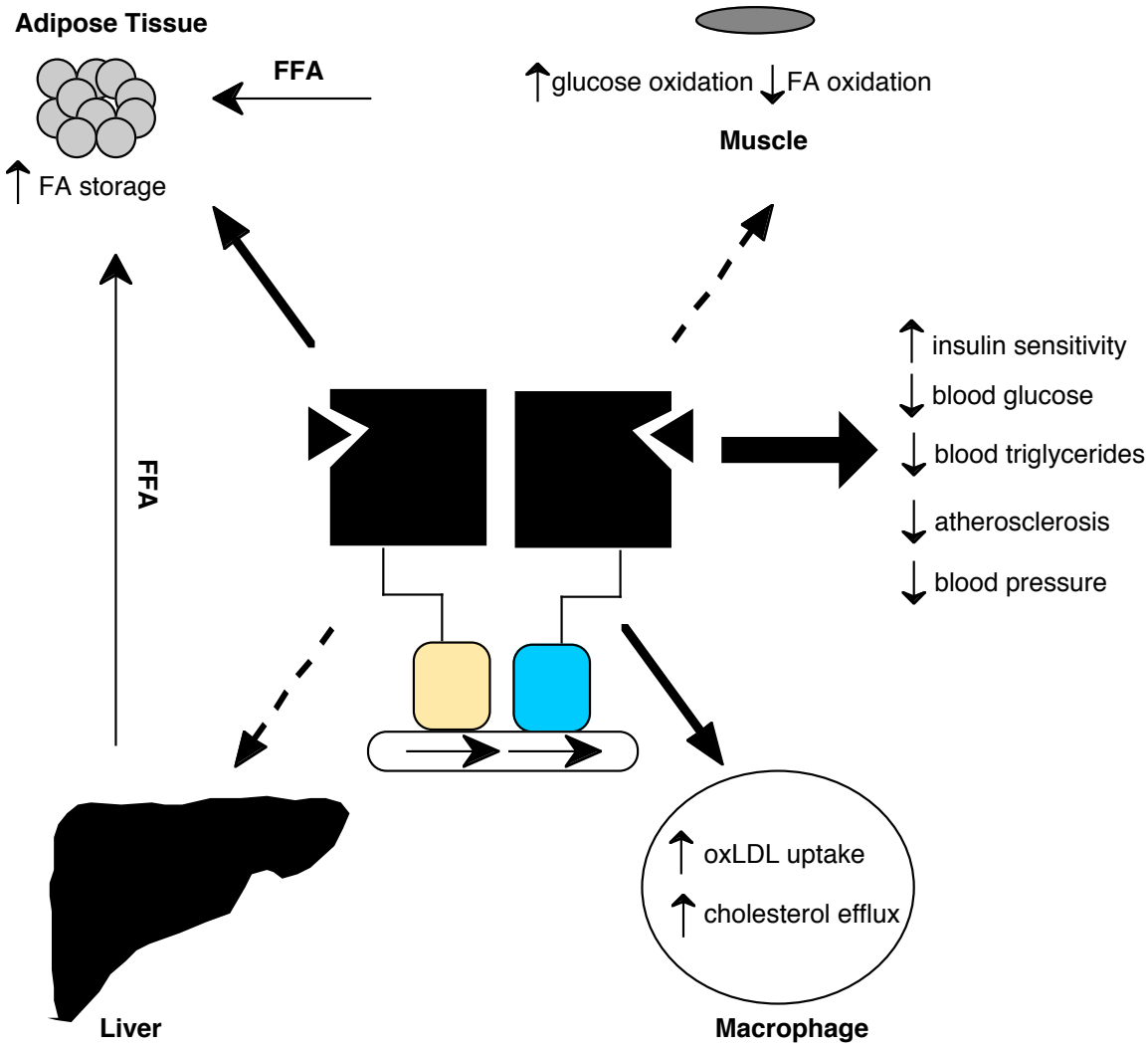

Figure 6. Metabolic consequences of PPAR activation – see text for details.

model, PPARγ activation in adipose tissue leads to the uptake of triglycerides and free fatty acids (FFAs) and hence a reduction in the serum level of FFAs. Normally, elevated FFAs in the serum inhibits glucose utilization by muscle and stimulates gluconeogenesis in the liver. Thus, the direct action of PPARγ in reducing serum FFA levels has the indirect effect of promoting the use of glucose as an energy source in muscle and inhibiting glucose production by the liver. The net result is lower blood glucose levels. Finally, it could be that part of the ability of PPARγ agonists to increase insulin sensitivity is due to activation of PPARα in liver and/or muscle, a hypothesis supported by experiments which demonstrate that TZDs still have beneficial effects on blood glucose levels in mice engineered to lack adipocyte (Burant et al., 1997). Finally, it cannot be ruled out that some of the anti-diabetic effects of TZDs are independent of PPARγ.

PPARγ and the Control of Cholesterol Trafficking in Macrophages

Cholesterol and lipid-loaded macrophages (known as foam cells) are a major part of the atherosclerotic plaque that builds up in the vascular wall of arteries. A report by Evans and colleagues identified CD36, the scavenger receptor that facilitates the uptake of ox-LDL, as a target gene of PPARγ (Tontonoz et al., 1998). They further identified 9-HODE and 13-HODE as components of the ox-LDL particle that can act as ligands for PPARγ. This suggested a positive feedback loop in which ox-LDL generates ligands that activate PPARγ, which in turn initiates gene expression events that lead to enhanced uptake of ox-LDL (Fig. 6). The end result of this would be enhancement of lipid loading in foam cells and hence one might predict that TZDs and other PPARγ agonists would be pro-atherosclerotic. However, data from both mice and humans suggest otherwise; in both instances TZDs prevent the development of atherosclerotic lesions (Li et al., 2000; Minamikawa et al., 1998). This discrepancy could be due to the recent finding that PPARγ also promotes the efflux of cholesterol from foam cells through upregulation of

the reverse cholesterol transporter ABCA1, which is a member of the ATP binding cassette (ABC) family of transporters(Fig. 6) (Chawla et al., 2001). In addition, a recent study suggests that, unlike the case with CD36, PPAR decreases expression of the ox-LDL scavenger receptor SR-A (Moore et al., 2001). The net result of all these activities of PPAR appears to be a decrease in lipid and cholesterol accumulation within foam cells.

PPAR and the Inflammatory Response

Several reports have indicated that PPAR ligands can inhibit the ability of certain cytokines to induce pro-inflammatory genes in macrophages. This activity was proposed to involve a transrepression mechanism involving PPAR-mediated inhibition of AP-1 activity (Jiang et al., 1998; Ricote et al., 1998). These studies were extended to intestinal epithelial cells; Wu and colleagues reported that activation of PPAR inhibits IL-8 gene expression in the Caco-2 colorectal cancer cell line via inhibition of NF- B. Moreover, TZDs reduced inflammation in a mouse model of colitis (Su et al., 1999). These experiments suggested that PPAR was an important regulator of the inflammatory response. However, in both instances the concentration of synthetic PPAR agonist that was required to cause these anti-inflammatory effects was approximately 100X fold in excess of the EC_{50} value for transactivation of PPAR . At such a high dose, these compounds are likely to have PPAR independent targets raising the issue of whether PPAR is truly a regulator of the inflammatory response. In support of this is the recent finding that high doses of TZDs will inhibit the expression of pro-inflammatory genes in both wild type and PPAR null macrophages (Chawla et al., 2001).

PPAR and the Control of Cell Cycle

Given the role of PPAR as an inducer of adipocyte differentiation, it is likely that the receptor could be involved in the regulation of signaling pathways that lead to

cessation of cell growth. Altiok *et al.* have shown that activation of PPAR in fibroblast cells results in G1 phase cell cycle arrest and growth inhibition (Altiok et al., 1997). The mechanism for the growth arrest was due to PPAR -mediated downregulation of protein phosphatase 2A (PP2A). Consistent with this, treated of cultured liposarcoma cell lines with PPAR ligands caused inhibited the growth of these cell lines (Tontonoz et al., 1997).

Cyclooxygenase and Colorectal Cancer

Colorectal cancer represents the second leading cause of cancer related deaths in the United States. In the normal colonic mucosa, undifferentiated stem cells located at the base of invaginated crypts give rise to cells that migrate toward the lumen as they further differentiate into specialized enterocytes; these cells are subsequently removed by apoptosis, extrusion, or by phagocytes underlying the epithelial layer. Understanding the molecular events that govern this process has important implications since genetic and epigenetic perturbations of pathways that tightly regulate the differentiating enterocyte lie at the foundation for the development of colorectal neoplasms. One important pathway that appears to be an important regulator of colon epithelial cell function is the cyclooxygenase (COX) pathway. Below is a brief a discussion of COX signaling and the evidence that COX may be important in the development of colorectal cancer.

Cyclooxygenase and Prostaglandin Synthesis

COX catalyses a key step in the formation of prostaglandins (PGs) (Fig. 7) (DuBois et al., 1998; Herschman, 1996; Herschman et al., 1995; Smith et al., 2000). Prostaglandins are formed by the oxidative cyclization of the central five carbons within twenty carbon polyunsaturated fatty acids such as arachidonic acid. The key regulatory step in this process is the enzymatic conversion of the fatty acid to PGG_2 and PGH_2 by COX. PGH_2 is subsequently converted to one of several related PGs, including

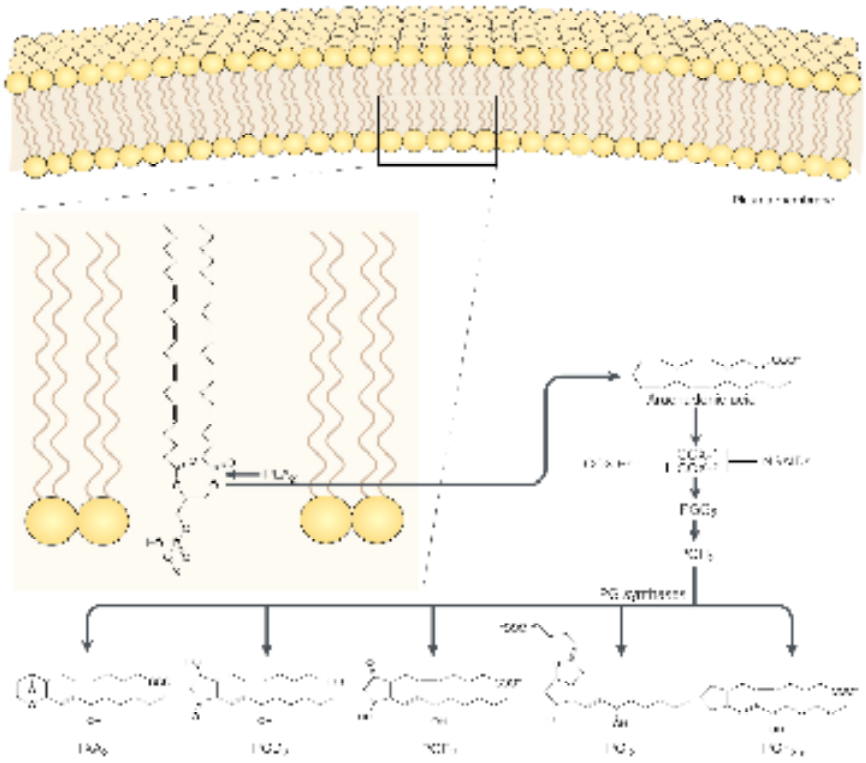

Figure 7. The cyclooxygenase signaling cascade – see text for details

PGE_2, PGD_2, $PGF_2\alpha$, PGI_2, and thromboxane A_2 (TxA_2), by the activity of specific PG synthases. Non-steroidal anti-inflammatory drugs (NSAIDs), a group of compounds that have been used for more than a century because of its analgesic and anti-inflammatory properties, were shown by Vane to inhibit PG biosynthesis due to direct inhibition of COX activity. PGs have important functions in almost every organ system and regulate such diverse physiological processes as immunity, reproduction, maintenance of vascular integrity and tone, nerve growth and development, and bone metabolism. PGs are synthesized in a broad range of tissue types and act as autocrine or paracrine mediators to signal changes within the immediate environment.

A Second Cyclooxygenase Enzyme

Until recently, only one isoform of COX (now known as COX-1) had been purified and cloned. However, in the late 1980's an inducible cyclooxygenase activity that was negatively regulated by glucocorticoids was discovered and postulated to be distinct from a constitutive COX activity (Raz et al., 1988) (Fu et al., 1990). Around the same time, two independent groups identified and cloned a second COX enzyme, COX-2, whose expression was highly induced in cells transformed with the oncogene *v-src* (Xie et al., 1991) or treated with phorbol esters (Kujubu et al., 1991). Subsequent research has suggested that COX-1 is responsible for "housekeeping" PG biosynthesis and is constitutively expressed in most tissues in the body. COX-2, on the other hand, is not normally expressed in most tissues but is induced by a wide spectrum of growth factors and pro-inflammatory cytokines in specific pathophysiological conditions (reviewed in (Smith et al., 2000))

Because COX-2 is highly induced at sites of inflammation, it was proposed that the anti-inflammatory and analgesic properties of traditional NSAIDs, which inhibit both COX-1 and COX-2, are largely due to their ability to inhibit COX-2 (Masferrer et al., 1994). In addition, one of the major side-effects of NSAIDs — erosion and ulceration of

the gastric mucosa — was thought to be due to their ability to inhibit COX-1 mediated PG production in the gastric epithelium. These studies eventually led to the design and synthesis of a new class of NSAIDs that specifically inhibit the COX-2 isoform (reviewed in (Marnett and Kalgutkar, 1999)). Currently available clinical evidence suggests that COX-2-specific inhibitors offer the therapeutic benefits of traditional NSAIDs without the associated toxicity (Emery et al., 1999; Laine et al., 1999). Two different COX-2 selective inhibitors, rofecoxib and celecoxib, are currently available for clinical use in the United States for the treatment of chronic arthritic conditions such as rheumatoid arthritis and osteoarthritis. How did the potential of COX-2 inhibition for the treatment and prevention of colorectal cancer become evident?

NSAIDs and Colorectal Cancer

One of the earliest hints that NSAIDs might be beneficial in the treatment of colon cancer came from a case report by Waddell and Loughry (Waddell and Loughry, 1983). They documented a patient whose rectal polyps disappeared while taking a combination of two commonly used NSAIDs — indomethacin and sulindac — for pain relief. Further clinical evidence that NSAIDs might be protective against colorectal cancer has come from large population-based studies. Both retrospective and prospective studies have suggested that chronic intake of aspirin or other NSAIDs over a 10–15 year period can lead to a 40–50% reduction in the relative risk of developing colon cancer (Giovannucci et al., 1995; Giovannucci et al., 1994; Thun et al., 1991) and reviewed in (DuBois et al., 1996). But the most convincing clinical evidence that NSAIDs can reduce the risk of colon cancer has come from a randomized, double-blind, placebo-controlled clinical trial in patients with FAP (Box 2) Patients with FAP have a germline mutation in one of the alleles of the tumor suppressor gene, adenomatous polyposis coli (APC) that causes them to develop adenomas throughout their gastrointestinal tract early in life (Kinzler et al., 1991). One or more of these polyps will

eventually develop into frank carcinoma unless the colon is removed surgically. Three different groups have documented the ability of sulindac to substantially reduce the size and number of colonic polyps in FAP patients, a trend that reversed when therapy was terminated (Labayle et al., 1991) (Nugent et al., 1993) (Giardiello et al., 1993). All of these studies used NSAIDs that inhibit both COX-1 and COX-2, but these results have now been repeated with the selective COX-2 inhibitor celecoxib. In this study, 400 mg of celecoxib given twice daily for 6 months reduced the number, size, and overall colorectal polyp burden in FAP patients by 32% compared with placebo(Steinbach et al., 2000).

Evidence for a Role for COX-2 in Colorectal Carcinogenesis

Although NSAIDs can have effects independent of their binding to the COX enzymes, a wealth of evidence has accumulated in the last decade to suggest that part of their anti-neoplastic activity is likely due to their ability to inhibit COX-2. The most direct evidence implicating COX-2 in colorectal tumorigenesis has come from genetic studies in mice. Oshima *et al.* determined intestinal polyp number in $Apc^{\Delta 716}$ mice (a mouse model similar to the APC^{Min} but in which there is a target truncation in the APC gene) in both wild-type and homozygous null COX-2 genetic backgrounds(Oshima et al., 1996). The number and size of polyps was reduced in the COX-2 null mice compared to COX-2 wild-type mice. Treatment of the $Apc^{\Delta 716}$ COX-2 wild-type mice with a novel COX-2 inhibitor or the NSAID sulindac also reduced polyp number.

There is also genetic evidence implicating a role for host COX-2 in carcinoma growth (Williams et al., 2000). The lewis lung carcinoma cell line grows more slowly when injected subcutaneously in the flank of $COX\text{-}2^{-/-}$ mice than in either the wild-type control or $COX\text{-}1^{-/-}$ mice. This last study suggests that COX-1 has a limited role in promoting cancer progression. Chulada *et al.* reached a different conclusion in

experiments demonstrating an equivalent reduction in tumor multiplicity in APC^{Min} mice null for either COX-1 or COX-2 (Chulada et al., 2000). They also showed that both COX-1 and COX-2 contribute to the total levels of PGE_2 in polyps from these mice. One interpretation of these data is that the total amount of PGs is the rate-limiting variable in polyp growth. It will be important to determine if a selective a COX-1 inhibitor can reduce polyposis in this model to the same degree as COX-2-specific inhibitors. In most studies with animal models, then, COX-2 inhibitors show equivalent or greater efficacy in polyp prevention compared with nonselective COX inhibitors. These data, coupled with the fact that COX-2 inhibitors offer a wider safer therapeutic window than traditional NSAIDs, suggests a limited potential for COX-1 selective or non-selective COX inhibitors in colorectal cancer prevention or treatment.

A study by Liu *et al*. is the first to demonstrate that over-expression of COX-2 is sufficient to induce cellular transformation (Liu et al., 2001). This group developed transgenic mice in which the murine mammary tumor virus promoter/enhancer controls COX-2 expression. Although virgin mice over-expressing COX-2 did not develop mammary tumors, multiparous mice showed significant increases in mammary gland carcinomas compared with age-matched controls. Recently, a similar study has reported that transgenic expression of COX-2 in basal keratinocytes results in epidermal hyperplasia and dysplasia, implying a direct link between COX-2 expression and the development of squamous cell carcinoma in the skin (Neufang et al., 2001).

How Does COX-2 Promote Tumor Development?

Because COX-2 has been localized to both tumor epithelial cells and adjacent stromal cells, COX-2-derived PGs may be acting on the malignant epithelial cells (a cell autonomous effect) or on the surrounding stroma (cell non-autonomous or "landscaping" effect (Kinzler and Vogelstein, 1998)) to promote tumor development. There is evidence to support both theories.

If COX-2 inhibitors reduce tumor growth solely though inhibition of COX-2-derived PGs acting on the stromal compartment, then one would predict that most colorectal cancer cell lines, irrespective of their COX-2 status, would be sensitive to therapy with COX-2 inhibitors. This does not appear to be the case: in the nude mouse xenograft model, tumor cells that express COX-2 appear to be more sensitive to treatment with selective COX-2 inhibitors (Sheng et al., 1997). Consistent with this, several studies suggest that forced expression of COX-2 in intestinal epithelial cell lines leads to changes in cellular pathways linked to carcinogenesis. For example, rat intestinal epithelial cells engineered to over-express COX-2 have elevated levels of the anti-apoptotic protein Bcl-2 and exhibit increased resistance to apoptosis induced by sodium butyrate (Tsujii and DuBois, 1995). In the Caco-2 cancer cell line, over-expression of COX-2 leads to an increase in cell migration and invasion that is associated with elevated levels of several members of the matrix metalloprotease (MMP) family (Tsujii et al., 1997). This same cell line also secretes higher levels of several angiogenic factors compared with vector-transfected control cells, and promotes the formation of endothelial cell tubes when co-cultured with human umbilical vein endothelial cells (Tsujii et al., 1998). In a colorectal cancer cell line that constitutively expresses COX-2, transfection with an antisense COX-2 cDNA reduces cellular proliferation, an effect that can be reversed by exogenous administration of PGD_2 derivatives (Chinery et al., 1999). The *in vivo* relevance of these findings has not yet been determined.

A series of reports have provided evidence that COX-2 can also promote tumorigenesis through direct actions on the stromal compartment. These studies have largely focused on the ability of COX-2-derived prostaglandins to stimulate tumor-associated angiogenesis. Using a model in which angiogenesis is assessed in sponge implants injected with various growth factors, Majima *et al.* were one of the first groups to demonstrate that COX-2 inhibitors could block neovascularization (Majima et al., 2000; Majima et al., 1997). COX-2 inhibitors also blocked the migration of human

microvascular endothelial cells and growth factor-induced corneal angiogenesis, effects that could be reconstituted with a TXA_2 agonist (Daniel et al., 1999). A related study reported strong COX-2 immunoreactivity in tumor neovasculature in human colon, breast, prostate and lung cancer biopsy tissue (Masferrer et al., 2000). In addition, corneal blood vessel formation in rats was potently suppressed by selective COX-2, but not COX-1, inhibitors. Jones *et al.* reported that both non-selective and COX-2 selective NSAIDs inhibit angiogenesis through direct actions on endothelial cells through both COX and non-COX mechanisms (Jones et al., 1999). Finally, a recent study provides genetic evidence that stroma-derived COX-2 can promote tumor growth by a landscaping mechanism (Williams et al., 2000). In this study, the growth of a lung cancer cell line was attenuated if engrafted on to COX-$2^{-/-}$ versus wild-type control mice. COX-2 expression within the stroma surrounding the tumor was localized primarily to fibroblasts, and cultured skin fibroblasts from the COX-$2^{-/-}$ mice exhibited defects in the basal secretion of several angiogenic growth factors. These experiments argue that COX-2 may modify tumor growth by limiting the ability of fibroblasts to support neovascularization within the microenvironment of a tumor.

Summary

PPAR□is a NHR that can potentially be regulated by COX metabolites. Furthermore, the receptor is expressed in the colon at levels nearly equivalent to that found in adipocytes (Fajas et al., 1997) and it is capable of regulating cell cycle pathways in fibroblast cells. Furthermore, although COX-2 derived prostanoids promote the development of colorectal tumors, the downstream PG receptors that are involved in this regulation are not known. Our original intent was to determine if COX metabolites are signaling via PPAR□during the development of colorectal cancer. In preliminary work we could find no evidence to indicate that such a signaling pathway existed. However, in the course of these initial experiments we noticed that activation of PPAR□in colorectal

cancer cells resulted in the inhibition of colorectal cancer cell growth. Based on these findings, our focus shifted to a study on the role of PPAR in the biology of colon epithelial cells.

CHAPTER II

MATERIAL AND METHODS

Cell Culture

The HCT 15, COLO 205, HCT 116, HT-29, CV-1, Cos7, and Caco-2 cell lines were purchased from ATCC. 293-EBNA cells were purchased from Invitrogen. The MOSER S (M-S) cell line was a gift from M. Brattain (University of Texas Health Sciences, San Antonio, TX). The FET cell line was a gift of W. Grady (Vanderbilt Unviersity, Nashville, TN) and the CBS cell line was a gift of H. Moses (Vanderbilt University, Nashviile, TN). The MIP 101 and Clone A cell lines were a gift from L. B. Chen (Dana Farber Cancer Institute, Boston, MA). The HCA-7 cell line was obtained from S. Kirkland (University of London, London, England).

All Cell lines were grown in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone), L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 □g/mL) in a 5% CO_2 atmosphere with constant humidity. However, for all experiments in which a PPAR or RXR ligand was added, cells were grown in the above media except regular 10% FBS was replaced with 10% charcoal stripped FBS (Hyclone).

Nuclear Receptor Ligands

All synthetic PPAR and RXR ligands were provided by Timothy Willson (GlaxoSmithKline, Research Triangle Park, NC) and dissolved in DMSO. The DMSO concentration of all experiments was kept constant at 0.1%. The following synthetic ligands were used in our studies to determine the specificity (*i.e.*, PPAR□dependent or independent) and selectivity (*i.e.*, PPAR subtype specific) of each induction or

repression: BRL49653 [thiazolidinedione PPARγ agonist(Lehmann et al., 1995)], GW7845 [tyrosine analogue PPARγ agonist(Suh et al., 1999)], GW9662 [irreversible PPARγ antagonist(Huang et al., 1999)], GW7647 [PPARα agonist (Brown et al., 2001)], GW1514[2] [PPARβ agonist (Oliver et al., 2001)] and LG100268 [RXR agonist]. Each compound was used at a concentration at which it is selective for the indicated isoform. 15-deoxy$\Delta^{12,14}$-PGJ_2 was purchased from Cayman Chemical.

Plasmids

For all plasmid constructions involving PCR reactions, Pfu Turbo Taq Polymerase (Stratagene) was used. Every construct generated was sequenced to ensure the absence of any unwanted mutations**.** hPPARα/pCMX and PPRE3-tk-luciferase (PPRE3-tk-luc) were obtained from Ron Evans (Salk Institute, La Jolla, CA), hPPARβ/pJ3 was obtained from G.A. Rodan (Merck, Sharp & Dohme Research Laboratories, West Point, PA), hPPARγ/pBKCMV was obtained from R. Mukherjee (Ligand Pharmaceuticals, San Diego, CA), GAL4-N-$coR_{1552-2453}$ and GAL4-$SMRT_{1252-1491}$ were obtained from L. Jameson (Northwestern Univ., Chicago, IL), and mRXR -α, -β, and -γ/pCMX were obtained from S.K. Dey (Univ. of Kansas, Kansas City, KS).

PPARγ plasmids: Full-length wild-type PPARγ was originally cloned into pBLUESCRIPT KS+ and pCB7. PPARγ/pBLUESCRIPT KS+ was used as a template to generate PPARγ K422Q using oligonucleotide-directed *in vitro* mutagenesis (Muta-Gene, BioRad). Both WT and K422Q PPARγ were cloned into pCDNA3.0 (Invitrogen) for use in transient transfection and EMSA experiments. VP16-PPARγ WT and VP16-PPARγ K422Q vectors were generated by inserting in-frame a fragment containing amino acid residues 154-474 of each receptor into the pACT vector (Promega), HA-tagged WT and K422Q PPARγ were generated by PCR using Pfu Turbo Taq Polymerase (Stratagene) and the proper non-tagged cDNA as a template. The 5' primer contained a *Xho*I site, the full-length HA epitope, and a partial region of PPARγ starting at codon 2. The 3' primer

contained a *Hpa*I site and a partial region of PPAR starting at codon 479 (stop codon). Each amplicon was digested and cloned into the *Xho*I/*Hpa*I site of the retroviral expression vector pMSCVpuro (Clontech). All plasmids were sequenced to avoid unwanted mutations.

TSC-22 plasmids: Full length TSC-22 was cloned using PCR from a cDNA library of M-S cells treated with 1 M BRL 49653 for 24 h. The product was cloned into the pPCR-Script cloning vector (Stratagene) and this plasmid was utilized as a template for all subsequent PCR reactions. For all primers, the 5' primer was designed to include a Not1 site and nucleotides encoding the full length HA or FLAG epitope. Each 3' primer was designed to include a BglII site. For HA TSC-22 WT or FLAG TSC-22 WT, the 5' primer included a partial region of TSC-22 starting at the second codon and the 3' primer included a partial region starting at codon 145 (stop codon). For HA TSC-22 DN, in which both Repressor Domains (RD) were deleted, the 5' primer included a partial region of TSC-22 beginning at codon 38. The 3' primer included a stop codon and a partial region starting at codon 102. For HA TSC-22 DNCON, both RD's were deleted and two highly conserved leucine residues in the leucine zipper domain were mutated to alanine (L91A and L97A). The 5' primer was identical to that used for HA TSC-22 DN. The 3' primer was also the same except for a mismatch at codons 91 and 97 such that the amplified product would contain the L91A and L97A mutations. See Fig. 5A for a schematic of all constructs generated. Each PCR product was digested with Not1/BglII and subcloned into the pcDNA3.1Zeo expression vector (Invitrogen).

RT-PCR for PPAR Subtypes

Total RNA was extracted with TRI REAGENT (Molecular Research Center Inc.) and first-strand complementary DNA (cDNA) was generated using 10 g of total RNA as template. Random Hexamer (3.3 Mol/L) was used to prime a standard reverse-

transcription (RT) reaction. Polymerase chain reaction (PCR) cocktail consisted of 10 mMol/L Tris-HCl, 50 mMol/L KCl, 2.0 mMol/L $MgCl_2$, 0.2 mMol/L each deoxynucleoside triphosphate, 2.5 U AmpliTaq Polymerase (Perkin-Elmer Corp) and 0.5 Mol/L of each of the following primers: PPAR 5'-GAGTTCATGCTTGTGAAGGATGC, 3'-CGATATCACTGGAGATCTCCGCC, human -actin 5'-TTGTAACCAACTGGGACGATATGG, 3'-GATCTTGATCTTCATGGTGCTAGG. Two l of cDNA template was added and thermocycling was performed according to the following profile: 94° C for 45 sec., 55° C for 45 sec., and 72° C for 2 min., repeated 40 times followed by a final extension at 72° C for 7 min. Analysis of amplicons was performed on a 2.0% TAE agarose gel containing 0.5 g/ml of ethidium bromide. A 100 bp ladder (Promega) was used as a size standard.

Antibodies

The following primary antibodies were used: monoclonal anti-PPAR (Santa Cruz; 1:500), rabbit polyclonal anti-PPAR [(Lim et al., 1999); 1:500], monoclonal anti-PPAR [P b11.80A obtained from GlaxoSmithKline (Su et al., 1998); 1:1000], monoclonal anti-HA antibody clone HA.11 (1:1000; Babco), and monoclonal anti-FLAG M5 (1:500; Sigma). The following secondary antibodies were used: HRP-donkey anti-rabbit (1:50,000) and HRP-donkey anti-mouse (1:50,000) (The Jackon Laboratory).

Western Blot Analysis

Exponentially growing cells were harvested in ice cold 1 X phosphate-buffered saline (1 X PBS) and cell pellets were lysed in RIPA buffer. Centrifuged lysates (50 g) from each cell line were fractionated on a 10% or 4-20% gradient SDS-polyacrylamide gel and electrophoretically transferred to a polyvinlylidene difluoride membrane (NEN). Membranes were blocked for 1 hour at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% powdered milk. The primary antibody was then added and incubated at room temperature for 2 h or overnight at 4°. This was

followed by incubation with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. Detection of immunoreactive polypeptides was accomplished using an enhanced chemiluminescence system (Amersham).

Detection of PPAR protein by Immunoprecipitation/Western Blot

The following protocol was used to detect PPAR protein in the studies described in Chapter III. Whole cell lysates were prepared in RIPA lysis buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, and 60 g/ml Aprotinin). PPARg was immunoprecipitated from lysates by incubation with 1 g affinity purified anti-peptide antibody raised against amino acids 2-20 of PPAR (Santa Cruz) in conjunction with 5 ml protein A conjugated agarose beads (Boehringer Mannheim). Immunoprecipitates were washed three times with RIPA lysis buffer, and eluted overnight with 50 l (200 g/ml) of the peptide from which the antibody was raised. Eluates were heat denatured in 4% SDS-10% -mercaptoethanol, fractionated by polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to a polyvinylidene difluoride membrane (NEN). Membranes were blocked for 1 hr at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% powdered milk (BLOTTO), and then incubated with the same antibody.

Transient Transfections

The following is a general protocol that was used for transient transfection of plasmids into various cell lines. Typical DNA amounts transfected were 1.2 g/well in 24 well plates, 3-4 g/well in 6 well plates, and 8 g/10 cm plate. The transfection reagents utilized were FUGENE 6 (Roche Molecular Bioscience) for transfections described in Chapters IV-VI and Cellfectin (Life Technologies) for transfections described in Chapter III. In each case, a lipid:DNA ratio of 3.5:1 was used. All

transfection mixes were initially made in OPTI-MEM media (Life Technologies). In general, transfections were replaced with complete media after 4-6 h.

Luciferase Assays

For luciferase assays, cell lines were plated at a density of 5.0-7.5 X 10^5/well in 24 well plates. M-S cells, HCT-15, and HCT-15-G25 cell lines were transfected with 0.66 μg/ml PPRE3-tk-luc, 0.66 μg/ml pRL-TK (Promega), and 0.66μg/ml of pCNDA3 (Invitrogen). The transfection mix was replaced with complete media containing either vehicle (0.1% DMSO) or the indicated nuclear receptor ligand. CV-1 cells were transfected with a mix containing 0.66 μg/ml PPRE3-tk-luc, 0.010 μg/ml pRL-SV40 (Promega), and 0.66 μg/ml of pCNDA3.0/WT PPARγ or pCDNA3.0/K422Q PPARγ HCT 15-pMSCV, HCT-15 PPARγ WT, or HCT-15 PPARγ K422Q cells lines were transfected with 0.66μg/ml PPRE3-tk-luc, 0.010μg/ml pRL-SV40, and 0.66μg/ml of pCDNA3.0. After 24-36 h, cells were harvested in 1 X luciferase lysis buffer. Relative light units from firefly luciferase activity were determined using a luminomoter (MGM Instruments) and normalized to the relative light units from renilla luciferase using the Dual Luciferase kit (Promega).

Cell Growth Measurements

The following is a general protocol for cell growth measurement used for all the cell lines this study. Briefly, cells were plated at a density of 5 X 10^4/well in 6 well plates and replaced the next day with DMEM media containing 10% charcoal stripped FBS and either vehicle, the indicated nuclear receptor ligand, or TGF-β1. Cells were exposed to fresh media and compound every 48 h. Cells were counted at the indicated times using a coulter counter. Each experiment was done in triplicate.

Anchorage Independent Growth Assay

HCT-15 and independent clones were plated at 3 X 10^4 cells/35 mm dish in DMEM supplemented with 10% FBS, 0.33% (w/v) agar, and either DMSO or the indicated PPAR agonist. After 7 days of growth the total number of colonies were determined using an Omnicon image analyzer.

Tumor Growth in Athymic Mice

Athymic mice (Harlan Sprague Dawley, Inc.) were injected subcutaneously in the dorsal flanks with $5X10^6$ cells of parental HCT 15 cells or HCT-15 cells expressing wild-type PPAR in a volume of 0.10 mL of 1XPBS. Dosing was begun 7-10 days post-injection for each cell line when the mean tumor volumes were approximately 75 mm^3. Mice were then orally gavaged 5 times/week with either vehicle (0.5% methylcellulose in 0.05 N HCl)) or 10 mg/kg of rosiglitazone (in a total volume of 0.10 mL per mouse). Rosiglitazone was formulated daily by first dissolving the compound in 0.1N HCL that had been pre-warmed to 40°C followed by the addition of an equal volume of 1% methylcellulose. The size of each tumor was determined by direct measurement of tumor dimensions. The volume was calculated according to the equation (V=[L x W2] x 0.5), where V = volume, L = length, and W = width.

Flow Cytometry

HCT-15, HCT-15-G25, HCT 15-pMSCV, HCT-15 PPAR WT, or HCT-15 PPAR K422Q cell lines were treated with 0.1% DMSO, or the indicated receptor ligand for 36 h. The DNA content of nuclei was determined by staining nuclear DNA with propidium iodide (50 g/mL) followed by measuring the relative DNA content of nuclei using a Facsort fluorescence-activated sorter (Becton Dickinson). The proportion of nuclei in each phase of the cell cycle was determined using the MODFIT DNA analysis software (Becton-Dickinson).

cDNA Microarray Screening

Human Named Genes GENEFILTERS Release I blots (Research Genetics) were probed with ^{33}PdCTP labeled cDNA synthesized from RNA isolated from exponentially growing M-S cells that had been exposed for 12h, 24 h or 6 days to 0.1% DMSO or 1 μM of the PPARγ ligand BRL49653. Alternatively, cDNA was synthesized from RNA isolated from exponentially growing M-S cells that had been exposed to 0.1% 4 mM HCl or 2 ng/ml TGFβ1. The cDNA was synthesized using Reverse Transcriptase(RT) primed with oligo(dT) primers. For the 6 day treatment, fresh media and compound was added every 48 h. Membranes were hybridized and washed according to manufacturer instructions. The blots were imaged using the Cyclone Storage Phosphor System (Hewlitt-Packard) and imported into the Pathways Software (Research Genetics) to identify differences in the intensity of cDNA spots between different treatments.

Oligonucleotide Microarray Screening

Total RNA was isolated from M-S cells exposed to 0.1% DMSO or 1 μM of the PPARγ ligand GW7845 for 24 h. Alternatively, total RNA was isolated from HCT 15-PPARγ WT cells exposed to 0.1% DMSO or 2.0 μM of the PPARγ ligand rosiglitazone for 24 h. The RNA was subsequently treated with DNAse I and cleaned up using the RNeasy mini kit (Qiagen). Samples were sent to the DNA Facility at the University of Iowa (Iowa City, IA), where the RNA samples were converted to biotinylated cRNA and probed to the Affymetrix Human GeneFL GeneChip arrays according to manufacturer's directions. Statistical analysis was done on the M-S cell treatment experiment (see Chapter IV) to try and estimate the total number of transcripts changed due to exposure to a PPARγ ligand. Using the difference call decision matrix algorithm, the Affymetrix chip

software identified a total of 89 genes (1.6% of transcripts) that were induced or repressed 2.5X or greater after treatment with the PPAR□ligand GW7845. Fourteen out of these 89 were tested for
confirmation by Northern blot hybridization, and 9/14 (60%) were confirmed as true positives. Using the confidence interval equation:

$$\hat{p} \pm 1.96\sqrt{p(1-p)/n} - \frac{1}{2n}$$

a 95% confidence interval was obtained for the estimate of "true positives" found in the sample of 14 genes based on Northern Blot. This 95% confidence interval can be used to estimate the interval for which 95% of the time will cover true value of proportion of true positives. This value can then be used as an estimate for the proportion of true positives from the original 89 genes identified by the Affymetrix software as increased or decreased from the control sample

Northern Hybridization Analysis

Total RNA for Northern blots was isolated using the TRI REAGENT (Molecular Research Center). Northern blot analysis was performed as described previously (Gupta et al., 2000). Exponentially growing M-S cells were treated with the indicated PPAR or RXR ligand for 24 h or 6 days. HCT 15-pMSCV, HCT 15-PPAR□WT, and HCT 15-PPAR□K422Q cells were treated with either 0.1% DMSO or 2.0 □M rosiglitazone for 24 h. Total RNA from each sample (20 □g) was fractionated on a 1.2% agarose-formaldehyde gel and transferred to a Hybond-NX nylon membrane (Pharmacia). Filters were pre-hybridized for 4 hr at 42ºC in Ultrahyb (Ambion). Hybridization was conducted in the same buffer in the presence of ^{32}P radiolabeled partial cDNA fragment of the indicated gene. In general, blots were washed 4 X 15 min at 50ºC in 2X SSC, 0.1% SDS and once for 30 min in 1X SSC, 0.1% SDS. Membranes were then exposed to

a phosphorimager screen and images were analyzed using a Cyclone Storage Phosphor System and Optiquant Software (Hewlitt-Packard). In Chapter IV, each membrane generated was also hybridized with a ^{32P}dCTP labeled probe for 1B15/cyclophilin (Medhurst et al., 2000) to normalize for differences in RNA loading.

cDNA Probes for Northern Blots

Partial cDNA fragments for adipophilin, Reg IA, keratin 20, BGP, and NCA were generated by PCR with M13 forward and reverse primers using a sequence validated human IMAGE cDNA clone (Research Genetics) of each gene as a template. Partial cDNA fragments for L-FABP, Gob-4, and TSC-22 were generated using RT-PCR and gene specific primers corresponding to base pairs (each from the translational start site) 56-375 for L-FABP, 283-550 for Gob-4, and 1-425 for TSC-22. The template for these PCR reactions was a random primed cDNA library of MOSER S cells treated with either 0.1% DMSO or 1 μM rosiglitazone. The L-FABP and Gob-4 PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and the TSC-22 product was cloned into pPCR-Script (Stratagene). All plasmids were sequenced to confirm gene identity. A partial fragment of CEA was obtained by restriction digest of full-length CEA cloned into pBLUESCRIPT (SK+) obtained from C. Stanners (McGill Cancer Center, Montreal Canada). The mouse Mox-2 cDNA was obtained from C. Wright (Vanderbilt University, Nashville, TN).

Cell Aggregation Assay

The protocol used was identical to the methods reported by Yan *et al*(Yan et al., 1997). Briefly, exponentially growing M-S cells were treated with 0.1% DMSO or the indicated ligand for 4 days, with fresh media and compound added after the first 48 h. Cells were then harvested by gentle trypsinization with 0.025% trypsin and 0.01%

EDTA. After resuspending the cells in 10 mM EDTA in 1 X PBS (at a density of 2 X 10^6/ml), pelleted cells were resuspended in 5 mM EDTA, 1 X PBS. This was followed by disaggregation that was achieved by repeated forceful passages through a pasteur pipette to generate single-cell suspensions. Aliquots were then transferred to a 15 X 100-mm test tube and shaken for the indicated time at room temperature (tubes were placed at a 45° angle). A sample was taken and the total number of cells and the number of single cells for each sample were counted using a hemacytometer. Each experiment was done in triplicate. For the antibody blocking experiments, Fab' fragments from normal rabbit IgG (Santa Cruz) or a rabbit polyclonal anti-CEA antibody (CEA Ab-2, NeoMarkers) were isolated using papain digestion followed by incubation with protein A-Agarose (Santa Cruz) to remove both undigested antibody and Fc fragments. The anti-CEA antibody reacts to CEA and CEA like proteins including BGP and NCA. Fab' fragments at a concentration of 1 mg/ml were added to the aggregation experiments when indicated.

PPAR Gene Mutation Detection

Mutations in the PPAR gene in the COLO 205, MIP101, and Clone A cell lines were detected using a combination of denaturing gradient gel electrophoresis (DGGE) and direct sequencing as described previously (Sarraf et al., 1999; Zhou et al., 2000). PPAR mutations in the HCT 15, MOSER S, HT-29, HCT 116, and HCA-7 cell lines were detected by automated dideoxy sequence analysis of PCR products that span the coding region of PPAR 1 using primers sets described previously (Yen et al., 1997).

Electro Mobility Shift Assay (EMSA)

EMSA's were done based on methods reported by Schulman *et al.* (Schulman et al., 1998) PPAR and RXR receptors were synthesized using a T7 Quick TNT *in vitro* Transcription/Translation Kit (Promega). 1.0 l of the PPAR receptor and either 0.10, 0.50, 0.75 or 1.0 l of RXR were added to a final reaction buffer volume of 20 l that

contained 1X binding buffer (20 mM HEPES [pH 7.5], 75 mM KCl, 2.0 mM dithiothreitol [DTT], 0.1% Nonidet P-40 [NP-40], 7.5% glycerol), 2.0 μg of poly(dI-dC), and 0.02 pmol of an ^{32}P-labeled oligonucleotide containing a PPRE derived from the acyl-coenzyme A (acyl-CoA) oxidase promoter (GTCGACAGGGGACC AGGACA A AGGTCA CGTTCGGGAGT). After 20 min. of incubation, the reactions were resolved on 5% nondenaturing acrylamide gels.

Mammalian Two-Hybrid

293-EBNA cells were transiently transfected with 0.33 μg/ml of pG5luc, 0.010 μg/ml of pRL-SV40, and the indicated combinations of either pVP16 (pACT; Promega), pGAL4 (pBind; Promega), VP16-PPARγWT, VP16-PPARγK422Q, GAL4-SMRT$_{1252-1491}$, or GAL4-N-coR$_{1552-2453}$ (each at 0.33 μg/ml) using Opti-MEM and FUGENE 6 at a lipid:DNA ratio of 3:1. Transfections were replaced with complete media containing 0.1% DMSO or 1.0 μM rosiglitazone for 48 h and measured for luciferase activity as described above.

Generation of Stable Cell Lines Using Retroviral Infection

Phoenix-Ampho cells [purchased from ATCC with prior approval of G. Nolan (Stanford University, Palo Alto, CA)] were transiently transfected with pMSCVpuro, pMSCV/HA-PPARγWT, and pMSCV/HA-PPARγK422Q using FUGENE 6 at a lipid:DNA ratio of 3.5:1. Approximately 72 h post-transfection, viral supernatants were collected, filtered, supplemented with 2 μg/ml of polybrene (Sigma) and used to infect exponentially growing HCT 15 cells. After 48 h, HCT 15 cells were split 1:5 into media containing 4 μg/ml puromycin (Sigma) to select for infected cells. After selection, all stable cell lines were grown in media containing 2 μg/ml puromycin prior to any experiments.

Generation of Stable Cell Lines Using Plasmid Transfection

HCT-15-G25: HCT-15 cells were transfected with 3 □g/ml pCB7-PPAR□mixed with 20 □g Cellfectin. Twenty-four hours following transfection cells were plated into 100 mm tissue cultures plates with DMEM containing 10% FBS and 300U/ml hygromycin B. Colonies from individual cells were selected which contained functional PPAR□activity which was assayed for by transient transfections with PPRE3-tk-luciferase and induction with BRL 49653. No PPAR□induction can be detected in either parental HCT-15 cells or cells transfected with the empty expression vector.

M-S TSC-22 Cell Lines: Four different vectors were utilized to generate eight unique pools of stable transfected cells: 1) pcDNA3.1Zeo (pools designated as M-S VECTOR clones A and B) 2) HA TSC-22WT/pcDNA3.1Zeo (M-S TSC WT clones A and B) 3) HA TSC-22dn/pCDNA3.1Zeo (M-S TSC DN clones A and B) and 4) HA TSC-22dncon/pcDNA3.1Zeo (M-S TSC DNCON clones A and B). *Transfection* - M-S Cells were transfected with the appropriate vector using FUGENE 6 at a lipid:DNA ratio of 3:1 and a DNA concentration of 5 □g/ml in OptiMEM media. After 6 h of transfection, normal media was added back and the cells were allowed to grow for another 72 h. *Selection* - Cells were then split into media containing 200 □g/ml zeocin (Invitrogen) and selected for 2 weeks. Resistant clones were combined into two unique pools and expanded. Zeocin was maintained in the media prior to all experiments at a dose of 100 □g/ml.

Immunoprecipitations

Anti-HA affinity matrix (100 □l; Roche Molecular Biosciences) was added to 500 □g of whole cell lysate from cos7 cells transfected with the indicated TSC-22 construct in NET-N Buffer (150 mM NaCl, 1 mM EDTA, 20 mM TrisHCl, pH 8.0, 0.5% NP-40, and 10% glycerol) and incubated at 4ºC for 4 h. Beads were then washed 3X with NET-N

Buffer. Protein were eluted by the addition of 1X SDS-loading buffer followed by a 5 min incubation at 100ºC.

In Situ Hybridization

In situ hybridization was performed as described previously (Lim et al., 1999). Sense or antisense ^{35}S-labeled cRNA probes were generated from human TSC-22. The probes had specific activities at 2 X 10^9 disintegration's per minute (dpm)/□g. Sections hybridized with the sense probes did not exhibit any positive autoradiographic signals and served as negative controls.

CHAPTER III

ACTIVATION OF PPARδ INHIBITS COLORECTAL CANCER CELL GROWTH

Introduction

Prior studies on PPARδ in the gastrointestinal tract have been limited to expression analysis. In the colon, PPARδ mRNA is expressed at levels nearly equivalent to that found in adipocytes (Fajas et al., 1997). Immunohistochemistry and *in situ* hybridzation data have suggested that PPARδ is primarily localized to epithelial cells in the colon. Within the epithelium, PPARδ is predominantly expressed in the post-mitotic, differentiated colonocytes facing the lumen (Lefebvre et al., 1999; Mansen et al., 1996). In order to determine the biological function(s) of PPARδ in the colon and what role the receptor may play in colorectal carcinogenesis, we examined the expression of PPARδ in a panel of colorectal cancer cell lines. We further determined whether activation of the receptor with a high-affinity, PPARδ selective agonist had any effects on the growth of colorectal cancer cell lines.

Results

PPARδ expression and transcriptional activity in a panel of human colorectal cancer cell lines

We selected four colon cancer cell lines (HCA-7, HCT-15, HCT-116, Caco-2) to assess PPARδ expression and endogenous activity. PPARδ mRNA (Fig. 8A) and protein (Fig. 8B) are present in all four of these cell lines. To determine if the PPARδ receptor is functional as a transcriptional activator we performed transient transfection assays in all four cell lines using a PPRE3-tk-luc reporter vector that contains a luciferase cDNA downstream of three tandem repeats of the PPAR response element (PPRE) from the

acyl-coA oxidase gene (Kliewer et al., 1994). Treatment of the transfected cells with a PPARγ-selective agonist (rosiglitazone) resulted in ~2.5-fold increase in luciferase activity in HCA-7, HCT-116, and Caco-2 cells, but no transactivation was observed in the HCT-15 cells (Fig 8C). The lack of transactivation in HCT-15 cells was not due to decreased transfection efficiency since activity from the control vector (SV40-Renilla) was equivalent in all four cell lines (data not shown). Furthermore, other components of the PPARγ signaling pathway (i.e. RXR) are intact in HCT-15 cells, since transient transfection with PPARγ cDNA results in expression of PPARγ protein which is functional in these cells (data not shown). These results suggested that endogenous HCT-15 PPARγ may have some defect that results in loss of transcriptional activation.

Activation of PPARγ inhibits human colorectal cancer cell growth *in vitro*

To evaluate the potential role of PPARγ in the biology of colon cancer cells we over-expressed PPARγ in HCT-15 cells since this cell line did not appear to contain a functional receptor. For this purpose, HCT-15 cells were stably transfected with a PPARγ expression vector and positive clones were selected by measuring PPARγ

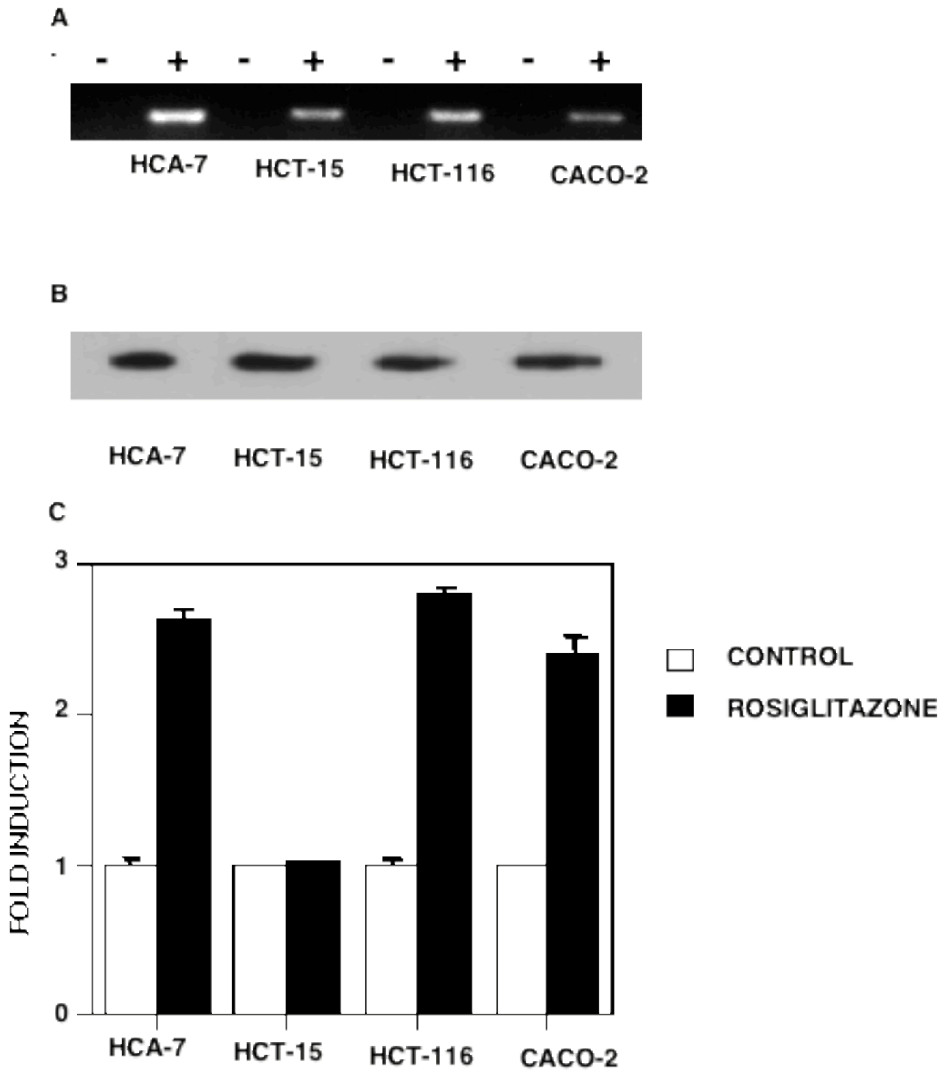

Figure 8. PPAR□is expressed and functionally active in colon cancer cells. (A) PPAR□is expressed at the mRNA level in colon cancer cells. Total RNA (10 □g) from either HCA-7, HCT-15, HCT-116, or Caco-2 cells was subjected to a reverse transcription reaction either with (+) or without (-) reverse transcriptase (RT). An aliquot (2 □l) from each reaction was subjected to 40 cycles of PCR. Amplification products were resolved in a 2% agarose gel. We confirmed the identity of the amplicon by restriction mapping and DNA sequence analysis. (B) PPAR□protein is present in colon cancer cells. Whole cell extracts (500 □g) were immunoprecipitated with a PPAR□peptide-specific affinity-purified antibody (1 □g) (Santa Cruz) for 4 hr. Immunoprecipitates were eluted with 50 □l PPAR□peptide (200 □g/ml) overnight. Eluted proteins were resolved on a 10% SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted with the same antibody. (C) Cells were transiently transfected with PPRE3-tk-luc (1 □g) and pRL-SV40 (0.01 □g) by lipid transfection. Transfections were treated with rosiglitazone (10 □M) or DMSO (control) for 12 hr. Cells were harvested and the Dual-luciferase assay was performed as described in the methods section. The means of normalized relative light units from 3 independent transfections are presented. *Error bars* = S.E.M.

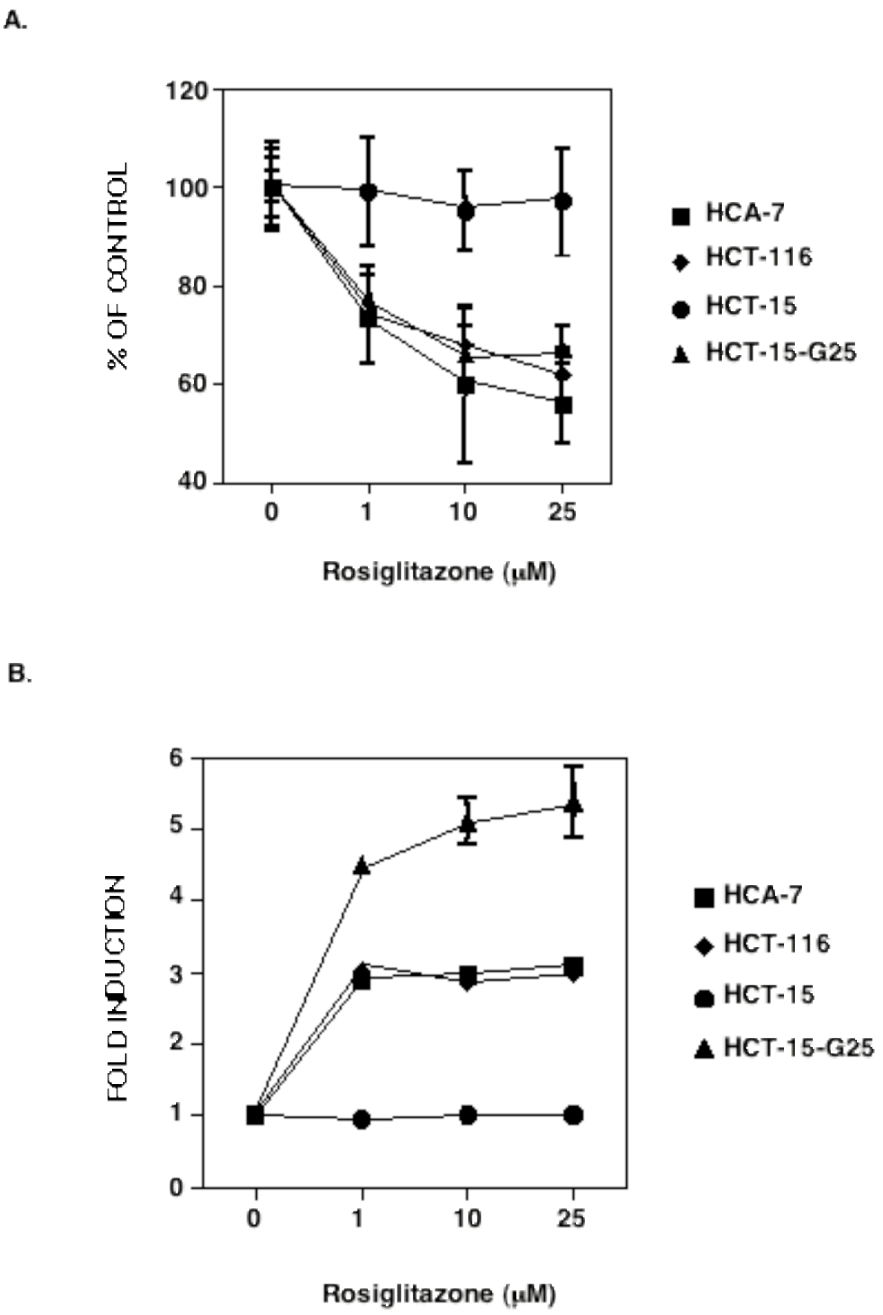

Figure 9. Rosiglitazone inhibits cell growth of colon cancer cells *in vitro*. (A) Cells (1 X 10^5 / well) were grown in the continuous presence of either 0, 1, 10, or 25 □M rosiglitazone with equal amounts of DMSO. After 5 days of growth, the number of cells/well was determined. The values shown represent the means of 3 independent wells normalized to the DMSO control, set at 100%. The experiment was carried out twice with similar results. (B) Cells were transiently transfected with PPRE3-tk-luciferase (1 □g), and pRL-SV40 (0.01 □g) by lipid transfection. Transfected cells were treated with either 0,1, 10, or 25 □M rosiglitazone for 12 hr, harvested and the Dual-luciferase assay was performed. *Error bars* = S.E.M.

transactivation of PPRE3-tk-luciferase. The clone with the highest level of PPAR functional activity (HCT-15-G25) was compared to the parental HCT-15 cells.

We first determined if the growth of cells that have functional endogenous PPAR receptors could be affected by treatment with a PPAR agonist. HCA-7, HCT-116, HCT-15, and HCT-15-G25 cells were treated with increasing doses of rosiglitazone. After 5 days of treatment, cell number was determined and we found that rosiglitazone (1 M) inhibited the growth of HCA-7, HCT-116, and HCT-15-G25 cells by approximately 25% (Fig. 9A). Concentrations of 10 and 25 M rosiglitazone resulted in a further inhibition of cell growth but this difference did not reach statistical significance. We also observed no effect on the growth of HCT-15 cells, even at a concentration of 25 M rosiglitazone. We next determined the effect of rosiglitazone (1-25 M) on the activation of PPAR in all four cell lines by transient transfection of the PPRE3-tk-luciferase vector. Importantly, in conjunction with its ability to inhibit cell growth at 1 M, rosiglitazone fully activates PPAR (Fig. 9B) at this concentration as well. Furthermore, we could not detect any PPAR activation in HCT-15 cells, even at 25 M rosiglitazone. We also sought to determine if these anti-proliferative effects could be replicated in the presence of putative endogenous PPAR ligand 15-deoxy ^{12,14}-J_2. The addition of this agonist to HCT-15 clones overexpressing functional PPAR results in a decrease in proliferation as measured by ^{3}H-thyminine incorporation when compared to the parental HCT-15 cell line. This ligand could not be used in the long-term assays utilized in this study due to toxicity to the cells (data not shown).

Finally, we determined if the growth inhibitory effects of PPAR ligands could be replicated in an anchorage independent growth assay since this represents a more accurate assessment of tumorigenicity. The growth of HCT-15-G25 cells on soft agar was inhibited by 50% in the presence of 10 M rosiglitazone compared to control cells (Fig. 10). Treatment of parental HCT-15 cells with rosiglitazone had no effect on anchorage independent growth, suggesting that specific activation of PPAR could inhibit

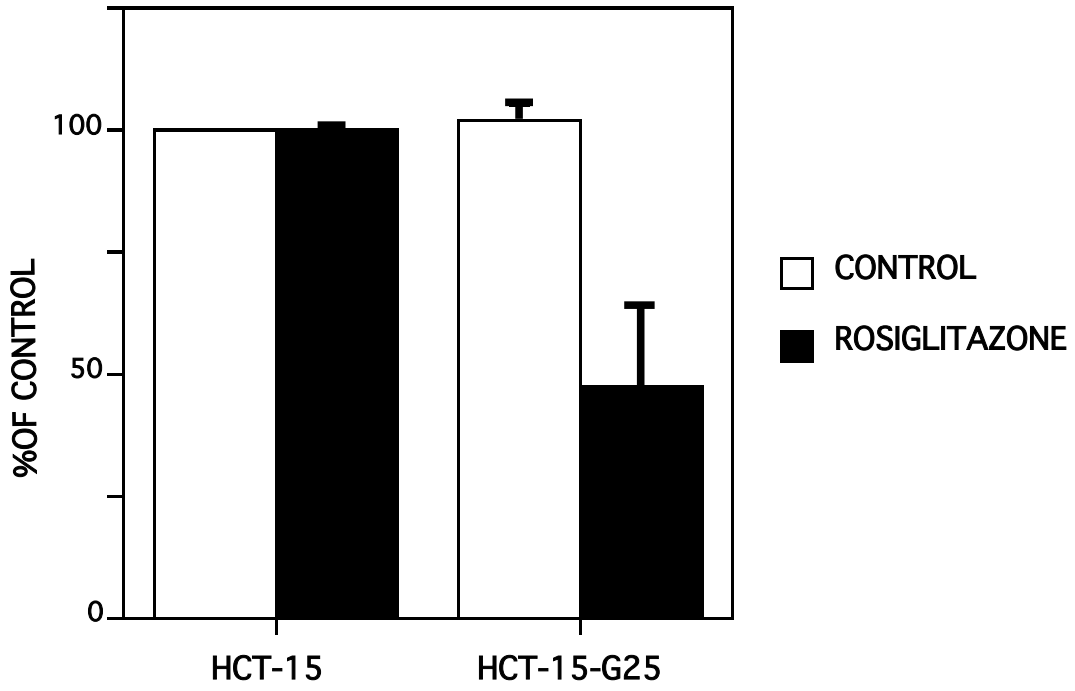

Figure 10. The PPAR□selective agonist rosiglitazone inhibits anchorage independent growth of cells that over-express functional PPAR□ 3 X 10^4 HCT-15 or HCT-15-G25 cells were plated into media containing 0.4% (w/v) agar, supplemented with either DMSO (control) or rosiglitazone (10 □M). After 7 days growth the number of colonies were determined. The values shown are the means of three independent experiments performed in triplicate. *Error bars* = S.E.M.

the growth of colon cancer cells in soft agar. As an additional control for this experiment, we also determined the effects of the PPAR activator WY-14,653. This compound is selective for the PPAR isoform at a concentration of 10 M while at higher concentrations (100 M) it will also activate PPAR. In agreement with this concentration dependent selectivity, inhibition of anchorage independent growth of the HCT-15G25 cell line was only observed in the presence of 100 m WY-14,653 and not at any of the lower concentrations in which WY-14,653 selectively activates PPAR. Furthermore, similar effects were seen in three independent HCT-15 clones that express ectopic PPAR.

Activation of PPAR inhibits human colorectal cancer cell growth *in vivo*

Finally, identical results were obtained *in vivo* using a nude mouse xenograft model of tumor growth. Athymic mice bearing tumors consisting of HCT 15 or HCT 15-G25 cells were treated by oral gavage with vehicle or 10 mg/kg body weight of rosiglitazone. This amount of rosiglitazone was chosen based on studies demonstrating this to be within the dose range of drug necessary to normalize glucose levels in mouse models of diabetes (Willson et al., 1996). A significant reduction in tumor volume was only seen in HCT 15 cells transfected with functional PPAR (Fig. 11). Several studies have identified PPARas a negative regulator of endothelial cell growth (and hence postulated to be anti-angiogenic *in vivo*), suggesting that some of the anti-neoplastic effects of the receptor could be through direct actions on the stromal compartment of a tumor (Bishop-Bailey and Hla, 1999; Murata et al., 2000; Xin et al., 1999). However, because we only see effective tumor reduction in carcinoma cells that express functional PPAR, our data would suggest that the anti-tumor activity of PPARis mainly a cell autonomous effect.

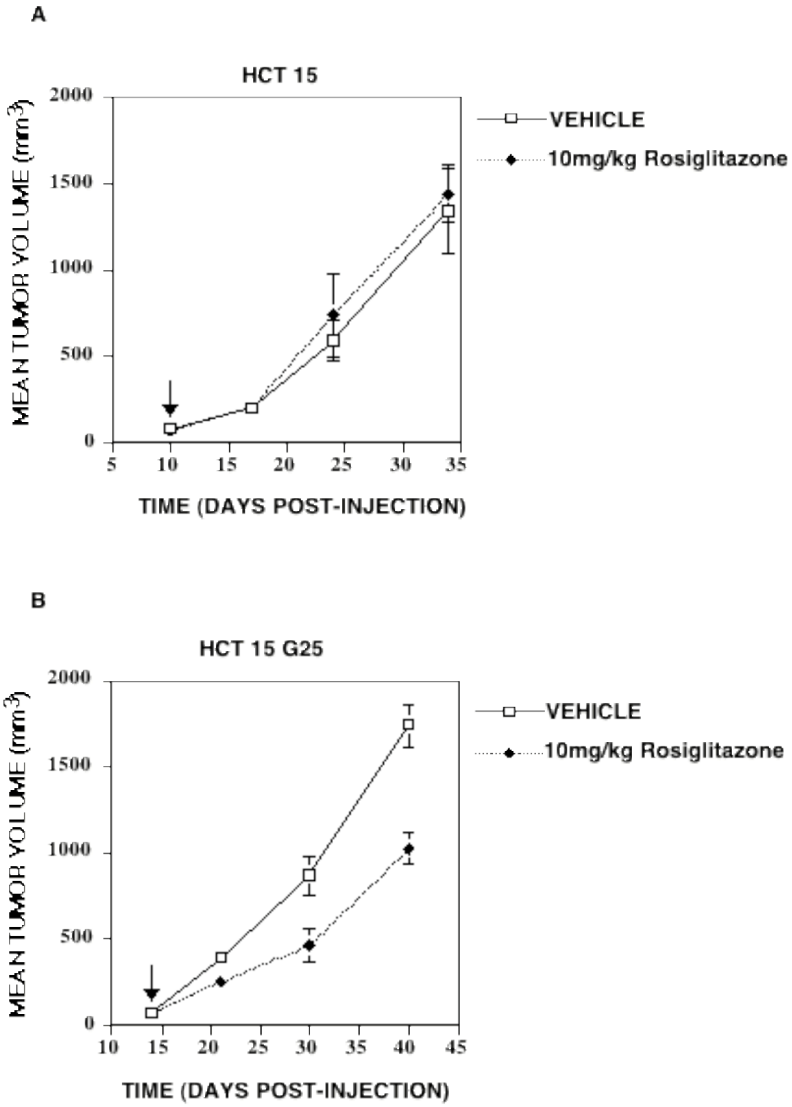

Figure 11. Rosiglitazone reduces the volume of tumors grown *in vivo* from HCT 15 cells expressing functional PPAR. Athymic mice were injected subcutaneously in the dorsal flanks with $5X10^6$ cells of HCT 15 or HCT 15-G25 cells in a total volume of 0.10 mL in 1XPBS. Treatment was begun 10-15 days post-injection for each cell line (indicated by arrow) when the mean tumor volume for cell line was approximately 75 mm^3. Mice were then orally gavaged 5 times/week with either vehicle (0.5% methylcellulose in 0.05 N HCl)) or 10 mg/kg of rosiglitazone (in a total volume of 0.10 mL per mouse). The size of each tumor was determined by direct measurement of tumor dimensions. The volume was calculated according to the equation ($V=[L \times W^2] \times 0.5$), where V = volume, L = length, and W = width. *Error bars* = S.E.M.

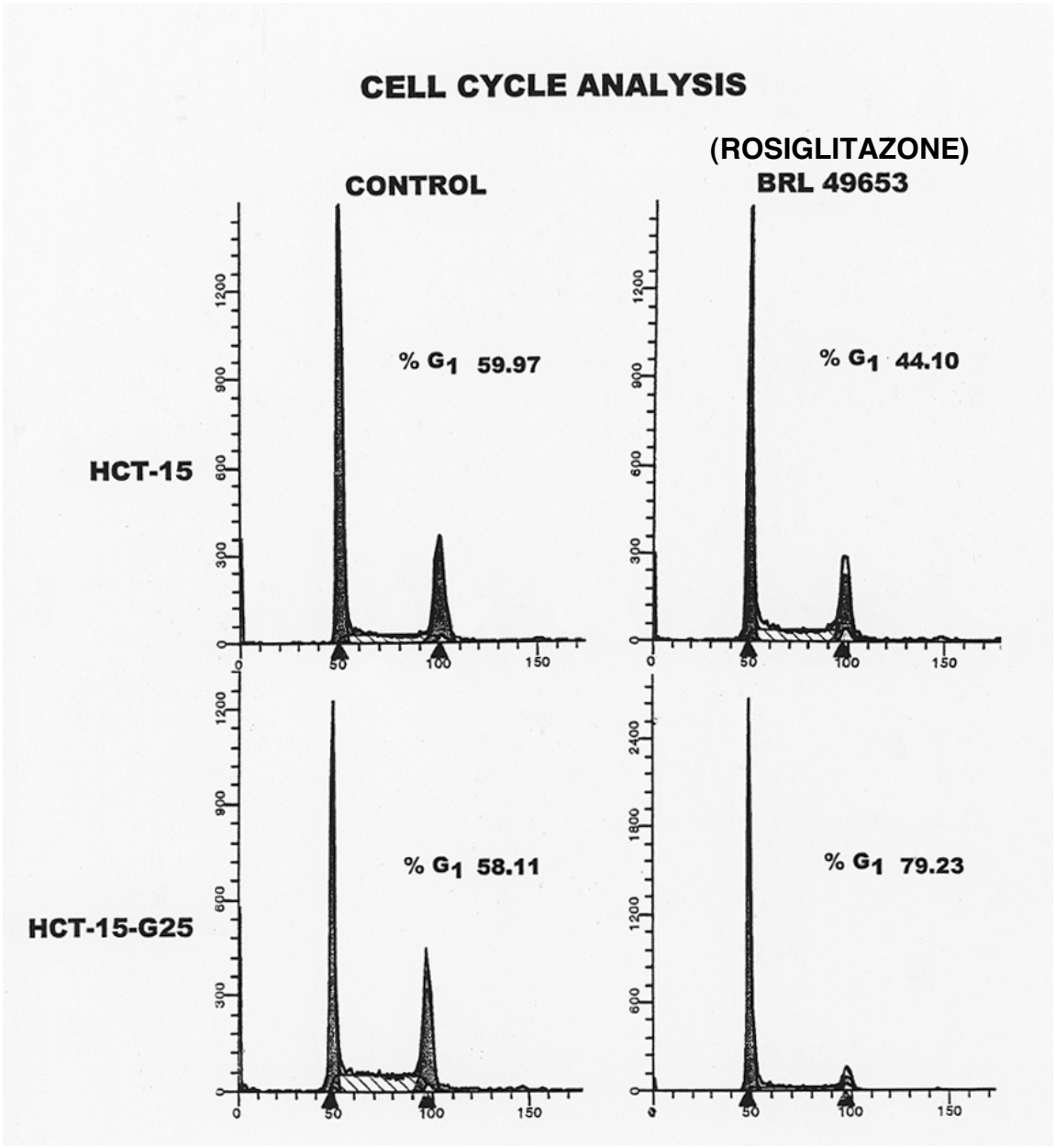

Figure 12. PPAR activation induces G_1 cell cycle arrest. Either HCT-15 or HCT-15-G25 cells (0.25 X 10^6) were plated into 6 well plates and allowed to grow to 30% confluence. Cells were then treated with either DMSO or rosiglitazone (10 M) for 36 h in serum free media. Cells were harvested, stained with propidium iodide, and analyzed for DNA content by FACS analysis. The values presented represent the number of cells in the G_1 phase of the cell cycle as a percentage of total cells.

Activation of PPARγ delays cell cycle progression

Activation of PPARγ could be inhibiting cell growth by inducing apoptosis or by inducing a delay in cell cycle progression. We found no evidence to indicate that rosiglitazone was inducing apoptosis in any of the cell lines examined in this study. Todetermine the effects of rosiglitazone treatment on cell cycle progression, either HCT-15 or HCT-15-G25 cells were treated with 10 μM rosiglitazone for 36 h. The cells were then harvested and stained with propidium iodide and the DNA content was determined by flow cytometry. Treatment of HCT-15-G25 cells with rosiglitazone resulted in a significant increase in the number of cells in the G_1 phase of the cell cycle (58.11 vs. 79.23, respectively) (Fig. 12). No increase in the percentage of cells in G_1 was observed in parental HCT-15 cells after treatment. These data suggest that PPARγ ligand binding may result in the activation or expression of genes that modulate cell cycle progression.

Conclusion

PPARγ plays an essential role in adipocyte differentiation and PPARγ activation induces G_1 cell cycle arrest in SV40 transformed adipogenic HIB1B cells (Altiok et al., 1997). However, the biological effects of PPARγ in colon epithelial cells are unknown. These data establish that human colorectal cancer cell lines express functional PPARγ. Moreover, activation of the receptor with a selective PPARγ agonist results in growth inhibition in colorectal cancer cells grown on plastic, in soft agar, and *in vivo* in the subcutaneous compartment of athymic mice. The decrease in cell growth is likely due to the ability of activated PPARγ to induce a delay in the G_1 phase of the cell cycle.

CHAPTER IV

TARGET GENES OF PPARδ IN COLORECTAL CANCER CELLS

Introduction

Although the central focus in the PPARδ field has been its role in lipid and glucose homeostasis, evidence is emerging that the receptor is important in regulating pathways beyond energy homeostasis (Kliewer and Willson, 1998). Our previous studies in Chapter III established that activation of PPARδ could inhibit the growth of colorectal cancer cells. Sarraf et al. have reported similar findings in a study demonstrating that activation of PPARδ induces growth inhibition and differentiation in a broad range of human colorectal cancer cells (Sarraf et al., 1998). In fact, activators of PPARδ have been shown to inhibit the growth of epithelial cells derived from diverse organs including prostate (Kubota et al., 1998; Mueller et al., 1998), lung (Chang and Szabo, 2000), stomach (Takahashi et al., 1999), and breast (Mueller et al., 2000). Whether or not the anti-neoplastic effects of PPARδ ligands operate *in vivo* remains controversial. For example, agonists of the receptor reduce pre-malignant intestinal lesions in rats treated with the carcinogen azoxymethane (Tanaka et al., 2001) but slightly increase colon polyps in mice that are predisposed to intestinal adenomas because of a mutation in the Adenomatous Polyposis Coli (APC) tumor suppressor gene (Lefebvre et al., 1998; Saez et al., 1998).

PPARδ may also be important in modulating the inflammatory response in colon epithelial cells. Ligands for PPARδ have been shown to inhibit the induction of pro-inflammatory cytokines in colorectal cancer cells through a NF-κB dependent mechanism (Su et al., 1999). In mouse models of inflammatory bowel disease, mice treated with PPARδ agonists exhibit decreases in several indices of inflammation. In addition, mice

that are heterozygous for the PPAR gene show an increased sensitivity to chemicals that induce colitis (Nakajima et al., 2001).

Thus, although PPAR regulates important facets of colon epithelial cell biology, the downstream transcriptional targets of PPAR in colorectal cancer cells are unknown. Clearly, the identification of these targets is the first step in elucidating the mechanisms by which the receptor modulates colonocyte physiology. In this study we sought to identify and characterize PPAR regulated genes in a human colorectal cancer cell line using microarray technology. PPAR selective targets included genes involved in the regulation of cell growth, colon epithelial cell maturation, and inflammation. In addition, three different members of the carcinoembryonic antigen (CEA) family were induced by PPAR . Consistent with this, exposure of colon cancer cells to a PPAR ligand induced an increase in Ca^{2+} independent, CEA-dependent homotypic aggregation, suggesting a potential role for PPAR in regulating interceullar adhesion.

Results

Evaluation of Cell Culture System to Monitor PPAR Target Genes

The M-S colon carcinoma line was evaluated for its suitability in studying gene expression changes in response to PPAR activation in intestinal epithelial cells. The cell line was found to express protein for all three PPAR subtypes (Fig. 13A). PPAR transcriptional activity was measured in cells transfected with the PPRE3-tk-luc reporter vector that contains a luciferase cDNA downstream of three tandem repeats of the PPAR response element (PPRE) from the acyl-coA oxidase gene (Kliewer et al., 1994). PPAR agonists from two different chemical families (rosiglitazone and GW7845) induced a dose dependent increase in reporter activity that could be blocked by co-treatment with the PPAR antagonist GW9662 (Fig. 13B). Either of these two PPAR agonists also induced a time-dependent decrease in cell number compared to vehicle treated cells; this

decrease in cell growth could be reversed by co-treatment with GW9662 (Fig. 14A). The PPARγ selective compound GW7647 or the PPARα selective ligand GW1514 had negligible effects on the growth of the M-S cells (Fig. 14B). Thus, activation of PPARγ activity in these cells induces a specific and significant decrease in cell number. This decrease in cell number was not due to an increase in apoptosis but rather a delay in the G_1 phase of the cell cycle (data not shown).

Identification of PPARγ Target Genes Using Microarrays

Two different microarray technologies were used to identify genes induced or repressed in the M-S cells exposed to rosiglitazone (see Chapter II for extended details). Membranes containing 5,184 non-control spotted cDNA's (Research Genetics) or oligonucleotide-based arrays that represent 5,600 unique genes (Affymetrix) were used. A short treatment time (24 h) was examined with the intent of identifying direct PPARγ target genes that are responsible for the growth inhibition and differentiation induced by activation of the receptor. An extended treatment time (6 days) was also evaluated to understand the gene expression pattern in cells that have already significantly growth arrested due to PPARγ activation.

Data from the cDNA filter arrays were found to contain a large number of false positives and used only to help interpret data obtained from the oligonucleotide-based arrays. Using the difference call decision matrix algorithm, the Affymetrix chip software identified a total of 89 genes (1.6% of transcripts) that were induced or repressed 2.5X or greater after treatment with the PPARγ ligand GW7845. Fourteen of these genes were selected for independent confirmation by Northern blot hybridization. Four of these genes [Adipophilin, Keratin 20, Non-Specific Cross Reacting Antigen (NCA), and Liver-Fatty Acid Binding Protein (L-FABP)] were selected based on their prior identification as PPARγ targets using cDNA filter arrays. These were the only four gene targets commonly identified by both array methods. The other nine genes were selected for

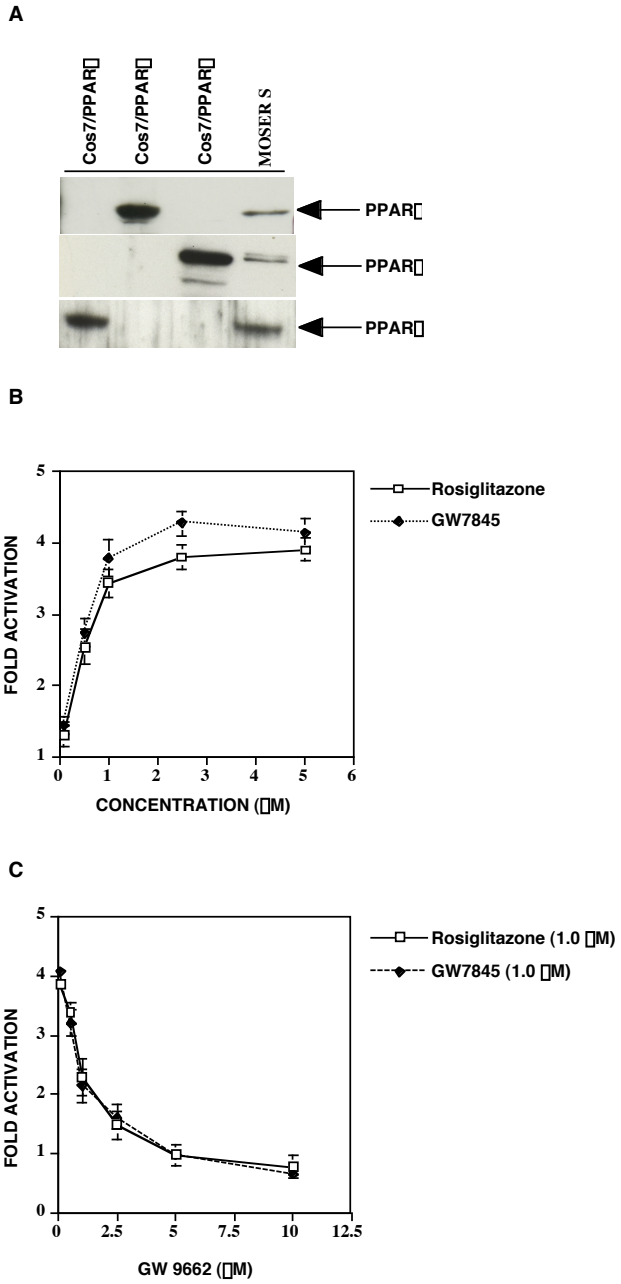

Figure 13. PPAR is expressed and transcriptionally active in the M-S colon carcinoma cell line. A) Protein lysates (50 g) from M-S cells or cos7 cells transiently transfected with PPAR, , or were analyzed by western blot using monoclonal anti-PPAR or antibodies and a rabbit polyclonal anti-PPAR antibody. B) M-S cells were transiently transfected with the PPAR reporter vector PPRE3-tk-luc and exposed to increasing doses of the PPAR agonists rosiglitazone or GW7845. Cells were harvested and the dual luciferase assay was performed. C) M-S cells transiently transfected with PPRE3-tk luc and treated with 1.0 M of rosiglitazone or GW7845 were exposed to increasing concentrations of the PPAR antagonist GW9662. Data are represented as fold activation over non-GW9662 treated cells. Cells were harvested and the dual luciferase assay was performed. Data points represent the mean of three independent experiments. *Error Bars* = S.E.M.

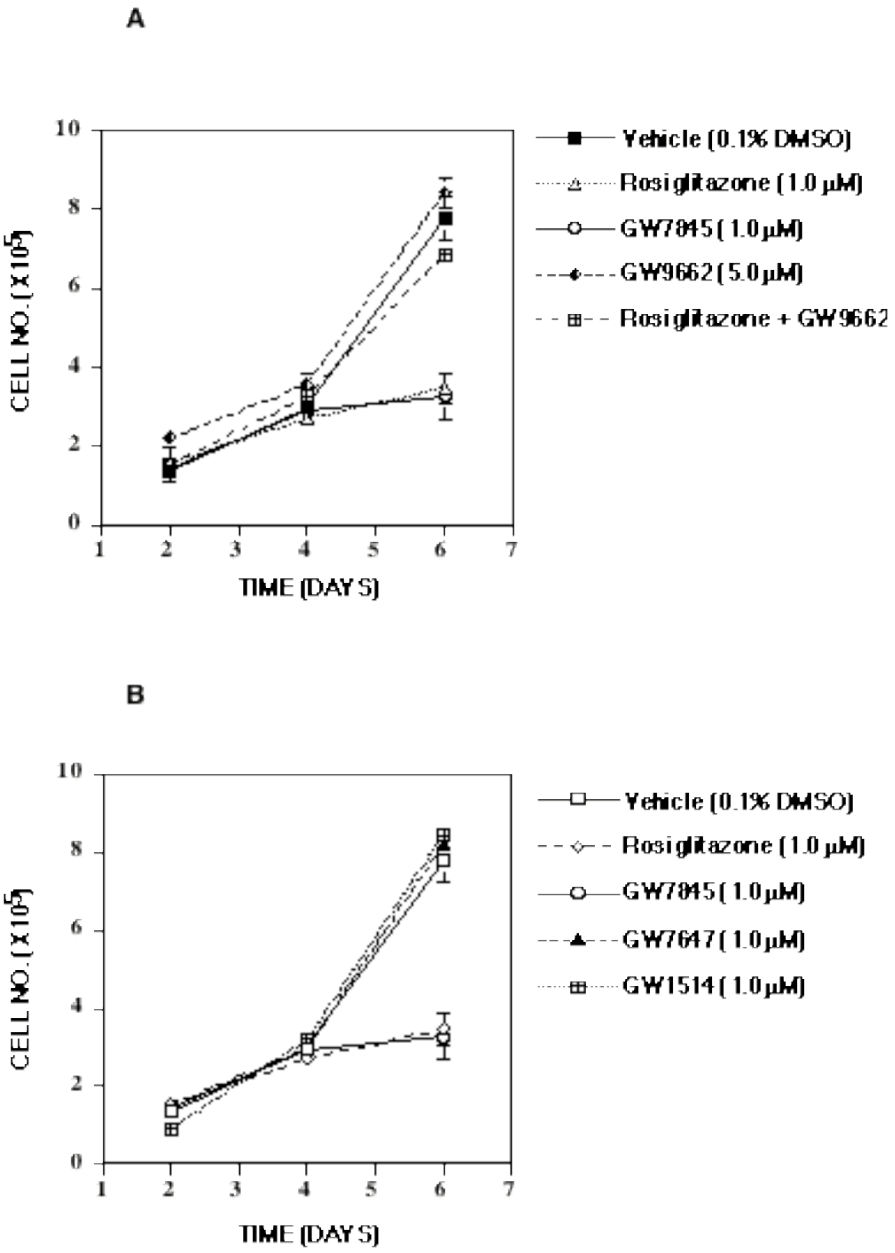

Figure 14. PPAR□specifically and selectively inhibits the growth of the M-S colon carcinoma cell line. A) M-S cells were treated with the PPAR□agonists rosiglitazone (1.0 □M) or GW7845 (1.0 □M), the PPAR□antagonist GW9662 (5.0 □M) or a combination of rosiglitazone and GW9662 and the number of cells were counted at Days 2, 4, and 6 post-treatment. B) M-S cells were treated with the PPAR□agonists rosiglitazone (1.0 □M) or GW7845 (1.0 □M), the PPAR□ agonist GW7647 (1.0 □M), or the PPAR□agonist GW1514 (1.0 □M) and the number of cells were counted at Days 2, 4, and 6 post-treatment. Each data point represents the mean of three independent experiments. *Error Bars* = S.E.M.

secondary confirmation based on a combination of cDNA probe availability and putative gene function. From this subset analysis, 9/14 genes (60%) on the Affymetrix chip could be verified by Northern blot hybridization (Table II). Based on these results, a confidence interval equation was used to estimate the proportion of true positives from the original 89 genes detected by the Affymetrix software (see Chapter II for details). The result was a 95% confidence interval of (0.31, 0.82). This can be read as 95% of the time the true value of the proportion of true positives will lie between (0.31, 0.82).

The nine identified genes fall into several different functional categories. Both Adipophilin (Heid et al., 1998) (also known as adipose differentiation-related protein) and L-FABP (Poirier et al., 2001) are involved in fatty acid transport or storage. Regeneration gene IA (Reg IA) encodes a 166 amino acid secreted protein that induces the proliferation of pancreatic and acinar cells (Okamoto, 1997). Neutrophil Gelatinase Associated Lipocalin (NGAL) is a 25 kilodalton protein that is thought to blunt inflammatory responses by binding to and sequestering hydrophobic molecules that serve as neutrophil chemoattractants (Kjeldsen et al., 2000). Two of the genes have been linked to colonic epithelial cell maturation. Gob-4 (also known as hAG-2) is a secreted protein whose expression is strongly associated with mature goblet cells in the intestine (Komiya et al., 1999). Keratin 20 is an intermediate sized filament protein that is strongly expressed in the most differentiated cell types within the mucosal epithelium of the intestine (Calnek and Quaroni, 1993; Moll et al., 1993). PPAR also induced three different genes, Carcinoembryonic Antigen (CEA), NCA, and Biliary Glycoprotein (BGP), that are all members of the CEA gene family. Proteins in this family have diverse functions in the regulation of cellular adhesion and differentiation (Obrink, 1997). Finally, five of the genes (Adipophilin, L-FABP, RegIA, Gob-4, and NGAL) were most strongly induced or repressed after 24 h of ligand treatment while Keratin 20, CEA, NCA, and BGP were all strongly induced only after 6 days of ligand treatment.

TABLE II

Summary of genes induced or repressed after exposure of M-S colon carcinoma cells to the PPARγ ligand rosiglitazone.

Gene Name	Fold Change[a]
Adipophilin[b]	4.4
Liver-Fatty Acid Binding Protein (L-FABP)[b]	2.7
Regenerating Gene IA (RegIA)[b]	0.40
Gob-4[b]	0.28
Neutrophil Gelatinase Associated Lipocalin (NGAL)[b]	2.8
Keratin 20[c]	6.7
Carcinoembryonic Antigen (CEA)[c]	2.5
Non Specific Cross Reacting Antigen (NCA)[c]	8.1
Biliary Glycoprotein (BGP)[c]	5.5

[a]Relative fold induction calculated by measuring the Digital Light Units (DLU)/mm^2 of each indicated mRNA in storage phosphor images of northern blots containing total RNA (20 μg) from cells treated with 0.1% DMSO or 1 μM rosiglitazone and hybridized with the appropriate labeled cDNA. Values were first normalized to the DLU/mm^2 of 1B15 and expressed as a ratio of rosiglitazone/DMSO.

[b]24 h Treatment

[c]6 Day Treatment

Characterizing the Specificity and Selectivity of Each Target Gene Induction/Repression

The synthetic PPAR ligands used in our studies may have effects on gene expression independent of their ability to bind to and activate the receptor. Furthermore, since there has been little evidence for differences in the binding affinities of any of the three PPAR subtypes to a particular PPAR response element (Juge-Aubry et al., 1997), each of the identified target genes might also be regulated by PPAR and . Such gene targets may potentially be less interesting since ligands for either of these two receptor isoforms have no effects on the growth or differentiation of the M-S cells. To address these issues, the expression of each of the nine putative PPAR target genes was examined in response to the full panel of synthetic ligands described in detail in Chapter II. All nine of the putative target genes could be induced or repressed by PPAR agonists from two different chemical families (rosiglitazone and GW7845) and the change in expression could be blocked by co-treatment with the PPAR antagonist GW9662 (Fig. 15-17). This suggests that the ability of PPAR agonists to regulate the genes identified in this study are likely due to their ability to bind to PPAR and not via a non-specific target. Both of the gene targets involved in lipid storage or transport (Adipophilin and L-FABP) were also induced by either the PPAR agonist GW7647 and/or the PPAR agonist GW1514 (Fig. 15). The other seven targets were only activated by ligands for PPAR (Fig. 16-17). In general, the putative function of genes selectively regulated by PPAR correlated with the biological phenotype induced by PPAR activation in colon epithelial cells. For example, only PPAR ligands (and not ligands for PPAR or) were able to regulate genes involved in growth control (*e.g.* Reg IA) or coloncyte maturation (*e.g.* Keratin 20) (Fig. 16).

The PPAR-RXR complex represents a type of RXR-dependent nuclear receptor heterodimer that has been defined as "permissive" because PPAR does not block the ability of RXR to bind ligand and modulate gene transcription independent of the ligand

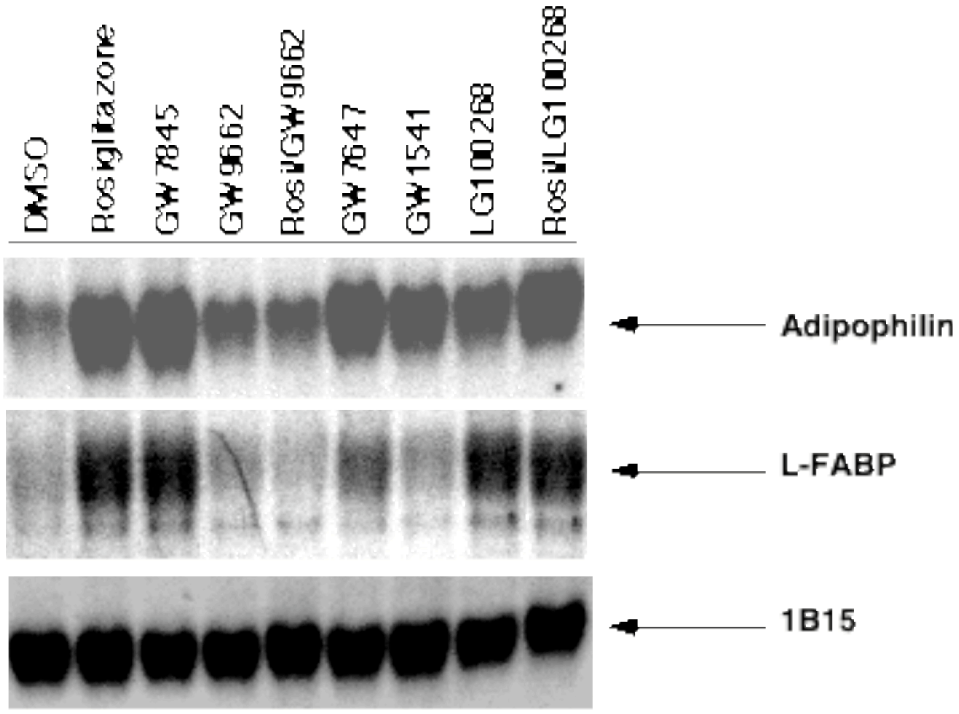

Figure 15. The PPAR target genes Adipophilin and L-FABP are also targets of PPAR and/or PPAR. M-S cells were treated with the following compounds for 24 h (dose and PPAR subtype selectivity in parentheses): rosiglitazone (1 M, PPAR), GW7845 (1 M, PPAR), GW9662 (5 M, PPAR antagonist), BRL + GW9662, GW7647 (1 M, PPAR), GW1514 (1 M, PPAR), LG100268 (0.5 M, RXR), and BRL + LG100268. Adipophilin, L-FABP mRNA levels was detected by Northern blotting (20 g total RNA/lane). Blots were probed with 1B15 to normalize for differences in RNA loading.

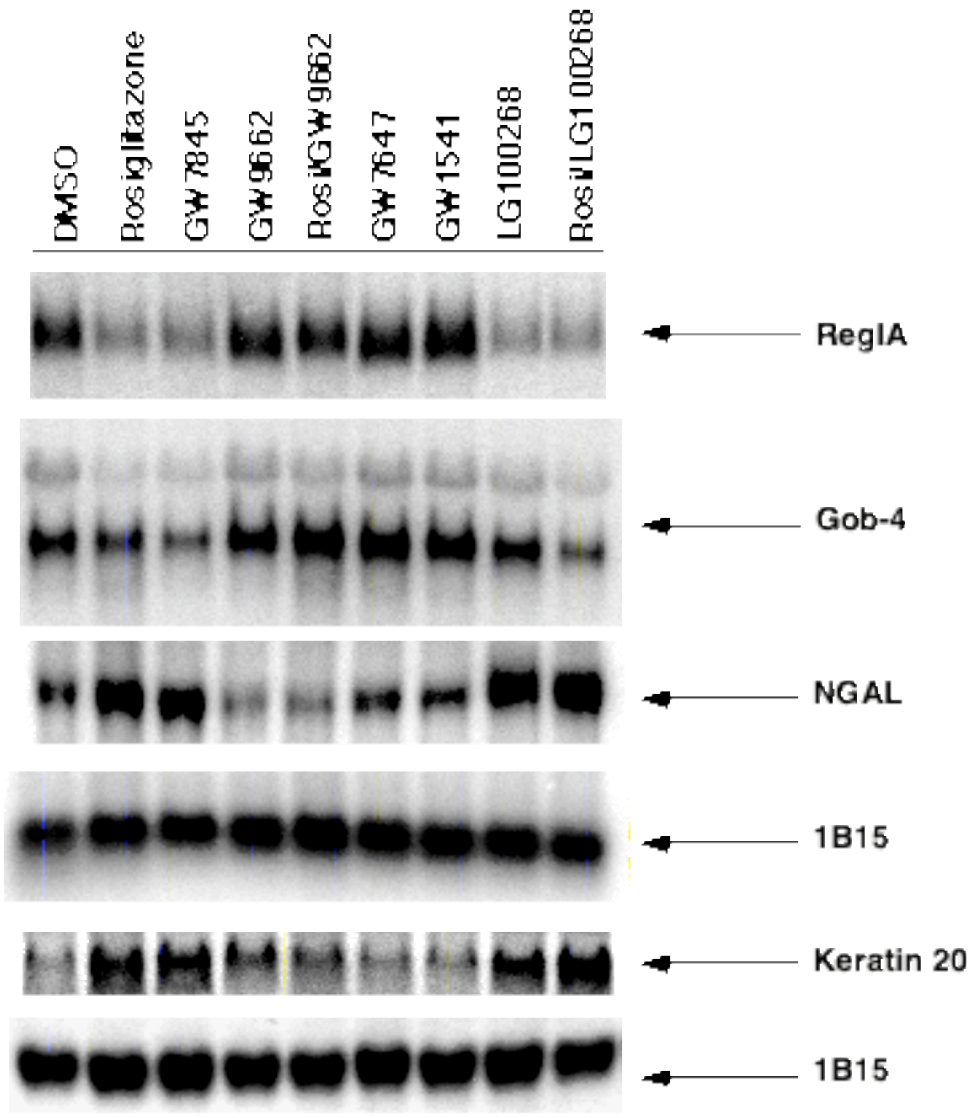

Figure 16. The PPARγ target genes Reg IA, Gob-4, NGAL, and Keratin 20 are specifically and selectively regulated by PPARγ. M-S cells were treated with the following compounds (dose and PPAR subtype selectivity in parentheses) for 24 h (Reg IA, Gob-4, and NGAL) or 6 days (Keratin 20): rosiglitazone (1 μM, PPARγ), GW7845 (1 μM, PPARγ), GW9662 (5 μM, PPARγ antagonist), BRL + GW9662, GW7647 (1 μM, PPARγ), GW1514 (1 μM, PPARγ), LG100268 (0.5 μM, RXR), and BRL + LG100268. The levels of mRNA for each gene were determined by Northern blot analysis. Blots were also probed with 1B15 to normalize for differences in RNA loading.

occupancy status of PPAR(Leblanc and Stunnenberg, 1995). We evaluated the ability of each target gene to be regulated independently by the RXR specific agonist LG100268 or synergistically by both LG100268 and rosiglitazone (Fig. 15-17). Of the nine gene targets, only the three CEA-family proteins could not be independently induced by LG100268 (Fig. 17). Finally, there was no evidence that PPAR and RXR ligands could synergistically regulate any of these nine target genes.

Activation of PPAR Induces an Increase in CEA-dependent Homotypic Aggregation

PPAR ligands selectively up-regulated three different members of the CEA family of proteins (Fig. 17). The CEA gene family is a member of the immunoglobulin gene superfamily and consists largely of highly glycosylated cell surface proteins. These are multi-functional proteins that can act as microbial receptors and regulators of cellular differentiation and adhesion (Obrink, 1997). In fact, CEA (Benchimol et al., 1989), NCA (Zhou et al., 1990), and BGP (Rojas et al., 1990) have all been implicated in Ca^{2+} independent homophilic binding to molecules on adjacent cells (versus cadherins that require Ca^{2+} for homophilic binding). Previous studies have demonstrated that CEA proteins can induce homotypic aggregation of colon cancer cells, suggesting a potential role for these proteins in regulating intercellular adhesion during intestinal development or in the adult intestinal epithelium (Benchimol et al., 1989). We determined whether PPAR ligands could induce cellular aggregation that is dependent on CEA or CEA-like proteins. Cells were forced into single suspension and allowed to re-aggregate over time in a Ca^{2+} free environment (to exclude cadherin dependent aggregation). Exposure of M-S cells to rosiglitazone or GW7845 induced a time dependent increase in the percent of aggregated cells (decrease in the percent of single cells) that could be reversed by co-treatment with the PPAR antagonist GW9662 (Fig. 18A). This increase in cellular aggregation was dependent upon CEA family proteins since Fab' fragments of a

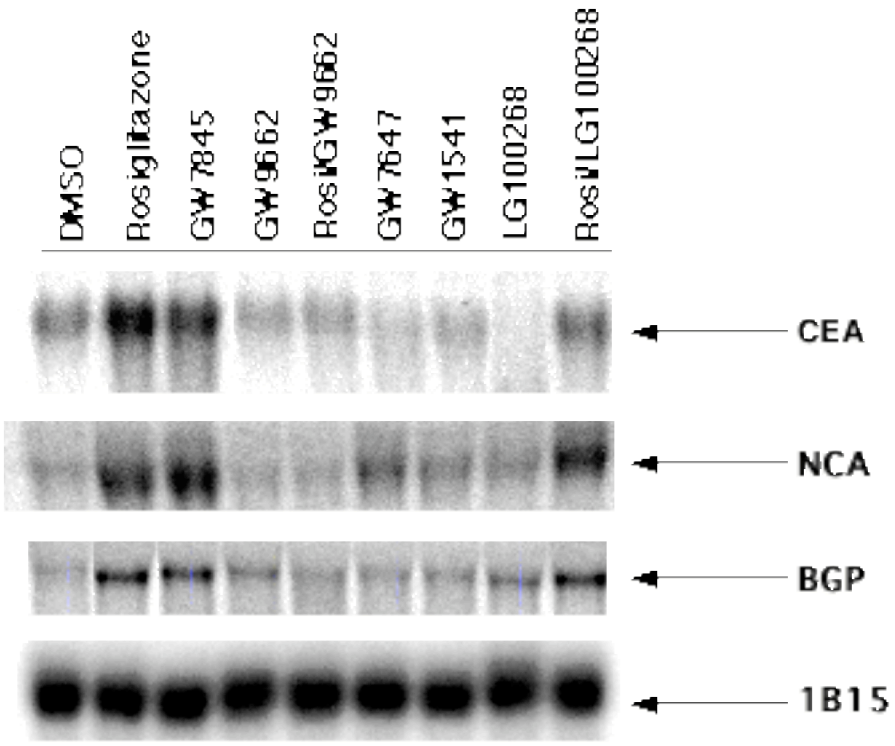

Figure 17. PPAR□induces three different members of the CEA family of proteins. M-S cells were treated with the following compounds for 6 days (dose and PPAR subtype selectivity in parentheses): rosiglitazone (1□M, PPAR□), GW7845 (1□M, PPAR□), GW9662 (5 □M, PPAR□antagonist), BRL + GW9662, GW7647 (1 □M, PPAR□), GW1514 (1 □M, PPAR□), LG100268 (0.5 □M, RXR), and BRL + LG100268. CEA, NCA, and BGP mRNA levels was detected by Northern blotting (20□g total RNA/lane). Blots were probed with 1B15 to normalize for differences in RNA loading.

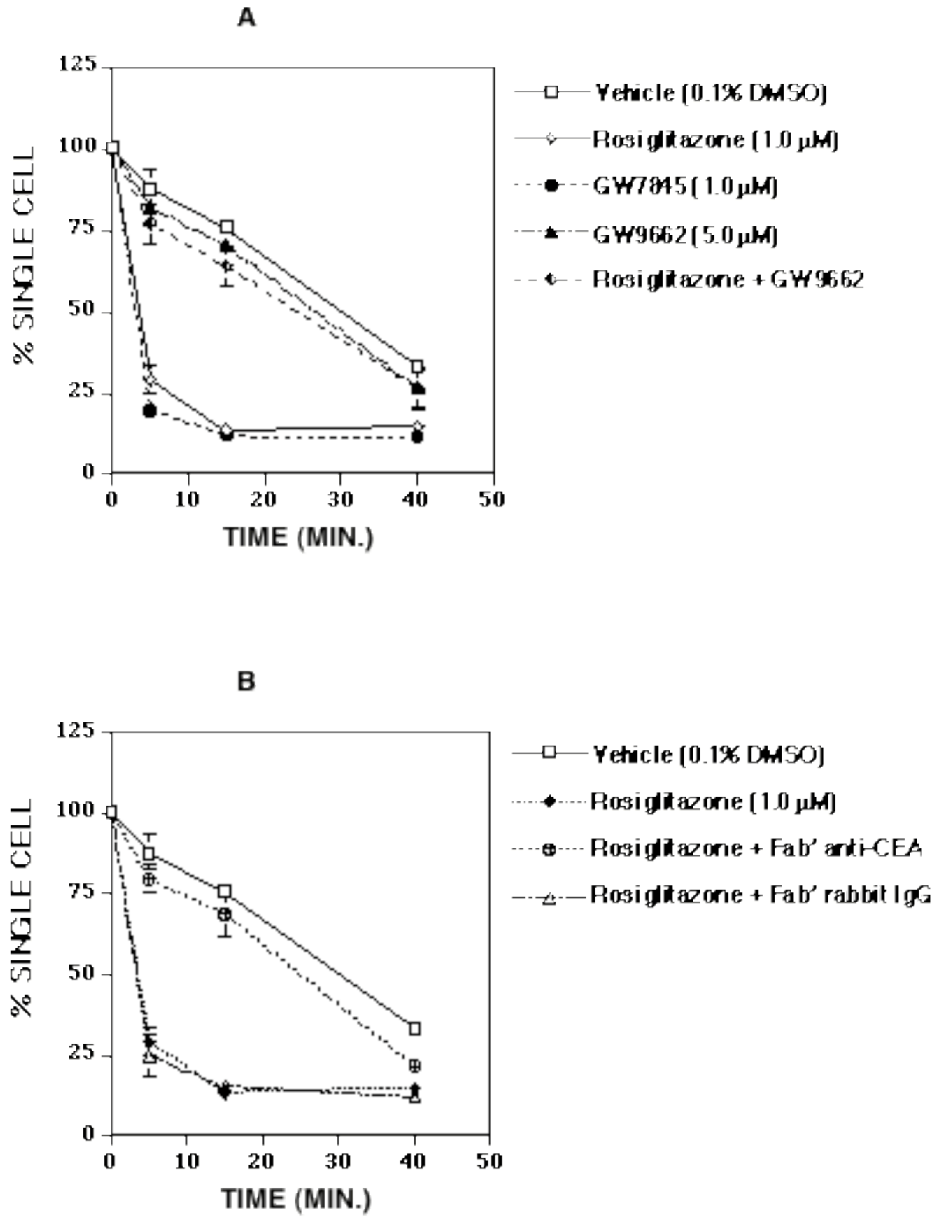

Figure 18. PPARγ ligands induce an increase in CEA-dependent aggregation of M-S colon carcinoma cells. M-S cells were treated with the 1.0 μM of the PPARγ agonists rosiglitazone or GW7845, 5.0 μM of the PPARγ antagonist GW9662, or a combination of BRL + GW9662 for 4 days. Cells were then forced into single cell suspension and allowed to re-aggregate in Ca^{2+} free conditions. B) M-S cells were treated with 1.0 μM of the PPARγ agonist rosiglitazone for 4 days. Cells were forced into single cell suspension and allowed to re-aggregate in the presence of Fab' fragments of normal rabbit IgG or a polyclonal antibody that recognizes CEA and CEA-like proteins. Each data point represents the percent of single cells (compared to vehicle treated) remaining and is the mean of three independent experiments. Error bars = S.E.M.

polyclonal antibody that recognizes CEA, NCA, and BGP could block the ability of PPARγ ligands to induce aggregation (Fig. 18B).

Conclusion

The genomic response to activation of PPARγ in non-adipocyte cell lines has not been well characterized. In this study we used microarray technology to identify PPARγ gene targets in colon epithelial cells. For each gene, the induction or repression was seen with two structurally distinct PPARγ agonists and the change in expression could be blocked by co-treatment with a specific PPARγ antagonist. A majority of the genes could be regulated independently by an RXR specific agonist. Genes implicated in lipid transport or storage [adipophilin, liver fatty acid binding protein (L-FABP)] were also activated by agonists of PPAR subtypes α and/or δ. In contrast, PPARγ selective targets included genes linked to growth regulatory pathways [regenerating gene IA (Reg IA)], colon epithelial cell maturation (gob-4 and keratin 20] and immune modulation [(neutrophil-gelatinase associated lipocalin (NGAL)]. Additionally, three different genes of the carcinoembryonic antigen (CEA) family were induced by PPARγ. Cultured cells treated with PPARγ ligands demonstrated an increase in Ca^{2+} independent, CEA-dependent homotypic aggregation, suggesting a potential role for PPARγ in regulating interceullar adhesion. Collectively, these results will help define the mechanisms by which PPARγ regulates intestinal epithelial cell biology.

CHAPTER V

A LOSS OF FUNCTION PPARγ ALLELE IN COLORECTAL CANCER CELLS CAUSED BY A MUTATION THAT DISRUPTS BASAL TRANSCRIPTIONAL REPRESSION

Introduction

In Chapters III and IV, we demonstrated that activation of PPARγ in human colorectal cancer cells induces growth inhibition both *in vitro* and *in vivo* and identified several downstream target genes of the receptor that regulate cellular proliferation and epithelial cell differentiation. Given these biological effects of PPARγ activation, one might predict that some colorectal cancer cell lines could develop resistance to PPARγ. In fact, in Chapter III we identified one colorectal cancer cell line, HCT 15, which was resistant to PPARγ ligand induced growth inhibition despite expressing robust levels of the receptor. Moreover, transfection of the wild type receptor could restore normal PPARγ signaling and cause these cells to growth inhibit after exposure to a PPARγ agonist.

To further explore the relationship between the loss of normal PPARγ signaling and the development of colorectal cancer, we screened a panel of established human colorectal cancer cell lines for responsiveness to PPARγ ligands and exonic mutations in the PPARγ gene. Four cell lines that were refractory to growth inhibition induced by activators of PPARγ harbored a previously unreported point mutation in the PPARγ gene at codon 422 (K422Q). Although the mutant PPARγ did not exhibit any defects in transcriptional responsiveness to either natural or synthetic ligands, only expression of the wild type (WT), and not mutant, receptor was able to rescue PPARγ ligand responsiveness in resistant cells. We further demonstrate that K422Q PPARγ may be functionally inactive due to an inability of the apo-receptor to repress the basal expression of a subset of PPARγ target genes.

Results

PPARγ ligand sensitivity and PPARγ gene mutations in a panel of human colorectal cancer cell lines

In our initial survey of the biological response of human colorectal carcinoma cells to PPARγ agonists, we noticed that some cell lines were resistant to the growth inhibitory effects of PPARγ ligands. A panel of eight cell lines (four sensitive and the four that were resistant) was chosen for further study. All eight cell lines expressed relatively equivalent levels of PPARγ protein (Fig. 19). The ability of the high-affinity, PPARγ subtype selective agonist rosiglitazone to induce growth inhibition in each of the eight lines was tested. Four of these cell lines (M-S, HCT 116, HCA-7, and HT-29) were growth inhibited in the presence of a PPARγ agonist, while the other four (HCT 15, MIP 101, Clone A, and COLO 205) were not affected (Table III).

There are a number of explanations for why a particular cell line could be resistant to activators of PPARγ despite expressing robust levels of the receptor. Since somatic loss of function mutations have been identified in a subset of colorectal tumors, we sought to determine if PPARγ ligand resistance in the four cell lines could be due to a loss of function mutation in the PPARγ gene. All four resistant lines contained a monoallelic point mutation in the PPARγ gene at codon 422 resulting in a change from lysine (Lys) to glutamine (Gln) (K422Q); this mutation was not found in the four sensitive cell lines (Table III). The correlation between the K422Q allele and lack of sensitivity to PPARγ ligands provided suggestive, but not definitive, evidence that this mutation caused the HCT 15, MIP 101, Clone A, and COLO 205 cell lines to be resistant to the growth inhibitory effects of PPARγ ligands.

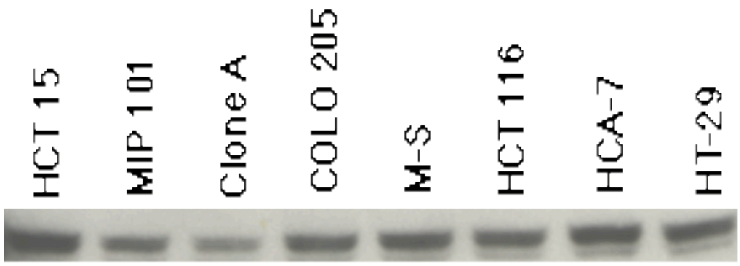

Figure 19. Expression of PPAR protein in human colorectal cancer cell lines. Whole cell lysates (50 g) from each of the eight indicated human colorectal cancer cell lines were fractionated on a 4-20% SDS-polyacrylamide gel. PPAR protein in each cell line was detected by western blot analysis using a monoclonal anti-PPAR antibody.

TABLE III

PPAR Ligand Sensitivity and PPAR Receptor Mutations in a Panel of Human Colorectal Cancer Cell Lines

Cell Line	% Growth Inhibition[a]	Codon Altered
HCT 15	103 +/-4.0	K422Q
MIP 101	96.0+/-2.1	K422Q
Clone A	98.2+/-3.0	K422Q
COLO 205	95.3+/-1.8	K422Q
M-S	44.4+/-0.5	
HCT 116	66.1+/-1.1	
HCA-7	69.0+/-2.5	
HT-29	62.3+/-1.8	

[a]Cell lines were treated for 6 days with 0.1% DMSO or 1.0 M rosiglitazone and the number of cells were counted using a coulter counter. Each experiment represents the mean of three independent experiments. Data are expressed as the number. of cells in the rosiglitazone treated samples as a percent of the total cell number in the DMSO treated samples +/- S.E.M.

Characterization of K422Q mutant allele

No previous studies documenting the sequence of the PPAR gene in various malignancies or from individuals at risk for diabetes or obesity have reported mutations at codon 422 of the receptor. K422 lies within the ninth -helix (H9) of the ligand binding domain of the receptor. Crystallographic studies of PPAR/RXR heterodimers suggest a role for H9 in receptor dimerization (Gampe et al., 2000). However, these studies found no direct role for K422 in any polar interactions found at the dimer interface. X-ray crystallography of PPAR homodimers revealed K422 to be located at the receptor surface and exposed to solvent, suggesting the possibility of involvement in co-factor interactions (Nolte et al., 1998). K422 is conserved in the PPAR cDNA's from all species reported in the NCBI Entrez nucleotide database, including the six different species shown in Fig. 20A. However, K422 is not conserved in either PPAR or , both of which encode a Gln at the homologous amino acid (Q413 and Q386 respectively) (Fig. 20A). Since codon 422 of PPAR in the resistant cell lines is mutated to an amino acid (Gln) which is normally present in the homologous positions of WT PPAR and , it is unlikely that the K422Q mutation disrupts an important structural interaction common to all three PPARs. In fact, it may be that the Lys at position 422 present in WT PPAR is responsible for an interaction unique to the subtype.

As no obvious function has been ascribed to K422, we first characterized what effects the K422Q mutation might have on WT receptor activity. There was no difference in the DNA binding activity of WT PPAR/RXR or K422Q PPAR/RXR on a PPAR response element (PPRE) from the acyl-coA oxidase promoter (Fig. 20B). Identical results were observed using RXR and RXR (data not shown). Transcriptional activity was assayed in cells transfected with either receptor cDNA and the PPRE3-tk-luc reporter vector that contains a luciferase cDNA downstream of three tandem repeats of the PPRE from the acyl-coA oxidase gene (Kliewer et al., 1994). There were no significant differences between WT and K422Q PPAR in the ability of either a synthetic

FIG. 2A

FIG. 2B

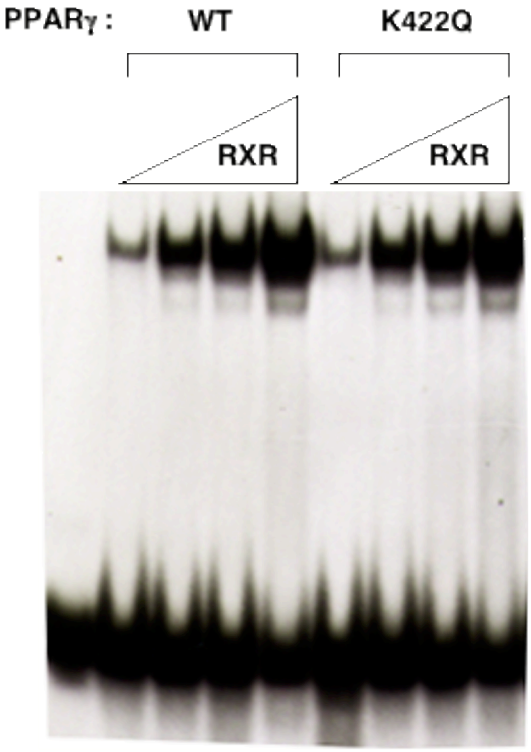

Figure 20 (A and B). DNA binding and Transcriptional activity of K422Q PPAR. (A) Amino acid sequence alignment of codons 412-432 of human PPAR compared to human PPAR subtypes and - and to PPAR from a range of species. K422 (boxed) is conserved in the PPAR from all species reported in GenBank, including the range of species shown here. (B) Electromobility Shift Assay (EMSA) of WT and K422Q PPAR. *In vitro* translated WT or K422Q PPAR was combined with increasing amounts of RXR. The receptor complexes were incubated with a ^{32}P labeled oligonucleotide containing the PPRE from the acyl-coA oxidase promoter for 20 min. followed by resolution on a 5% non-denaturing polyacrylamide gel and detection by autoradiography. The first lane on the left is a sample with probe only.

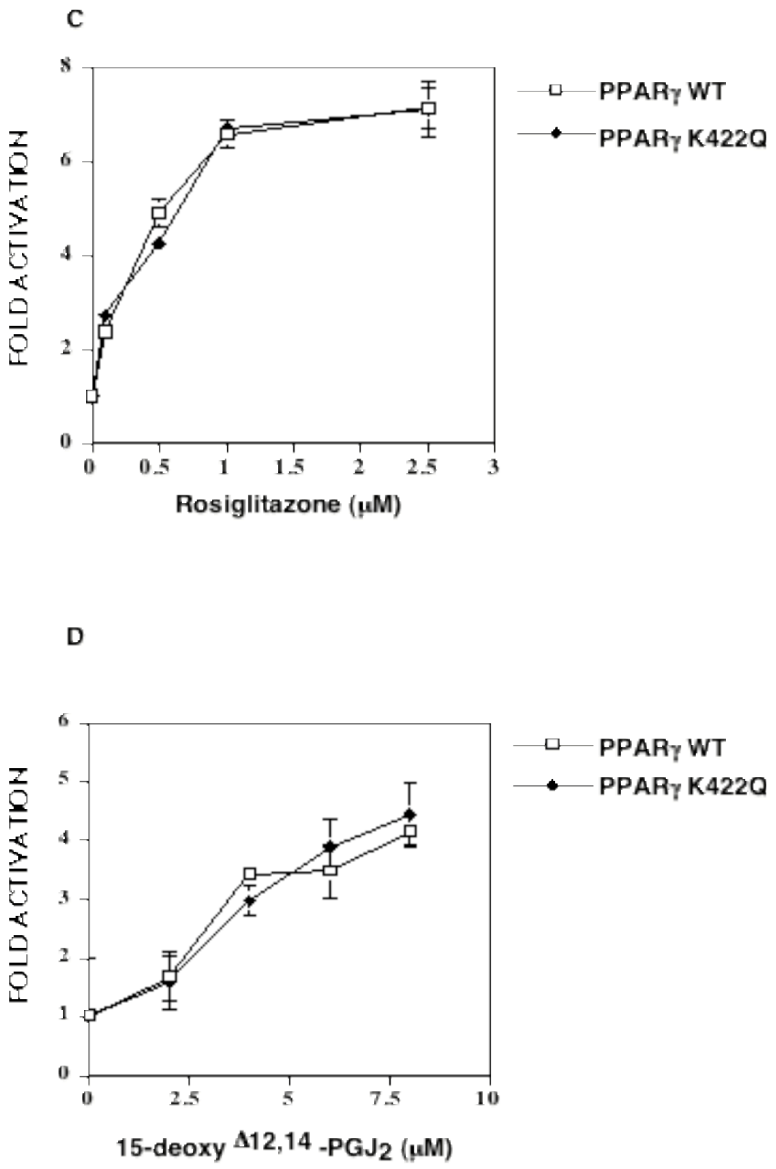

Figure 20 (C and D). DNA binding and Transcriptional activity of K422Q PPAR. (C and D) CV-1 cells were transiently transfected with PPRE3-tk-luc, pRL-SV40, and WT or K422Q PPAR/pcDNA3.0 and treated with increasing doses of either (C) rosiglitazone or (D) 15-deoxy^{12,14}-PGJ_2 for 24-36 h. Cells were harvested and the dual luciferase assay was performed. Data are represented as fold activation over vehicle treated cells and represent the mean from three independent experiments each done in triplicate. *Error bars* = S.E.M.

Wild type, but not K422Q, PPARγ can rescue PPARγ ligand unresponsiveness in resistant cells

Because WT and K422Q PPARγ showed equivalent activity in DNA binding and transactivation assays, it was not clear if the presence of the K422Q mutant allele was the reason the resistant cells were refractory to the growth inhibitory effects of PPARγ ligands. To directly test this hypothesis, one of the resistant cell lines, the HCT 15 cells, was retrovirally transduced with HA-tagged WT or K422Q PPARγ and assayed for PPARγ ligand-induced growth inhibition. Three different pooled stable cell lines—HCT 15-pMSCV (vector), HCT 15-PPARγ WT and HCT 15 PPARγ K422Q—were generated. Both the HCT 15-PPARγ WT and HCT 15-PPARγ K422Q cell lines expressed equivalent levels of the WT or mutant receptor protein (Fig. 21A). As observed with the transiently transfected receptors, there were no differences in ligand induced transactivation between WT and K422Q PPARγ in the stable cell lines (Fig. 21B).

Exposure of HCT 15-PPARγ WT, but not HCT 15-pMSCV or HCT 15-PPARγ K422Q, cells to a synthetic (rosiglitazone) or natural (15-deoxy$\Delta^{12,14}$-PGJ_2) PPARγ agonist induced a dose-dependent decrease in cell number (Fig. 22A and B). Similarly, only HCT 15 cells expressing the WT (but not mutant) receptor could undergo a partial arrest in the G1 phase of the cell cycle after extended exposure to a PPARγ agonist (Fig. 22C). Thus, despite the fact that both WT and K422Q PPARγ have comparable DNA binding and *trans*-activation activities, only the WT receptor could rescue the functional resistance of the parental HCT 15 cells. These data provide direct evidence that the HCT 15—and by extension MIP 101, Clone A, and COLO 205—cell lines did not undergo growth inhibition after ligand activation of PPARγ due to the presence of the K422Q allele.

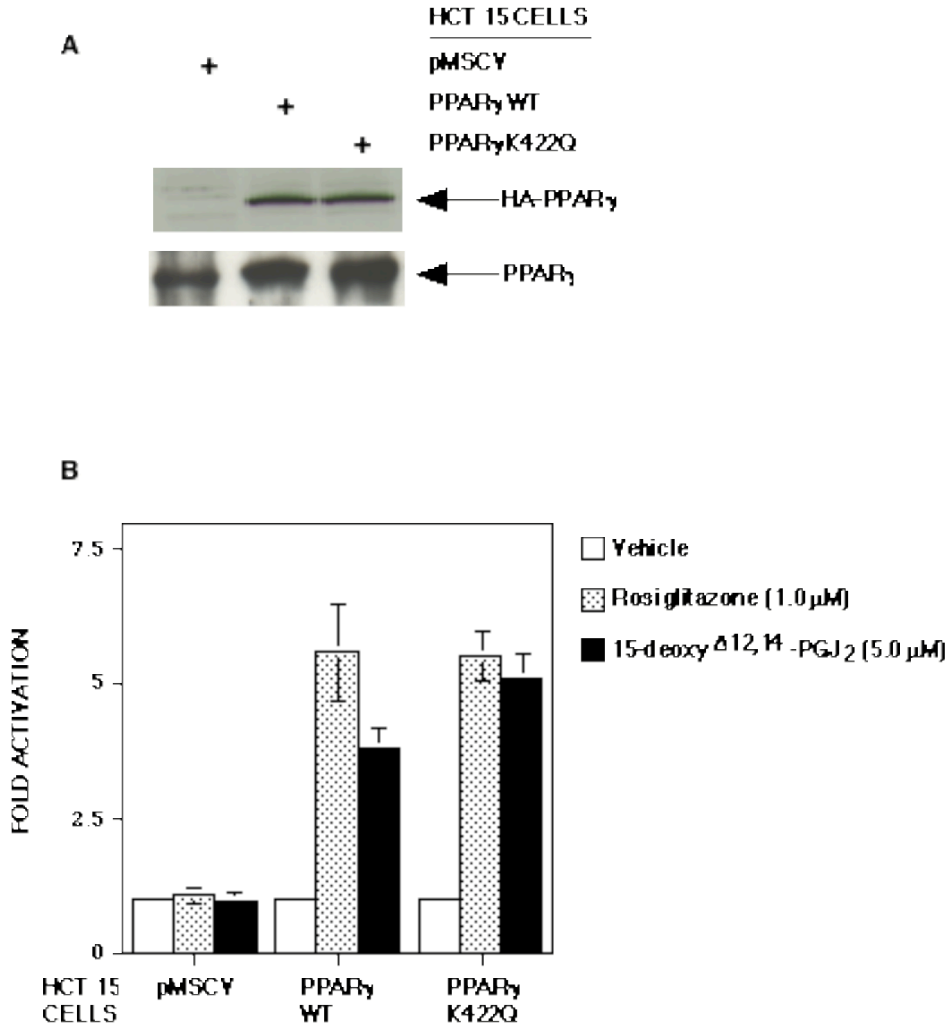

Figure 21. Generation of HCT 15 colorectal cancer cells expressing WT or K422Q PPAR by retroviral transduction. HCT 15 cells were infected with retrovirus expressing no insert (pMSCV), HA-tagged WT PPAR, or HA-tagged K422Q PPARand stable cell lines were generated using puromycin selection. (A) Protein expression of PPARin infected HCT 15 cells. Whole cell lysates (50 g) from exponentially growing HCT 15-pMSCV, HCT 15-PPARWT, and HCT 15-PPARK422Q were probed for PPAR protein levels by Western blot analysis using anti-HA or anti-PPARmonoclonal antibodies. (B) Transcriptional activation of PPARin infected HCT 15 cells. HCT 15-pMSCV, HCT 15-PPARWT, and HCT 15-PPARK422Q cell lines were transiently transfected with PPRE3-tk-luciferase and pRL-SV40 and treated with the indicated PPARligand for 36 h. Cells were harvested and the dual luciferase assay was performed. Data are represented as fold activation over vehicle treated cells and represent the mean from three independent experiments each done in triplicate. *Error bars* = S.E.M.

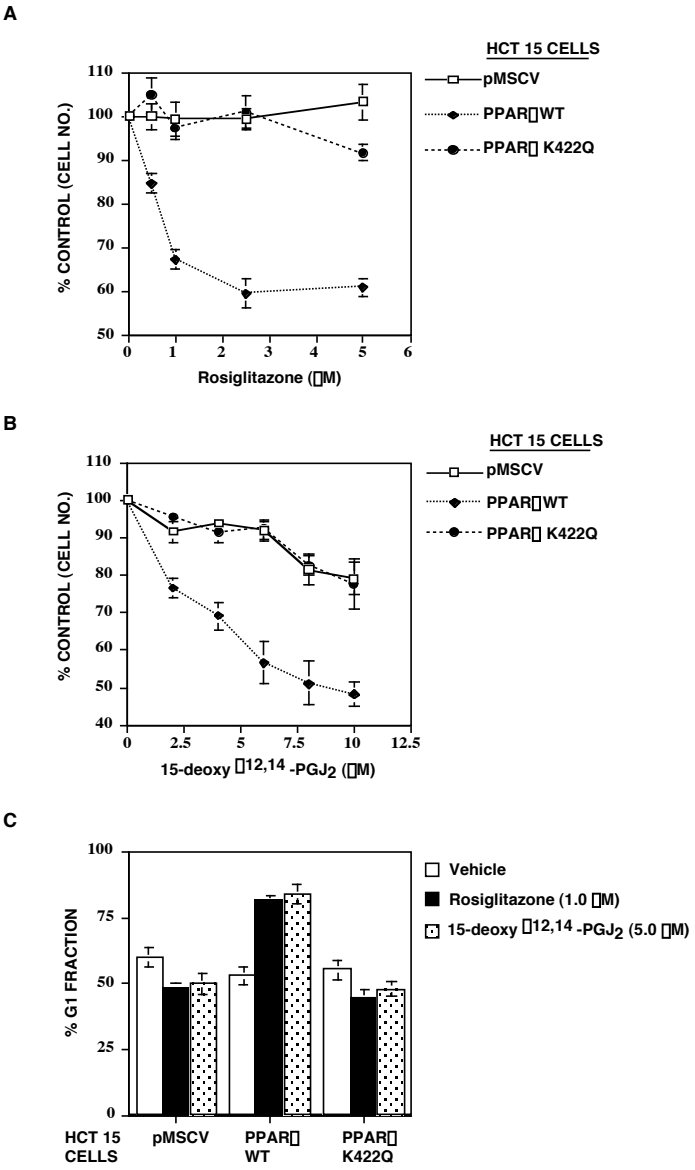

Figure 22. Expression of WT, but not K422Q, PPAR causes the previously resistant HCT 15 cell line to become sensitive to PPAR agonist-induced G_1 delay and growth inhibition *in vitro*. (A and B) HCT 15-pMSCV, HCT 15-PPAR WT, and HCT 15-PPAR cell lines were treated with increasing doses of either (A) rosiglitazone or (B) 15-deoxy^{12,14}-PGJ_2 for six days. The number of remaining cells was counted using a coulter counter and the values are expressed as a percent of vehicle treated cells. Data represent the mean of three independent experiments each done in triplicate. *Error bars* = S.E.M. (C) HCT 15-pMSCV, HCT 15-PPAR WT, and HCT 15-PPAR cell lines were treated with vehicle, rosiglitazone (1.0 M) or 15-deoxy^{12,14}-PGJ_2 (5.0 M) for 48 h. Cells were harvested, stained with propidium iodide, and analyzed for DNA content by fluorescence-activated cell sorter analysis. The values represent the number of cells in the G_1 phase of the cell cycle as a percentage of total cells and represent the mean of two independent experiments each done in triplicate. *Error bars* = S.E.M.

The K422Q apo-receptor cannot repress the basal expression of target genes

To understand why the presence of the K422Q mutation causes functional resistance to PPARγ agonists, we determined the effects of this mutation on the ability of the receptor to regulate the expression of endogenous PPARγ target genes. This would allow a more accurate assessment of receptor function (versus an assay measuring transactivation of an artificial promoter). To initially identify genes regulated by PPARγ in the HCT 15 cell line background, HCT 15-PPARγ WT cells were treated with vehicle or rosiglitazone and differences in gene expression were detected by oligonucleotide microarray analysis. Four genes (Adipophilin, Gob-4, TSC-22, and Mox-2) identified in this screen were selected for further study. We have previously identified adipophilin and Gob-4 as PPARγ regulated genes in the M-S colorectal cancer cell line (see experiments in Chapter IV) (Gupta et al., 2001). As mentioned earlier, adipophilin (also known as adipose differentiation related factor) is a protein involved in fatty acid storage (Heid et al., 1998) while Gob-4 is a secreted protein associated with mature intestinal goblet cells (Komiya et al., 1999). TSC-22 is a putative leucine zipper containing transcription factor originally identified as a TGF-β inducible gene (Shibanuma et al., 1992) and we have data establishing that the gene is selectively and specifically induced by PPARγ in colorectal cancer cells (See Chapter VI for details). Mox-2 has not previously been reported as a PPARγ regulated gene; it is a member of a homeobox gene family that plays a role in mesoderm formation (Candia et al., 1992; Candia and Wright, 1995).

The expression levels of these four PPARγ target genes was determined by northern blot hybridization in HCT 15-pMSCV, HCT 15-PPARγ WT, and HCT 15-PPARγ K422Q cell lines treated with vehicle or rosiglitazone (Fig. 23). There was a 3-fold difference between WT and K422Q PPARγ in ligand-dependent induction of a target gene (*i.e.* adipophilin). The mutant receptor showed no defect in ligand-dependent

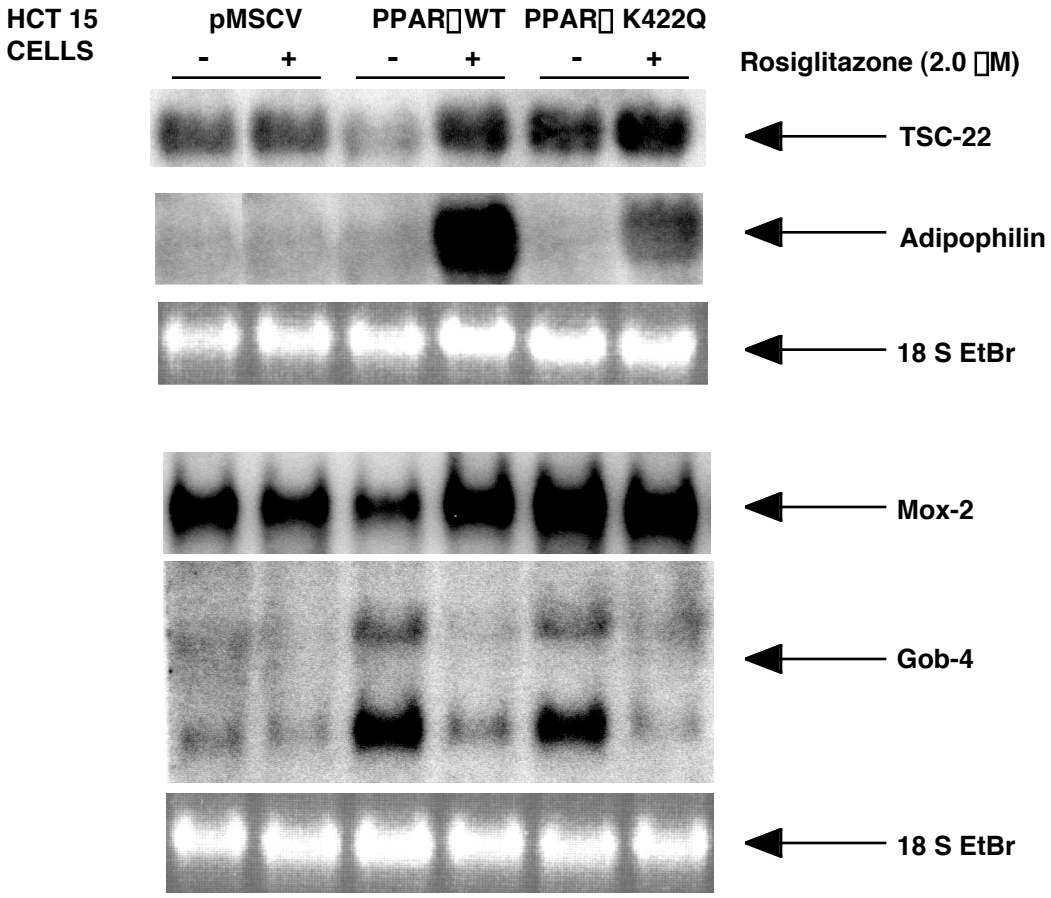

Figure 23. K422Q PPARγis defective in repressing the basal expression of target genes in the absence of exogenous ligand. HCT 15-pMSCV, HCT 15-PPARγWT, and HCT 15-PPARγcell lines were treated with vehicle or 2.0 μM of rosiglitazone for 24 h. The mRNA levels of the PPARγtarget genes TSC-22, Adipophilin, Mox-2, and Gob-4 were measured by Northern blot hybridization (20 μg total RNA/lane). A picture of ethidium bromide stained 18S rRNA for each blot is shown to indicate equal loading of RNA in each sample.

repression of a target gene (i.*e*. Gob-4). However, as compared to WT apo-PPAR, the most striking defect of the K422Q mutation was an inability of the apo-receptor to repress the basal expression of two PPARtarget genes, TSC-22 and Mox-2. To our knowledge, this is the first example documenting the ability of apo-PPARto repress the expression of a target gene.

The ability of some members of the nuclear hormone receptor superfamily, notably the retinoic acid receptors (RARs) and thyroid hormone receptors (TRs), to actively repress gene expression in the absence of ligand has been well documented (reviewed in (Glass and Rosenfeld, 2000; Hu and Lazar, 2000)). This activity is dependent on their ability to bind to transcriptional coregulators termed corepressors. Two of the best-characterized corepressors are N-CoR (nuclear receptor corepressor)(Horlein et al., 1995) and SMRT (silencing mediator for RAR and TR)(Chen and Evans, 1995). These proteins recruit histone deacetylases via interactions with the mSin3 proteins and this complex transrepresses a target gene by causing the hypoacetylation of histone N-terminal tails (Heinzel et al., 1997; Nagy et al., 1997). The binding surface of TR and RXR that interacts with N-CoR and SMRT has been mapped to H3, H4, and H5 of the ligand binding domain (Hu and Lazar, 1999). Although prior studies have failed to demonstrate the ability of either N-CoR or SMRT to bind DNA-bound apo-PPAR, both of these proteins can bind to PPARin solution, suggesting their possible involvement in apo-PPARmediated transcriptional repression(Zamir et al., 1997). Thus, we tested whether apo-K422Q PPARis unable to repress the expression of TSC-22 or Mox-2 due to a defect in corepressor binding. However, in a mammalian two-hybrid assay, there was no difference between WT and K422Q PPARin their ability to bind to, or exhibit ligand-dependent release from, N-CoR or SMRT (Fig. 24).

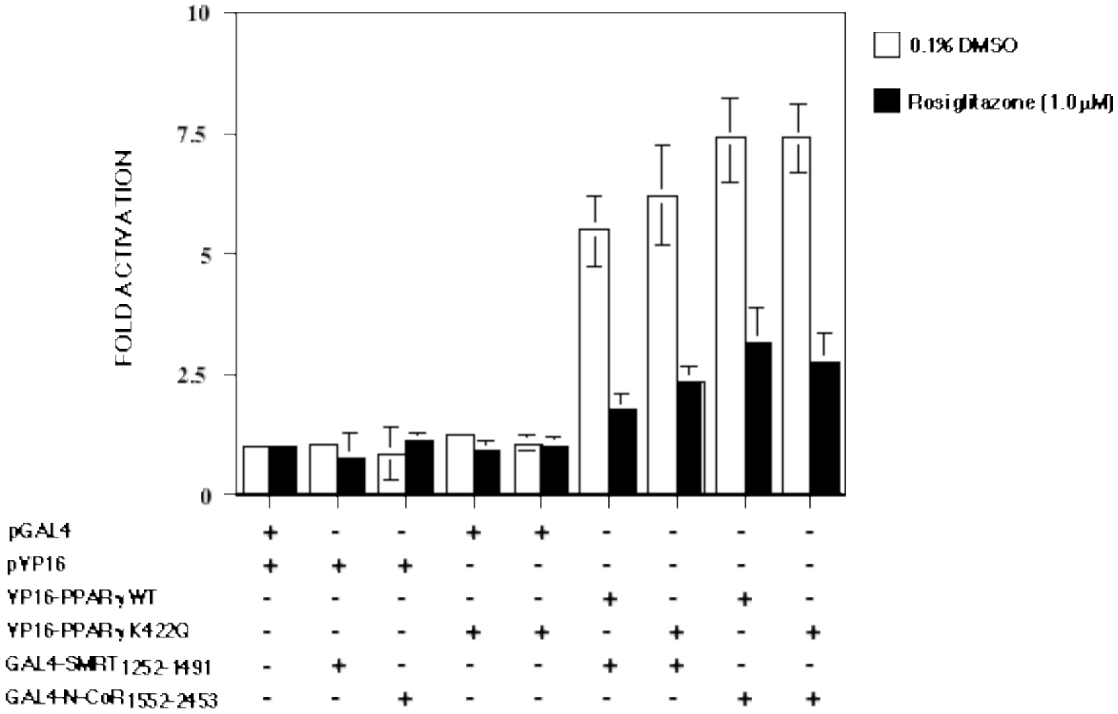

Figure 24. There is no difference between WT and K422Q PPAR in binding affinity to the co-repressors N-CoR or SMRT in solution. 293-EBNA cells were transiently transfected with pG5luc, pRL-SV40, and the indicated combinations of either pVP16, pGAL4, VP16-PPAR WT, VP16-PPAR K422Q, GAL4-$SMRT_{1252-1491}$, or GAL4-N-$coR_{1552-2453}$. Transfections were replaced with complete media containing 0.1% DMSO or 1.0 M rosiglitazone. After 48 h, cells were harvested and the dual luciferase assay was performed. Data are represented as fold activation over vehicle treated cells and represent the mean from three independent experiments each done in triplicate. *Error bars* = S.E.M.

Conclusion

We have identified four human colon cancer cell lines that are refractory to the growth inhibitory properties of PPARγ ligands. Sequence analysis of the PPARγ gene revealed that all four cell lines contain a previously unidentified point mutation in the ninth α-helix of the ligand binding domain at codon 422 (K422Q). This mutation was not found in 4 other cell lines that are all responsive to PPARγ activation. The mutant receptor did not exhibit any defects in DNA binding or RXR heterodimerization and was transcriptionally active in an artificial reporter assay. However, retroviral transduction of the mutant receptor was unable to rescue PPARγ ligand resistance while parallel studies demonstrated that the WT receptor could rescue this deficiency. Further analysis of the expression of PPARγ target genes in cells expressing the WT or K422Q mutant allele revealed that this mutation disrupts the ability of PPARγ to repress the basal expression of a subset of genes in the absence of exogenous ligand. These data suggest that this region of PPARγ may be involved in an interaction(s) necessary for non-ligand dependent transcriptional repression. They further imply that this type of repression may represent an important component of the anti-neoplastic activity of PPARγ

CHAPTER VI

PPARγ AND TRANSFORMING GROWTH FACTOR-β PATHWAYS INHIBIT COLON EPITHELIAL CELL GROWTH BY REGULATING LEVELS OF TSC-22

Introduction

The pathways that induce colon epithelial cell growth and differentiation are complex and multi-genic (Bach et al., 2000; Stappenbeck et al., 1998). In Chapters III and IV, we established that PPARγ is a regulator of colorectal cancer cell growth and differentiation. Moreover, in Chapter VI we describe experiments that demonstrate that some colon cancer cell lines have evolved mechanisms to bypass normal PPARγ signaling, emphasizing the importance of this pathway in colonocyte biology.

Another well-defined regulator of intestinal epithelial cell biology is the Transforming Growth Factor-β (TGF-β) signaling pathway. The TGF-β family of growth factors regulate a plethora of biological processes including embryonic development, wound healing, angiogenesis, proliferation and differentiation of cells (reviewed in (Blobe et al., 2000)). This latter function has been well defined in the colon, where TGF-β is a potent inhibitor of colonic epithelial cell growth (Barnard et al., 1989; Kurokowa et al., 1987). Loss of normal TGF-β responsiveness occurs commonly during the development of colorectal cancers associated with microsatellite instability and genetic lesions that disrupt the TGF-β pathway have been identified, including loss of function mutations in the TGF-β type II receptor (Grady et al., 1999; Markowitz et al., 1995), SMAD4, and SMAD2 (Eppert et al., 1996). Under these circumstances continued expression of TGF-β paradoxically leads to enhanced tumor growth through stimulation of angiogenesis, extracellular matrix production, and immunosuppression (Cui et al., 1996).

Thus, although both PPARγ and TGF-β are key regulators of epithelial cell biology, and while resistance to both pathways occurs in a subset of colorectal carcinomas, the molecular mechanisms by which either pathway induces growth inhibition and differentiation are incompletely understood. In preliminary experiments, we noticed a striking similarity in the cellular response to either TGF-β1 or PPARγ ligands. This led us to hypothesize that there may be a common subset of downstream target genes that both pathways regulate. Here we have identified Transforming Growth Factor Simulated Clone-22 (TSC-22) as a target gene of both pathways in colon epithelial cells. Functional studies with wild type and dominant negative forms of TSC-22 suggest that the gene is an important downstream component of PPARγ and TGF-β signaling during intestinal epithelial cell differentiation.

Results

Cellular Response of Colorectal Cancer Cells to PPARγ and TGF-β1

We noticed a striking similarity in the cellular response to either TGF-β1 or PPARγ ligands in colorectal cancer cells with intact PPARγ and TGF-β signaling pathways. For example, in the M-S, CBS, and FET colon carcinoma lines, exposure to the high affinity PPARγ ligand rosiglitazone (Lehmann et al., 1995) or TGF-β1 results in accumulation of cells in the G_1 phase of the cell cycle (data not shown) and a decrease in cell growth (Fig. 25A). Moreover, in all three cell lines, addition of either agent results in an increase in the p21 (Fig. 25B) and keratin 20 (data not shown). Elevated levels of the cyclin dependent kinase inhibitor p21(Tian and Quaroni, 1999; Tian and Quaroni, 1999) and keratin 20 (Calnek and Quaroni, 1993) are associated with intestinal epithelial differentiation in various model systems and p21 is a well-characterized TGF-β regulated gene(Elbendary et al., 1994). Because both PPARγ and TGF-β1 regulate such a wide spectrum of physiological processes, the genomic response to either pathway is complex.

This makes it difficult to identify those target genes of either pathway that play functionally important roles in cell growth and differentiation. We hypothesized that one way to identify relevant target genes of PPAR and TGF-1 in the setting of colon epithelial cell growth and differentiation is to focus on the common subset of downstream target genes regulated by both pathways.

Identification of TSC-22 as a PPAR and TGF- Target Gene in Colon Epithelial Cells

Microarray analysis was utilized to determine the genomic response of one of these lines, the M-S cells, after exposure to rosiglitazone or TGF-1 for 12 or 24 h. A subset of genes was commonly regulated by either pathway, including several members of the keratin and carcinogenic embryonic antigen (CEA) superfamilies. However, time course experiments suggested that these genes were not likely to be direct targets of PPAR or TGF- and may represent endpoints (rather than effectors) of the differentiation process. One promising candidate rapidly induced by either treatment was TSC-22, a gene originally identified as a TGF-1 stimulated gene in osteoblast cells (Shibanuma et al., 1992). Northern Blot analysis confirmed that either PPAR or TGF- could induce TSC-22 in the M-S, CBS, and FET cell lines (Fig. 26). We further became interested in studying this gene when *in situ* hybridization of TSC-22 in the normal colon demonstrated that its expression was enriched in the post-mitotic epithelial compartment of the normal human colon, where the most differentiated enterocytes reside (Fig. 27).

Transcriptional Regulation of TSC-22 by PPAR and TGF-1

The M-S cells were chosen as a system to study TSC-22 regulation by PPAR or TGF-1 and to test the hypothesis that TSC-22 plays a role in the ability of PPAR or TGF-1 to induce colon epithelial cell differentiation. The induction of TSC-22 by either

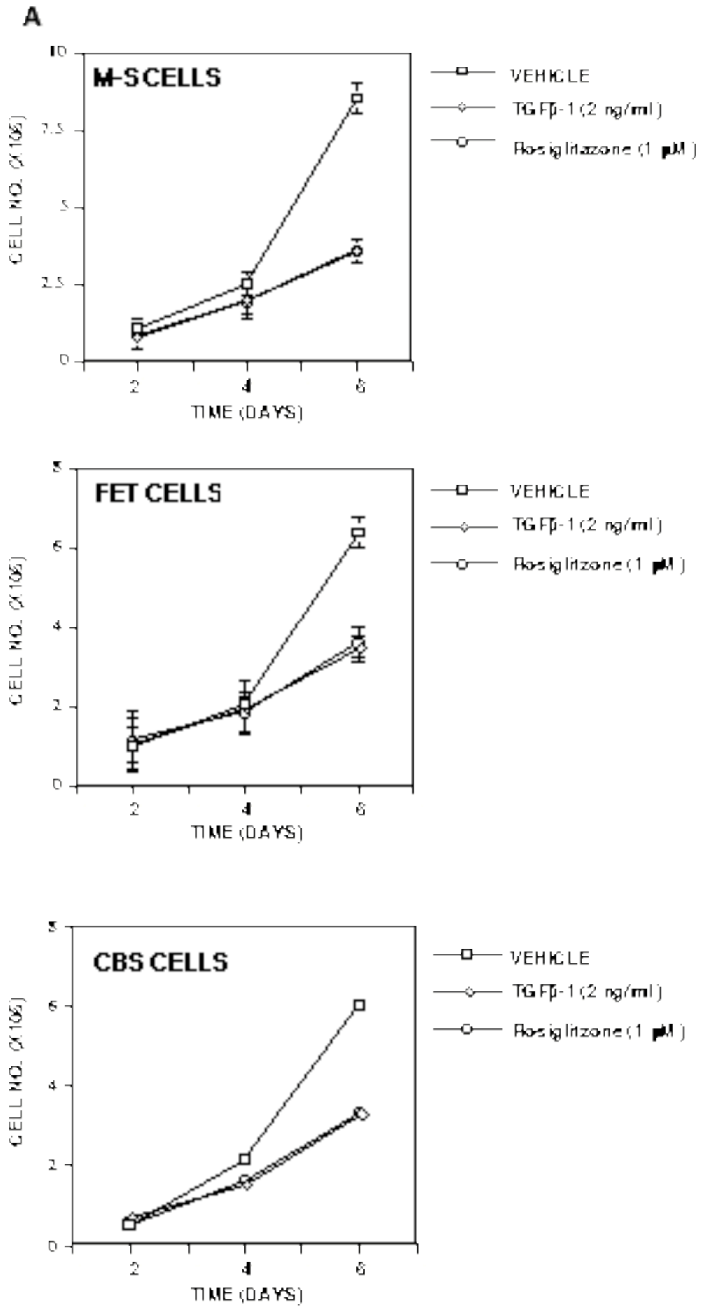

Figure 25A. The PPAR□ligand rosiglitazone or TGF-□1 induces growth inhibition and increases in protein levels of p21 in a panel of colon epithelial cell lines. A) M-S, FET, or CBS cell lines were treated with vehicle (0.1% DMSO + 0.1% BSA), 1 □M rosiglitazone or 2 ng/ml TGF-□1 and cells were counted at days 2, 4 and 6 post-treatment. Each data point represents the mean of two independent experiments, each done in triplicate. *Error bars* = s.e.m

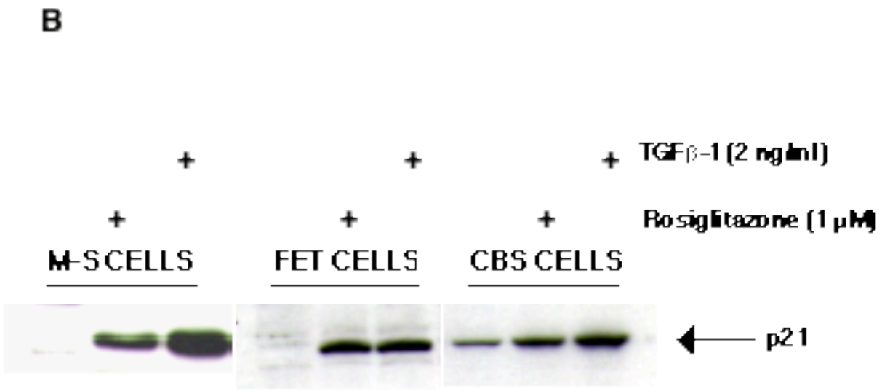

Figure 25B. The PPAR□ligand rosiglitazone or TGF-□1 induces growth inhibition and increases in protein levels of p21 in a panel of colon epithelial cell lines. (B) M-S, FET, and CBS cell lines were treated with vehicle, 1 □M rosiglitazone, 2 ng/ml TGF-□1 for four days after which total protein lysates and probed for levels of p21 by immunoblot.

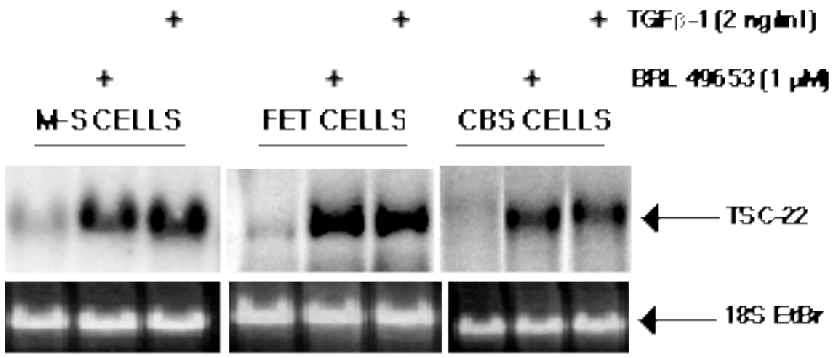

Figure 26. TSC-22 is a downstream target of both PPAR and TGF-1 in colon epithelial cells. M-S, FET, and CBS cell lines were treated with vehicle, 1M rosiglitazone, or 2 ng/ml TGF-1 for 12 h after which total RNA was collected and probed for TSC-22 levels by northern blot (20g RNA/lane).

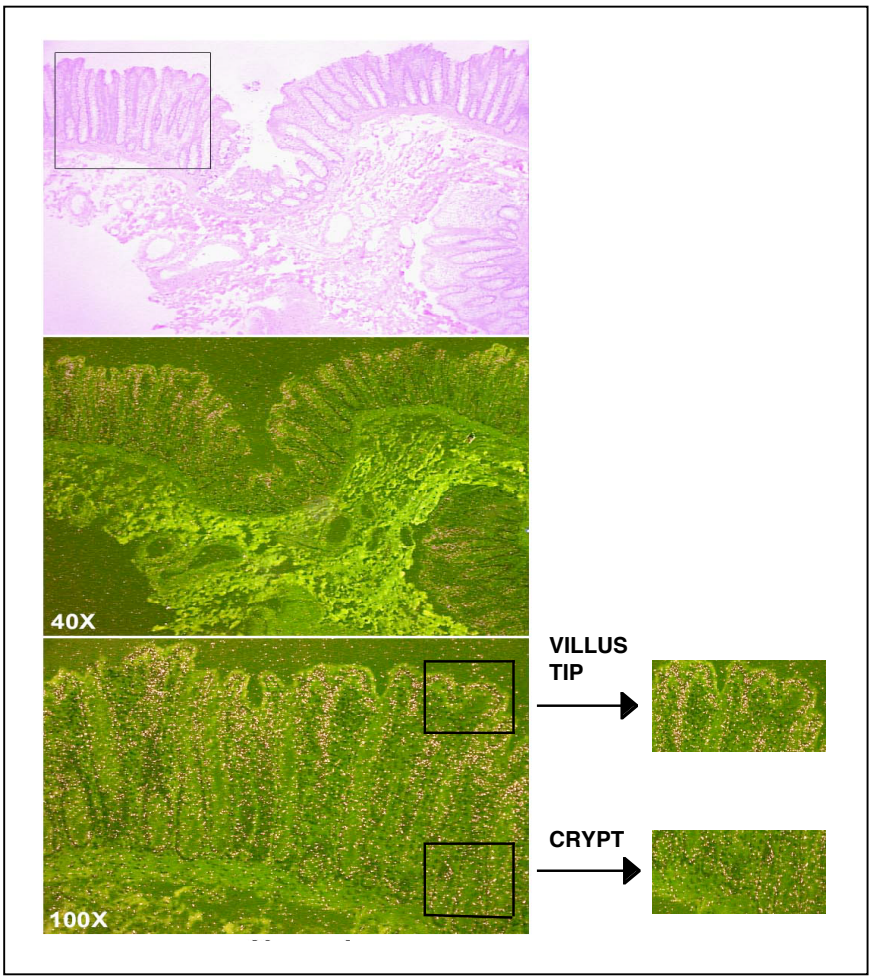

Figure 27. TSC-22 is localized to the post-mitotic epithelial compartment of the normal human colon. The surface of the human intestine is divided into an epithelial mucosal layer and submucosal layer containing supportive connective tissue, lymphatics, and vasculature. Within the mucosal layer, undifferentiated stem cells located at the base of invaginated crypts give rise to cells that migrate toward the lumen as they further differentiate into specialized enterocytes. Sections of normal colon were probed with anti-sense TSC-22 using *in situ* hybridization and a representative section is shown.

PPARγ or TGF-β1 was both time- and dose-dependent (Fig. 28A and B). TSC-22 has been shown to be a direct target gene of TGF-β1 (Shibanuma et al., 1992). We were interested in determining whether it is also a direct target of PPARγ as well. Cells were pre-treated with the protein synthesis inhibitor cycloheximide or the RNA Polymerase II inhibitor 5,6-Dichloro-b-D-ribofuranosyl-benzimidazole (DRB) followed by treatment with rosiglitazone. DRB co-treatment blocked the ability of PPARγ to induce TSC-22 suggesting that the ability of PPARγ to increase steady-state RNA levels of the gene was dependent on *de novo* transcription rather than through an increase in mRNA stability (Fig. 29A). Furthermore, while cycloheximide slightly induced TSC-22, co-treatment of rosiglitazone and cycloheximide led to a super-induction (Fig 29B), suggesting that *de novo* protein synthesis was not required for PPARγ to induce TSC-22. This last result suggested that PPARγ was not inducing TSC-22 by first increasing the levels of TGF-β and in fact rosiglitazone was not able to induce TGF-β1 in the parental M-S cells (data not shown).

As TSC-22 has not previously been shown to be a target of PPARs, we wanted to determine the specificity and selectivity of this induction. Cells were treated with two structurally distinct PPARγ ligands [the thiazolidinedione-based agonist rosiglitazone or the tyrosine-based agonist GW 7845 (Cobb et al., 1998)], an irreversible PPARγ antagonist [GW9662 (Huang et al., 1999)], or rosiglitazone plus GW9662. TSC-22 was induced by both PPARγ agonists and the induction by rosiglitazone could be blocked by co-treatment with the PPARγ antagonist (Fig. 30). Cells were also treated with a PPARα selective ligand [GW 7647(Brown et al., 2001)], a dual PPARα/γ ligand [GW 2433(Kliewer et al., 1997)], an RXR specific ligand (LG100268) or a combination of rosiglitazone and LG100268. Despite the fact that M-S cells express both PPARα and PPARδ (data not shown), neither of these other two PPAR isoforms were able to regulate TSC-22 expression (Fig. 30).

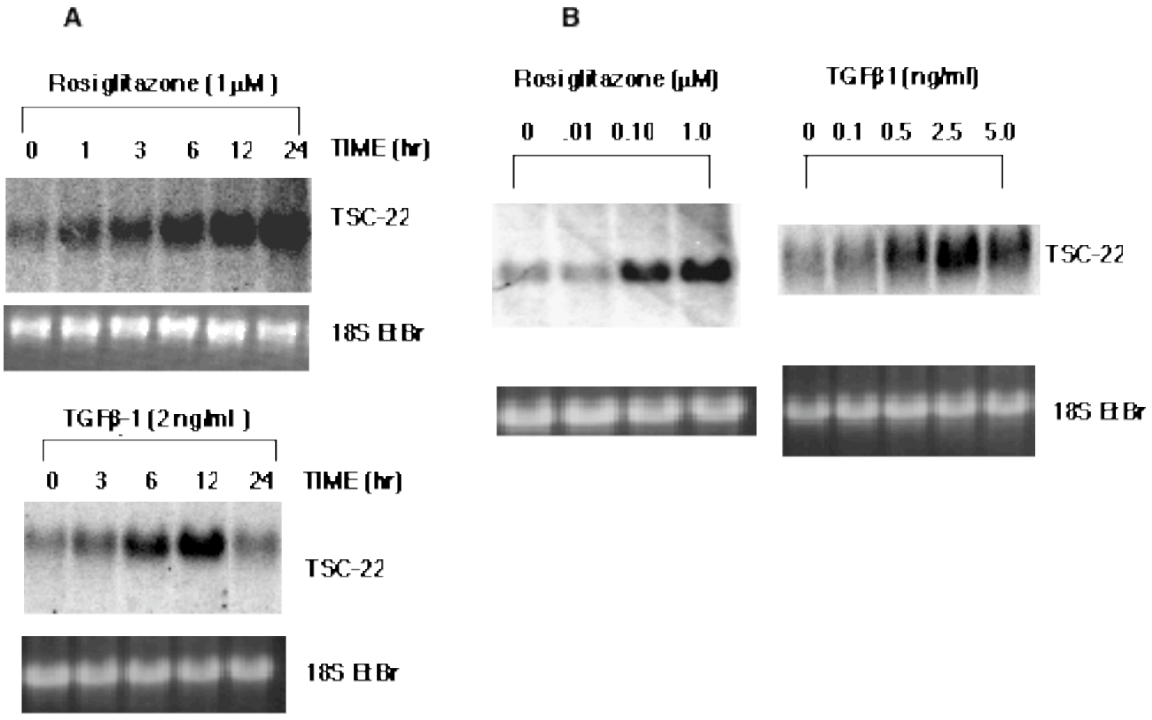

Figure 28. Time and dose dependent induction of TSC-22 by PPAR□and TGF-□1. A) Exponentially growing M-S cells were treated with 1 □M rosiglitazone or 2 ng/ml TGF-□1 and cells were harvested for RNA isolation at the indicated time points. TSC-22 mRNA levels was detected by Northern blotting (20□g total RNA/lane). B) M-S cells were treated with increasing doses of rosiglitazone (24 h) or TGF-□1 (12 h) and TSC-22 mRNA levels were detected by Northern blotting (20□g total RNA/lane).

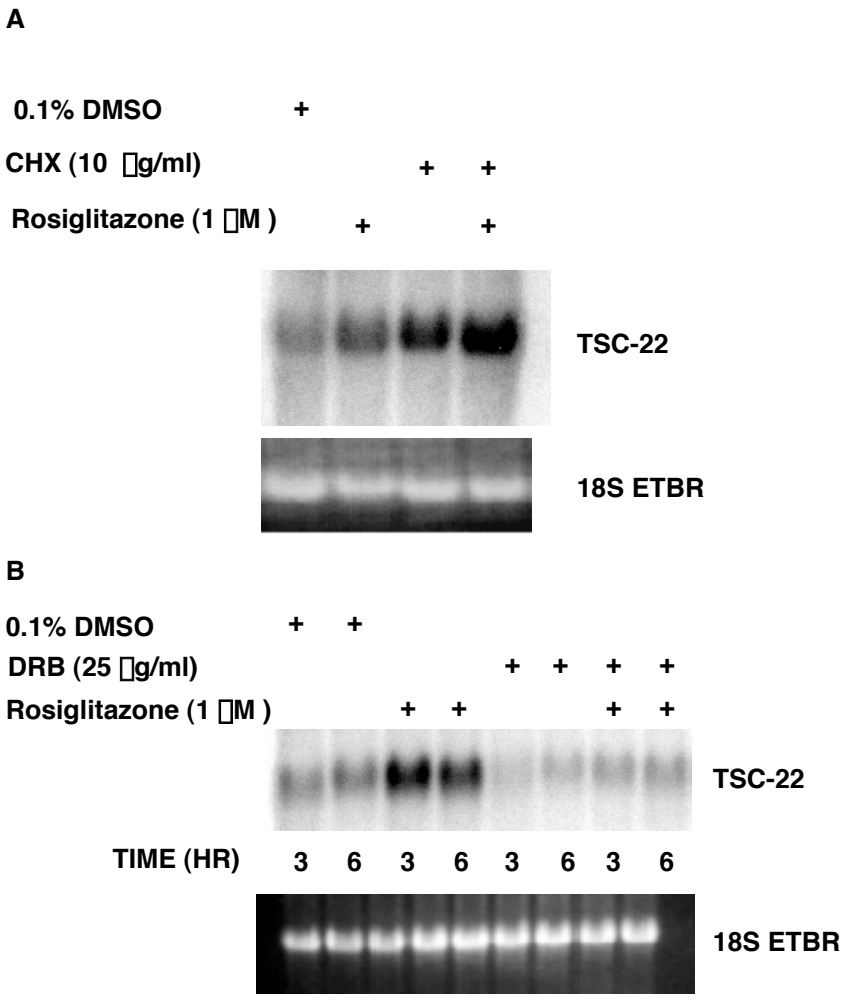

Figure 29. TSC-22 is a direct target of PPARγ. MOSER cells were pre-treated with 0.1% DMSO, 10 μg/ml Cycloheximide (CHX), or 25 μg/ml of DRB for 30 min. followed by treatment with 1 μM BRL 49653 for 3 h (CHX) or 3 and 6 h (DRB). TSC-22 mRNA levels was detected by Northern blotting (20μg total RNA/lane).

The Ability of PPARγ to Induce TSC-22 is Independent of TGF-β1

To further clarify the issue of whether the ability of PPARγ to induce TSC-22 and inhibit colon epithelial cell growth was dependent on the TGF-β pathway, the M-R cell line was utilized. The M-R line is a subclone of the parental M-S cells that are relatively refractory to the growth inhibitory effects of TGF-β. PPARγ, but not TGF-β1, could induce expression of TSC-22 in the M-R cells (Fig. 31A). Finally, an agonist of PPARγ inhibited the growth of either cell line by equivalent amounts, while the M-S-R cells (as has been previously reported) were relatively resistant to TGF-β induced growth inhibition (Fig. 31B).

Overexpression of Wild Type TSC-22 Inhibits Colon Epithelial Cell Growth and Induces Elevated Levels of p21

The functional role of TSC-22 in mediating any of the biological effects induced by either PPARγ or TGF-β is unknown. To address this issue we focused on two experimental strategies: 1) to determine if expression of TSC-22 in the M-S cells could recapitulate any of the phenotypic changes induced by PPARγ or TGF-β1 and 2) to determine whether inhibition of normal TSC-22 function blocks the phenotypic changes induced by either treatment. Kester *et al*. have defined 4 functional domains within TSC-22 (Kester et al., 1999) (Fig. 32A). Using both GST-pull down and mammalian two-hybrid assays, they demonstrated that TSC-22 could form homodimers via the leucine zipper (LZ) domain. They also showed that TSC-22 has transcriptional repressor activity when fused to a heterologous DNA-binding domain and identified two domains within TSC-22, Repressor Domains (RD) 1 and 2, which were in large part responsible for this effect. Finally, they were able to demonstrate that titration of a mutant TSC-22 in which both RD1 and 2 were deleted could inhibit the repressor activity of the wild-type (wt) protein and thus act as a dominant-negative (dn) inhibitor. Based on these results, we constructed three different HA-tagged TSC-22 constructs for stable introduction of this

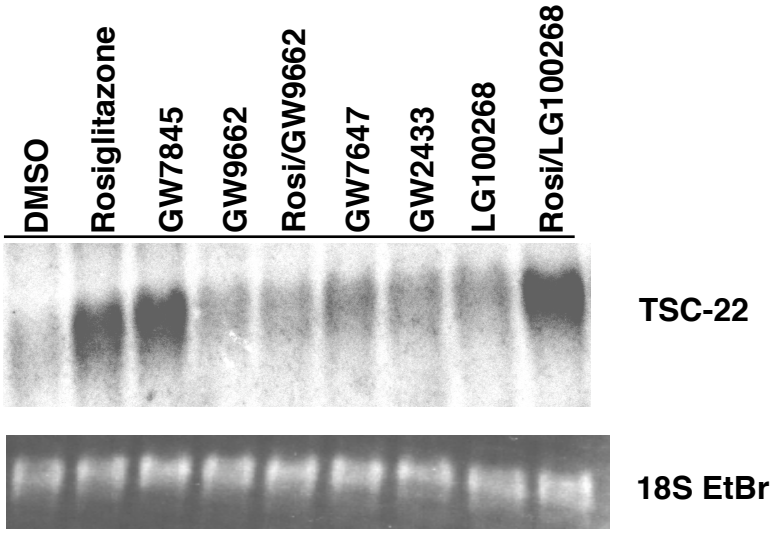

Figure 30. TSC-22 is specifically and selectively induced by PPAR M-S cells were treated with the following compounds for 24 h (dose and PPAR subtype selectivity in parentheses): rosiglitazone (1 M, PPAR), GW7845 (1 M, PPAR), GW9662 (5 M, PPAR antagonist), Rosi + GW 9662, GW7647 (1 M, PPAR), GW2433 (1 M, PPAR and PPAR), LG100268 (0.5 M, RXR), and Rosi + LG100268. TSC-22 mRNA levels were detected by Northern blotting (20 g total RNA/lane).

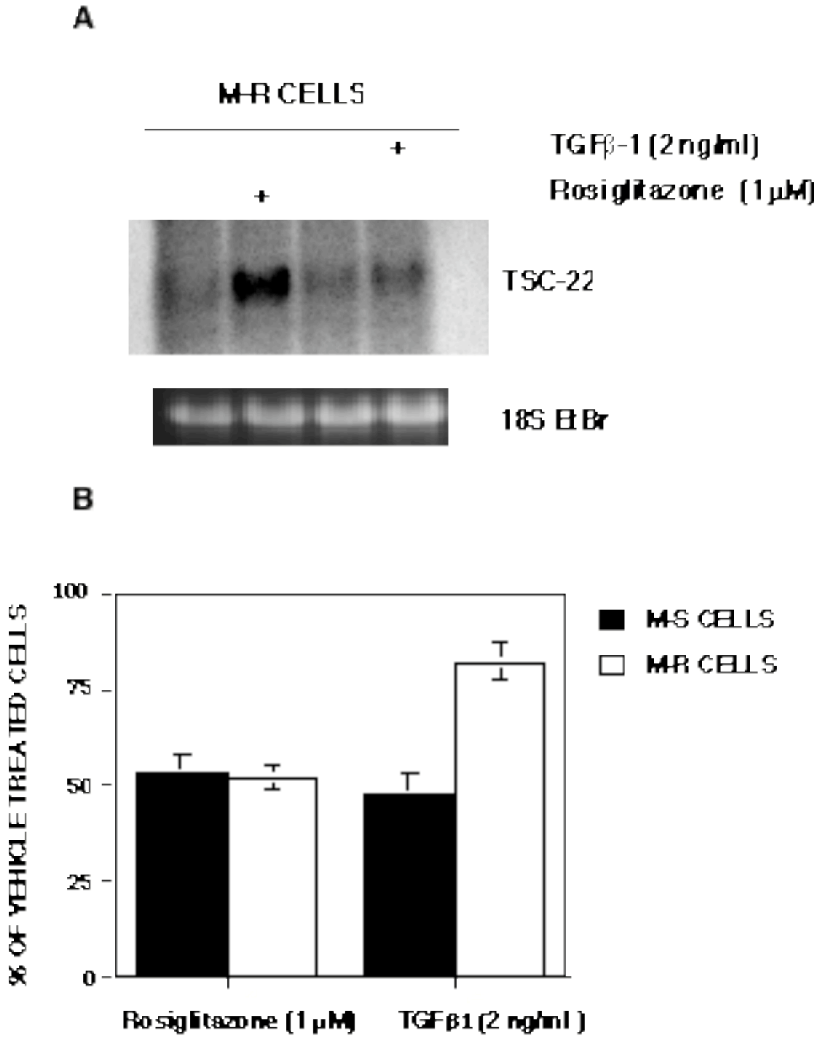

Figure 31. The induction of TSC-22 by PPAR□is not dependent on an intact TGF-□1 signaling pathway. A) M-R cells (a naturally identified TGF-□1 resistant clone) were treated with 1 □M rosiglitazone or 2 ng/ml TGF-□1 for 12 h. TSC-22 mRNA levels were detected by Northern blotting (20□g total RNA/lane). B) M-S and M-R cells were treated with vehicle (0.1% DMSO + 0.1 % BSA), 1□M rosiglitazone, or TGF-□1 for 6 days after which time the number of viable cells were counted. Values are expressed as percent of vehicle treated cells. Each experiment was performed in triplicate and the values represent the mean of two independent experiments. *Error bars* = s.e.m.

gene into the M-S cells: TSC-22 wt, TSC-22 dn (in which both RD 1 and 2 were deleted) and TSC-22 dncon (which, in addition to both RD domains being deleted, contains mutations in two highly conserved leucine residues within the LZ domain) (Fig. 32A). Co-immunoprecipitaton experiments confirmed that TSC-22 dn, but not TSC-22 dncon, could dimerize with TSC-22 wt (Fig. 32B). This last construct was made to help properly interpret any potential artifacts due to expression of TSC-22 dn that were independent of its ability to dimerize and inhibit the function of the wild-type protein.

Each of these three constructs (plus empty vector) was used to generate eight unique pools of stably transfected cells. Each of the pooled cell lines was found to express relatively equivalent protein levels of the integrated cDNA (Fig. 32C and D). The two different pools of M-S cells expressing TSC-22 wt were found to have significantly reduced growth rates compared to vector transfected cells (Fig. 33A). They also displayed higher levels of p21 protein but showed no difference in the levels of keratin 20 protein (Fig. 33B).

Overexpression of Dominant Negative TSC-22 Partially Inhibits PPARγ ligand and TGF-β1 Induced Growth Inhibition and p21 Induction

We next tested the ability of TSC-22 dn and TSC-22 dncon to block the ability of PPARγ or TGF-β to inhibit the growth of M-S cells. In the two different pools of cells expressing TSC-22 dn, the ability of PPARγ or TGF-β1 to inhibit growth was reduced by approximately 60% (Fig. 34A). Importantly, no differences in the inhibitory activity of either PPARγ or TGF-β1 were seen when comparing vector and TSC-22 dncon transfected cells (Fig. 34A). Northern blot analysis confirmed that PPARγ and TGF-β1 could still induce TSC-22 in these cell lines (data not shown). Finally, the ability of rosiglitazone or TGF-β1 to induce p21, but not keratin 20, was greatly diminished in M-S cells expressing TSC-22 dn but not TSC-22 dncon (Fig. 34B).

Conclusion

PPAR and TGF- are key regulators of epithelial cell biology. However, the molecular mechanisms by which either pathway induces growth inhibition and differentiation have remained incompletely understood. In this study, we identified Transforming Growth Factor Simulated Clone-22 (TSC-22) as a target gene of both pathways in intestinal epithelial cells. TSC-22 is member of a family of leucine zipper containing transcription factors with repressor activity. Although little is known regarding its function in mammals, the *Drosophila* homolog of TSC-22, *bunched*, plays an essential role in fly development. The ability of PPAR to induce TSC-22 was not dependent on an intact TGF 1 signaling pathway and was specific for the isoform. Localization studies revealed that TSC-22 mRNA is enriched in the post-mitotic epithelial compartment of the normal human colon. Cells transfected with wild-type TSC-22 exhibited reduced growth rates and increased levels of p21 compared to vector transfected cells. Furthermore, transfection with a dominant negative TSC-22 in which both repressor domains were deleted was able to reverse the p21 induction and growth inhibition caused by activation of either the PPAR or TGF- pathways. These results place TSC-22 as an important downstream component of PPAR and TGF- signaling during intestinal epithelial cell differentiation.

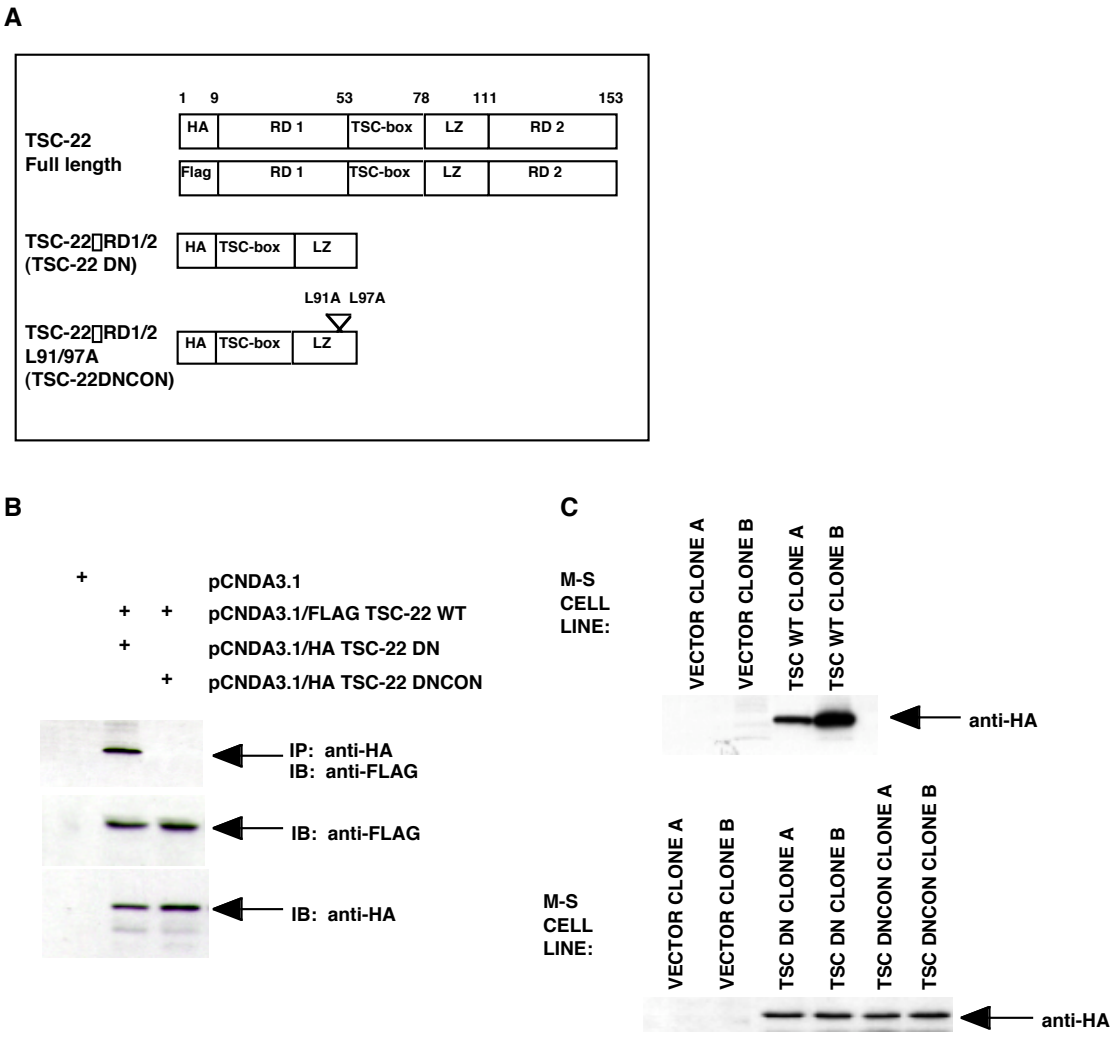

Figure 32. Expression of Full-length and Mutant TSC-22 constructs. A) Schematic of TSC-22 constructs outlining the following domains: RD1 and 2 (Repressor Domains 1 and 2), the TSC-box, and LZ (leucine zipper domain). TSC-22ΔRD1/2 (TSC-22 DN): The RD 1 and 2 domains were deleted. TSC-22ΔRD1/2L91/97A (TSC-22 DNCON): The RD1 and 2 domains were deleted and two highly conserved leucine residues in the LZ domain were mutated to alanine (L91A and L97A) to disrupt its ability to dimerize with WT TSC-22. B) TSC-22 DN, but not TSC-22 DNCON, can dimerize with TSC-22 WT. Cos7 cells were transiently transfected with the indicated plasmid and lysates were used for straight immunoblotting (50 μg total protein/lane) or immunoprecipitaton (500 μg total protein/lane) followed by immunoblotting. C and D) Overexpression of full-length and mutant TSC-22. Immunoblot analysis to detect stable expression of C) TSC-22 WT and D) TSC-22 DN and TSC-22 DNCON in individual pooled clones of zeocin resistant M-S cells. For both gels, 50 μg of total protein was loaded per lane and probed with an anti-HA antibody.

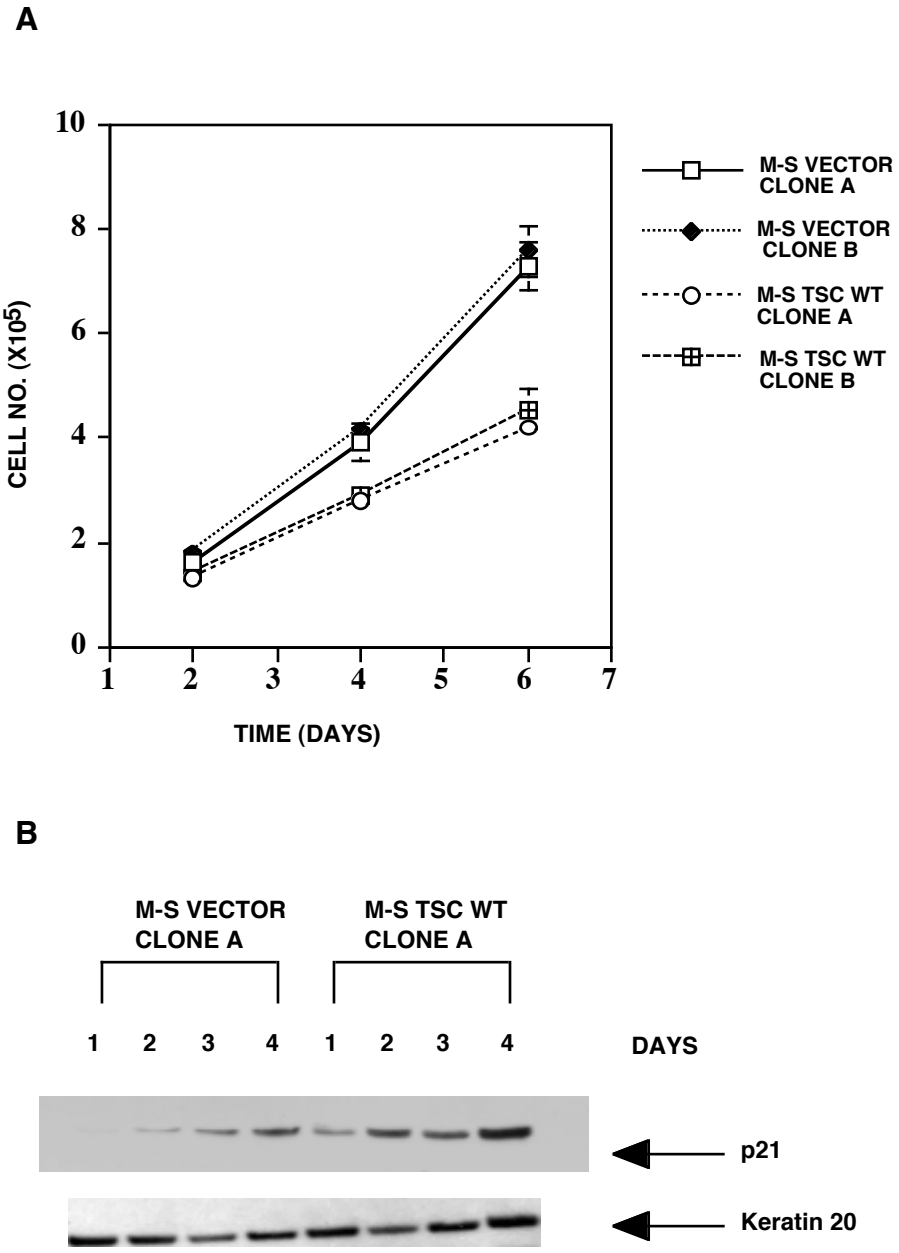

Figure 33. Wild type TSC-22 inhibits cell growth and leads to increased levels of p21, but not keratin 20. A) Each pooled line was plated and viable cells were counted at 2, 4, and 6 days. Each experiment was done in triplicate and each data point represents the mean of two independent experiments. *Error bars* = s.e.m. B) M-S Vector clone A and M-S TSC WT clone A cell lines were plated and protein lysates were collected at days 1-4. 50 □g of total protein was loaded on each lane and membranes were probed with anti-p21 or anti-keratin 20 antibodies.

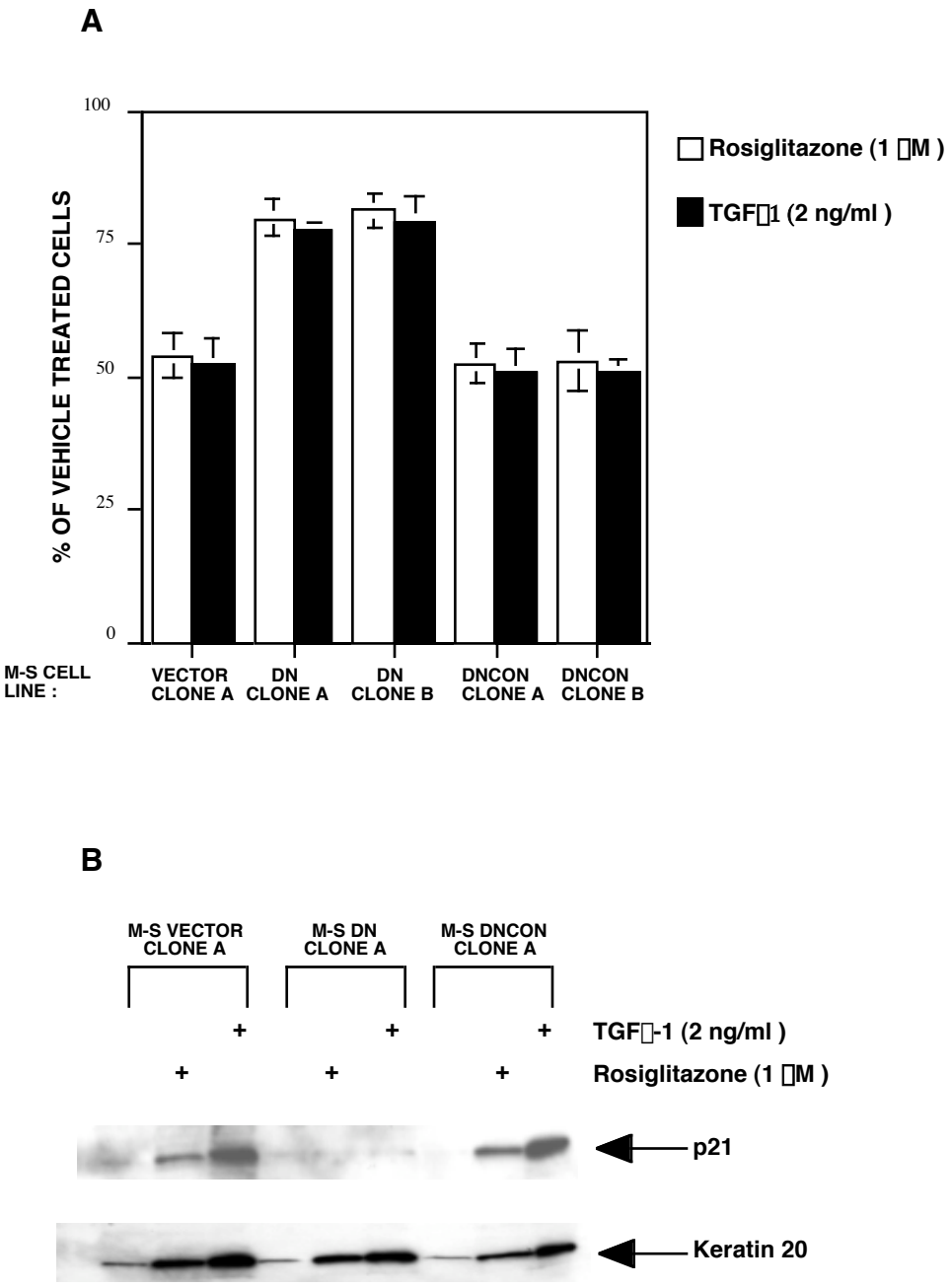

Figure 34. Dominant negative TSC-22 blocks the ability of PPARor TGF- to induce p21 and inhibit cell growth. A) Each indicated cell line was plated and the number of viable cells were counted at day 6 following treatment with 1 M rosiglitazone or 2 ng/ml TGF-1. Values are represented as % of vehicle treated cells and represent the mean from two independent experiments. *Error bars* = s.e.m. B) Each indicated cell line was treated with vehicle, 1M rosiglitazone, or 2 ng/ml of TGF-1 and protein lysates were collected after 4 days. 50 g of total protein was loaded and membranes were probed with anti-p21 or anti-keratin 20 antibodies.

CHAPTER VII

DISCUSSION

Activation of PPARγ Inhibits Colorectal Cancer Cell Growth

The nuclear hormone receptor PPARγ has traditionally been defined as a central regulator of adipocyte differentiation and most of the known target genes of the receptor are involved in lipogenesis and peripheral glucose utilization. In the set of experiments described in Chapter III PPARγ expression was detected in four different colon cancer cell lines and we observed activation of PPARγ mediated transcription in three of the four cell lines. We further demonstrate that activation of PPARγ in these cell lines inhibits cellular proliferation both *in vitro* and *in vivo*. The decrease in cell growth is likely due to the ability of the agonist occupied receptor to induce an increase in the G_1 phase of the cell cycle. As mentioned in Chapter I, Altiok *et al*. have reported that PPARγ activation can lead to G_1 cell cycle arrest in both fibroblast and SV40 transformed adipogenic HIB1B cells by a mechanism involving the down regulation of protein phosphatase 2A (PP2A). However, treatment of the HCT-15-G25 cells with rosiglitazone does not decrease the level of PP2A present in these cells (data not shown), suggesting that an alternative pathway for cell cycle arrest is involved in colorectal cancer cells.

We have also identified a cell line, the HCT 15 cells, which does not exhibit PPARγ functional activity and are resistant to PPARγ ligand induced growth inhibition both *in vitro* using an anchorage independent growth assay soft agar and *in vivo* using the nude mouse xenograft assay. However, we can restore ligand sensitivity by introducing a functional PPARγ receptor. Based on these experiments, it is unlikely that the anti-proliferative effect of the PPARγ synthetic agonist rosiglitazone is through a non-PPARγ target since the HCT 15 cell line is only sensitive to the compound when we introduce a functional receptor.

In summary, these initial experiments define PPARγ as an inhibitor of colorectal cancer cell growth. It does not appear that activation of the receptor is inducing trans-differentiation into adipocytes since treatment of these colorectal cancer cell lines with a PPARγ ligand did not induce an increase in lipid accumulation (as measured by Oil Red O staining). However, it is possible that activation of the receptor is important in initiating a terminal differentiation pathway during the normal development of an adult intestinal epithelial cell. Our initial studies led us to consider two major issues: 1) To determine the underlying mechanisms for the anti-proliferative effects caused by PPARγ activation in colon cancer cells and 2) To determine whether there is selective pressure for cells to harbor dysfunctional PPARγ during the development of colorectal carcinoma. Experiments described in Chapters IV-VI and discussed below attempt to address these issues.

Target Genes of PPARγ in Colorectal Cancer Cells

The genomic response to activating ligands for PPARγ in colorectal cancer cells is not known. In the experiments described in Chapter IV, we have used microarray technology to identify PPARγ target genes in the M-S colon carcinoma line. A majority of what is known about PPARγ and its target genes in other cell types has been gathered through the use of high affinity synthetic PPARγ agonists. However, these compounds are likely to have receptor-independent effects, particularly at high drug concentrations. For example, high micromolar levels of troglitazone or ciglitazone will inhibit inflammatory related genes in macrophages null for PPARγ (Chawla et al., 2001). Genes identified in this study are likely to be true PPARγ targets because their change in expression was seen in response to low concentrations of PPARγ agonists from two different chemical families and the effect could be blocked by co-treatment with a PPARγ antagonist.

Of the nine PPARδ regulated genes identified here, only L-FABP (Poirier et al., 1997) and CEA (Sarraf et al., 1998) have previously been proposed as PPAR targets. The three CEA family genes and keratin 20 were only induced after more than 48 h of ligand treatment with maximal induction occurring after six days. Because of this, it seems unlikely that PPARδ directly regulates these four genes. On the other hand, adipophilin, L-FABP, Reg IA, Gob-4, and NGAL were all induced or repressed as early as 6 h following ligand treatment and the change in expression could be blocked by co-treatment with the protein synthesis inhibitor cyclohexamide (data not shown). Thus, these five genes are likely direct PPARδ target genes. Only L-FABP has been reported to contain a functional PPRE in its 5' regulatory region (Poirier et al., 1997). It is not known how many of the other genes identified in this study also contain functional PPREs. This would be particularly interesting to know for the two genes, Reg IA and Gob-4, which are repressed by PPARδ activation. No negative *cis* PPAR response elements that dictate ligand dependent repression have been reported. It is possible that PPARδ represses these two genes via a transrepression mechanism that involves competition for limiting amounts of coactivators (Li et al., 2000).

Most colon cancer cells express all three PPAR subtypes. Why activation of PPARδ, but not PPARα or PPARγ, is capable of inducing inhibition of colon cancer cell growth and differentiation is not known. Presumably, there are select target genes that only PPARδ is capable of regulating. Experiments reported here support such a hypothesis. Adipophilin and L-FABP, genes with roles in "traditional" PPAR related functions such as fatty acid transport and storage, are not selectively regulated by PPARδ. In contrast, genes that regulate cell proliferation (Reg IA) or are linked to colonocyte maturation (keratin 20) are selectively induced by PPARδ agonists. The molecular basis for this type of specificity may be due to some combination of unique *cis*-acting sequences within certain target gene promoters and ligand dependent interactions with isoform specific co-factors. Castillo *et al.* reported that the ability of PPARδ to uniquely

induce adipoycte differentiaton was due to the N-terminal AF-1 domain of the receptor (Castillo et al., 1999). This group further determined that this region binds to a cofactor, PCG-2, that does not interact with other PPAR subtypes. It would be of interest to know if this same domain of PPAR is necessary for the selective activation of target genes reported here. There may also be unique epithelial cell co-factors that play a role in dictating PPAR subtype specificity.

Two of the more interesting PPAR target genes identified in this study are Reg IA and NGAL. Reg IA encodes a 166 amino acid secreted protein that was cloned on the basis of its rapid induction in islets of Langerhans cells that were forced to regenerate following depancreatization (Terazono et al., 1988). Reg gene products bind to a cell surface receptor and have been shown to stimulate the proliferation of both pancreatic -cells and ductal cells (Kobayashi et al., 2000). Recently, it was shown that transgenic mice expressing Reg IA in islet cells develop multiple tumors including cervical lymphoma, ovarian adenocarcinoma and hepatoceullar carcinoma (Yamaoka et al., 2000). The non-pancreatic effects of Reg IA in this system were presumably due to the high serum levels of Reg IA in the transgenic mice. Reg IA is over-expressed in a large percentage of colorectal tumors (Rechreche et al., 1999) and is also strongly expressed in the regenerating crypt epithelial cells of patients with ulcerative colitis(Shinozaki et al., 2001). Furthermore, Reg IA levels negatively correlate with survival rates following curative surgery of patients with colorectal cancer (Macadam et al., 2000). In tissue culture, Reg I levels negatively correlate with the differentiation status of colon cancer cells (Bernard-Perrone et al., 1999). Collectively, these data argue that excessive Reg IA may act as a factor that inappropriately stimulates intestinal epithelial proliferation and regeneration in pathological settings. The fact that PPAR negatively regulates Reg IA expression may in part explain the ability of the receptor to reduce the malignant potential of colon cancer cells or the level of tissue remodeling during chronic inflammation of the intestine. Because Reg IA is a secreted protein that can be detected

in the blood (Carrere et al., 1998), serum levels of Reg IA might be also a useful clinical indicator of the efficacy of PPAR ligand therapy in patients with colorectal cancer or inflammatory bowel disease. Finally, Reg IA over-expression in pancreatic islet cells induces diabetes (presumably via stimulation of excess islet cell regeneration)(Yamaoka et al., 2000). Therefore, the observation that PPAR ligands limit pancreatic cell injury and depletion in rats predisposed to insulin resistance (Buckingham et al., 1998) may be due to its ability to down-regulate Reg IA expression in the pancreas.

NGAL is a member of the lipocalin family (Kjeldsen et al., 2000). Members of this family are characterized by their ability to bind and transport lipophilic molecules such as retinoids. It is possible that NGAL may be involved in pathways that regulate PPAR ligand availability. Although NGAL was originally isolated as a component of the neutrophilic granule, it is now known to be expressed in epithelial cells from multiple organs (e.g. lung, stomach, colon) that are exposed to microrgansims (Cowland and Borregaard, 1997). Consistent with this, NGAL can bind bacterial formylpeptides and may act as a scavenger receptor to limit the pro-inflammatory effects of these molecules (Bratt et al., 1999). Other pro-inflammatory ligands that NGAL can bind to and potentially sequester are LTB_4 and platelet activating factor. The cyclooxygenase signaling pathway is often induced at sites of inflammation and produces ligands that can act as endogenous activators of PPAR . The ability of ligand activated PPAR to induce anti-inflammatory genes such as NGAL may represent a negative feedback loop to limit the extent of the inflammatory response.

The role of CEA and related genes NCA and BGP in colon epithelial cell biology are complex. In the normal colon, CEA, NCA, and BGP are expressed in the most differentiated epithelial cells exposed to the lumen (Frangsmyr et al., 1999). It has been suggested that CEA proteins might regulate rapid adhesive interactions between microvilli. Alternatively, since CEA-surface proteins can act as receptors for bacteria and viruses, these proteins could play an important role in epithelial-microbial

interactions. It is possible that PPARγ might regulate either of these processes in the normal colon by up-regulating CEA family proteins. In colorectal tumors (which often have very high levels of CEA) and in the embryonic intestine, CEA is found on adjacent cell membranes (Benchimol et al., 1989). In these circumstances, it has been proposed that CEA is an important component of the intercellular adhesive forces that allows the epithelium to develop into a multi-layered array. This would suggest that unregulated levels of CEA in the normal adult colon could be pro-oncogenic and in fact overproduction of CEA blocks the differentiation program of rat myoblast cells(Eidelman et al., 1993). We have shown that PPARγ ligands induce an increase in CEA-dependent intercellular adhesion. This could point to a role for PPARγ in maintaining proper tissue architecture during embryonic development of the intestine. It might also explain the pro-tumorigenic effects of PPARγ ligands in some model systems.

A Loss of Function Allele in Colorectal Cancer Cells Caused by a Mutation that Disrupts Basal Transcriptional Repression

In Chapter III we described experiments that demonstrate that activation of PPARγ inhibits cell growth and promotes the differentiation of colorectal cancer cells. In Chapter V, we describe the identification of four colorectal cell lines that are resistant to PPARγ ligand induced growth inhibition. Each cell line contained a point mutation in one allele of the PPARγ gene in that resulted in a change from Lys to Gln at codon 422 (K422Q). We also provide functional evidence to indicate that the ligand activated mutant receptor is unable to inhibit the growth of colorectal cancer cells, establishing K422Q PPARγ as a pathogenic, loss of function mutation. Our results support earlier findings by Sarraf *et. al* who identified 4 out of 55 primary colorectal tumors that harbored loss of function somatic mutations in either exon 3 or 5 of the PPARγ gene (Sarraf et al., 1999). Clearly, a subset of colon epithelial cells undergo selection for loss

of function mutations in PPAR as a component of the genetic lesions that eventually cause the development of colorectal carcinoma.

Information on the four patients from whom the HCT 15, MIP101, Clone A, and COLO 205 cell lines were derived is limited. Thus, it is unknown whether the K422Q mutation is a germline mutation or if it only occurred in the primary colorectal tumors of these individuals. The K422Q mutation was not found in an earlier study screening for PPAR gene mutations in 55 primary colorectal tumors. A more recent study failed to detect any PPAR gene mutations in a large number of clinical samples including cancers derived from the breast, colon, prostate, and lung (Ikezoe et al., 2001). The conclusion of the study was that loss of function mutations of the receptor is extremely rare in cancer. However, this study limited its screen to exons 3 and 5 of PPAR The K422Q mutation lies in exon 6 and our results emphasize that studies designed to screen for PPAR gene mutations in cancers should be extended to include exons spanning the entire coding region. We are currently screening clinical samples of colorectal cancer to determine the incidence of the K422Q mutation in primary colorectal tumors. It will also be of interest to know if the K422Q mutation is associated with metabolic syndromes such as insulin resistance or obesity for which germline PPAR mutations have previously been identified (Barroso et al., 1999; Ristow et al., 1998).

The K422Q mutation in the four resistant cell lines was only identified in one allele of the gene while the other allele encoded for wild-type receptor. Similarly, in the earlier report by Sarraf and colleagues, the four PPAR mutations found in primary colorectal cancers were all monoallelic with no evidence for loss of heterozygousity. However, it is not clear in these instances if the remaining WT receptor is even being expressed. In the HCT 15 cells, 10 independent PPAR cDNA fragments that span exon 6 were cloned and sequenced using RT-PCR; all 10 clones contained the K422Q mutation (data not shown). This would suggest that, at least in this cell line, the WT receptor is not expressed or is present at very low levels. In fact, in tumors that contain

one mutated allele of PPARγ, the other allele might be silenced through alterative mechanisms (*e.g.* promoter methylation).

Nevertheless, PPARγ does not appear to fit the classical Knudtson "two hit" hypothesis in which both alleles of a tumor suppressor are genetically inactivated. As opposed to studies which are limited only to analysis of DNA from primary colorectal cancers, our experiments with established cell lines has allowed us to conduct functional experiments which clearly establish that colon cancer cells with one mutant and one wild-type PPARγ allele are resistant to PPARγ ligand induced growth inhibition. In theory, this loss of normal PPARγ signaling could occur through a dominant-negative or haploinsuffiency mechanism. It is possible that in a situation where both WT and K422Q PPARγ are co-expressed in the same cell line, the K422Q receptor could out-compete the WT receptor for a limiting number of binding sites (*e.g.* to RXR or to specific DNA elements) and thus "inhibit" the function of the WT receptor. Alternatively, it could simply be a gene dosage effect. *Pparγ*$^{+/-}$ embryonic stem cells have a reduced capacity to differentiate into adipocytes as compared to WT cells, establishing that haploinsufficiency can occur for the PPARγ locus(Rosen et al., 1999).

The molecular mechanisms by which PPARγ regulates colorectal carcinoma growth remain obscure. Because the K422Q mutation blocks the ability of the receptor to repress basal transcription in the absence of ligand, our data infer that an important component of the anti-neoplastic activity of PPARγ is as a transcriptional repressor of certain target genes. We have identified two genes, TSC-22 and Mox-2, that K422Q PPARγ is incapable of repressing in the absence of exogenous ligand. Mox-2 is a homeobox gene important for mesoderm formation. However, because there is nothing known about the signaling pathways that Mox-2 is involved in, it is difficult to speculate as to why a lack of Mox-2 repression (or the lack of Mox-2 inducibility in response to a PPARγ ligand) may contribute to tumor growth.

TSC-22, on the other hand, has previously been identified as a negative regulator of carcinoma cell growth (Nakashiro et al., 1998) and we have evidence suggesting that PPAR inhibits colorectal cancer cell growth in part through its ability to induce TSC-22 (See Chapter VI). This would help explain why the HCT 15 cells undergo growth inhibition in response to PPAR ligands only when the WT receptor is expressed, since only in this case is there an actual amplification of TSC-22 expression upon ligand exposure. However, there are many more PPAR target genes than those examined in this study, and it may be that selection for loss of PPAR function in colon carcinoma is unrelated to control of cell cycle and proliferation. For example, PPAR can regulate the inflammatory response in colorectal cancer cells and it may be that some tumors undergo selection for the K422Q mutation in order to have higher basal levels of certain anti-inflammatory genes as means to evade the immune system.

The importance of nuclear hormone receptor mediated transcriptional repression is just now being fully realized. In the syndrome of thyroid hormone resistance, severity of the disease has in some instances correlated with the degree of binding between co-repressor and the mutant TR (Yoh et al., 1997). In addition, mice with all TRs deleted have a less severe phenotype than mice with deletions of thyroid hormone, suggesting that activities of unliganded TR (one of which would be basal repression) are important in normal physiology (Gothe et al., 1999). However, to our knowledge no prior studies have documented any biological role for PPAR mediated gene repression in the absence of exogenous ligand (as compared to the transrepression activity of PPAR, in which addition of ligand inhibits the induction of certain pro-inflammatory genes (Li et al., 2000)). In fact, it may be that the type of transcriptional repression described here is only important for certain biological activities of PPAR. For example, we found no difference in the ability of WT or K422Q PPAR to induce fibroblasts to differentiate into adipocytes (data not shown), emphasizing the distinct mechanisms by which PPAR induces adipocyte versus colonocyte differentiation.

It is unlikely that the repression described here is dependent on binding to either N-CoR or SMRT since there was no difference in binding affinity between either of these two proteins and WT or K422Q PPAR. However, it is not clear if N-CoR and SMRT are physiologically relevant PPAR binding proteins or whether PPAR is even capable of repressesing gene transcription when bound to a typical PPRE. Unlike the case with the TR, transfection of PPAR does not repress transcription of a reporter gene driven by its cognate DNA response element. Consistent with this, Zamir *et al*. demonstrated that while both PPAR and TR could bind to these two corepressors in solution, only TR could do so when bound to DNA (Zamir et al., 1997).

We do not currently know why the K422Q mutation blocks the ability of apo-PPAR to repress TSC-22, Mox-2, and potentially other unidentified target genes. This type of repression could either be "active" in which a PPAR-corepressor complex is mediating the repression. In this case, K422Q mutation might disrupt binding to an as yet unknown PPAR corepressor. Alternatively, it could be a "passive" repression in which case WT but not K422Q PPAR can saturate specific DNA sequences in a target gene 5'regulatory region or bind to limiting amounts of certain transcription factors or co-factors that would then prevent transcriptional activation.

PPAR and TGF-1 Pathways Inhibit Colon Epithelial Cell Growth by Regulating Levels of TSC-22

The complex mechanisms by which the undifferentiated stem cells of the intestine give rise to differentiated cells with specialized functions remain incompletely understood. Some of the key pathways that govern this process in the intestine have been identified from understanding genetic lesions found in colorectal cancer (Chung, 2000). Loss of function mutations have been identified in genes involved in the TGF- signaling pathway and PPAR. Activation of either of these pathways in cell culture models can lead to growth inhibition and the induction of markers of differentiation. These

experiments emphasize the biological relevance of these two pathways in intestinal epithelial cell biology. In the studies described in Chapter VI, we used microarray technology to identify TSC-22 as a target gene of both PPAR and TGF- in intestinal epithelial cells. We further show using wild type and dominant negative forms of TSC-22 that this gene plays an important role in the ability of either PPAR or TGF- to inhibit cell growth.

There are several reasons why the results presented here are likely to be biologically relevant. First of all, TSC-22 is localized to the most differentiated epithelia in the normal human colon. PPAR and TGF-1 have previously shown to be most strongly expressed *in vivo* in the most differentiated epithelial cells of the colonic mucosa (Avery et al., 1993; Mansen et al., 1996). Thus, all three of these genes appear to be co-localized in the normal human colon to the most differentiated enterocytes. Secondly, PPAR and TGF-1 were shown to regulate TSC-22 in multiple colon cancer cell lines, emphasizing the fact that the present findings are not simply limited to one unique colon cancer cell clone. In addition, the functional experiments in which a dominant negative TSC-22 could reverse the growth inhibition caused by PPAR ligands or TGF-1 are likely due to its ability to inhibit normal TSC-22 function. This is because no such reversal was seen with a control dominant negative construct that cannot dimerize with and inhibit wild-type TSC-22. We also provide evidence that there is specificity to this putative signaling system. For example, TSC-22 was not induced by PPAR or (neither of which induces epithelial cell differentiation). In addition, the induction of TSC-22 does not appear to be a general phenomenon of agents that inhibit cell growth since the RXR ligand LG100268 did not induce TSC-22 (See Fig. 30) but does inhibit the growth of multiple cell types, including the M-S cells (data not shown). Finally, TSC-22 did not have any effect on the expression of keratin 20 suggesting that TSC-22 is involved in one facet of the differentiation program (control of cell growth) but is not involved in pathways that lead to changes in the expression of certain structural proteins.

TSC-22 is highly conserved during evolution, with the human protein sequence being 98.5% identical to the mouse and rat proteins(Jay et al., 1996). TSC-22 has been shown to a have a solution structure similar to members of the bZIP family of transcription factors (Seidel et al., 1997). Similar to bZIP proteins, TSC-22 contains a leucine zipper domain and, analogous to the basic domain in bZIP family members, has a highly conserved sequence known as the TSC domain or box. In addition, TSC-22 is also homologous to the *Drosophila melanogaster* gene *shortsighted* (shs) or *bunched* which is thought to play an important role in oogenesis and eye, wing, and peripheral nervous system development (Dobens et al., 1997; Treisman et al., 1995) (Dobens et al., 2000). There is also evidence suggesting TSC-22 can negatively regulate the growth of cancer cells. For example, anti-sense expression of TSC-22 in a salivary gland carcinoma line inhibits the growth of these cells *in vivo* (Nakashiro et al., 1998) and over-expression of TSC-22 appears to sensitize carcinoma cells to certain anti-cancer chemotherapeutic drugs (Omotehara et al., 2000).

Evidence to date suggests that TSC-22 is part of a family of proteins that act as transcriptional repressors. As mentioned earlier, TSC-22 has been shown to homodimerize and exhibit transcriptional repressor activity when fused to a heterologous DNA binding domain (Kester et al., 1999). In fact, our results with the TSC-22 dn protein in which both repressor domains were deleted provide functional evidence in a biological system for the importance of these domains. This notion is further supported by the finding that the TSC-22 *Drosophila* homolog, *bunched*, is a powerful repressor of the enhancer trap reporter A359 (Dobens et al., 2000). It is also possible that TSC-22 may modify gene expression through protein-protein interactions. Recently, a TSC-22 homologue (termed THG-1) was cloned and found capable of forming heterodimers with TSC-22 (Kester et al., 1999)(THG-1 was not found to be expressed in the M-S cells). Exactly how either a TSC-22 homodimer complex or a TSC-22:THG-1 heterodimer complex can regulate the expression of a particular target gene is unknown.

One possible target gene of TSC-22 may be p21. However, at this point it is unclear whether TSC-22 increases p21 levels directly or indirectly. Although our work provides evidence that TSC-22 is involved in the signaling pathway by which both PPAR and TGF- induce p21, there are other mechanisms by which activation of either pathway may modulate p21 expression. For example, TGF- has been shown to activate p21 proximal promoter activity via two consensus Sp1 binding sites (Datto et al., 1995; Li et al., 1998). How (or if) TSC-22 may act to enhance basal Sp1 transcriptional activity is unknown. Clearly, a major goal of future work will be to identify genes that are directly regulated by TSC-22 and determine the mechanism(s) by which TSC-22 modulates the expression of these genes. Determining TSC-22 target genes in the intestine will lead to a much better understanding of the exact role of this protein during colon epithelial differentiation.

In summary, the data described in Chapter VI point to a previously unidentified role for the putative transcriptional repressor TSC-22 as a regulator of intestinal epithelial cell differentiation. A large percentage of advanced colorectal tumors lose their responsiveness to growth inhibition induced by TGF-. It will be of interest to determine what percentage of these colorectal tumors also contain mutations in PPAR. If this is not found to be a common occurrence, then our present studies predict that treatment with activators of PPARmay provide a way to bypass the loss of normal TGF- signaling that occurs during the progression of these tumors.

CHAPTER VIII

FUTURE AIMS

Future Aims

Although the work presented here has helped clarify the role of PPARγ in the growth and behavior of colon epithelial cells, many questions remain unanswered. For example, what are the factors that govern PPAR subtype specificity in colorectal cancer cells? The normal intestinal mucosa, as well as most colorectal cancer cell lines, express all three PPAR isoforms. In Chapter IV we demonstrated that some target genes could be activated by all three PPAR subtypes while other genes could only be regulated by PPARγ. Domain swapping experiments between PPARγ and PPARα or-δ could help establish what region of PPARγ confers the target gene specificity we observed. The identified domain could then be used as bait in a two-hybrid screen to identify PPARγ unique transcriptional cofactors. Moreover, it would be interesting to know what the differences are in the regulatory region of the different target genes such that only some can be activated by PPARγ and not the other receptor subtypes.

In Chapter V, we identified a mutation that appears to block the ability of the apo-PPARγ to repress basal transcription. A future focus in this area could be to dissect how PPARγ normally regulates TSC-22 and Mox-2 and to identity the proteins (if any) that differentially bind to WT and K422Q PPARγ. The results from these experiments should lead to a better understanding of how PPARγ can repress target gene expression and of the biological importance of this type of repression in the control of colorectal cancer cell growth and differentiation.

In Chapter VI, we report data that suggests that TSC-22 plays an important role in the ability of PPARγ or TGF-β to regulate colon epithelial cell growth. However, almost nothing is known about how TSC-22 regulates cell proliferation. What are the target

genes of TSC-22? Exactly how important is TSC-22 in regulating the growth responses of PPARγ or TGF-β? Are the findings presented in Chapter VI limited to a few colorectal cancer cell clones or do they represent a more general phenomenon? Studies examining PPARγ and TGF-β signaling in TSC-22-/- mice (and cells derived from these mice) could help answer these questions.

The best way to determine what role PPARγ plays in the normal differentiation of coloncytes may be the use PPARγ knock out mice. Although PPARγ-/- mice are embryonic lethal, tissue specific gene targeting strategies could be used to ablate PPARγ only in the colon. Experiments using these mice would undoubtedly help clarify the physiological function of PPARγ in the colon.

Selective PPARγ Modulators

Finally, it should be emphasized that questions focused on mechanisms of PPARγ transcription have important implications. One could envision, for example, that the ability of PPARγ to regulate insulin sensitivity might be due to its interactions with a certain set of target genes and coregulators that are distinct from those involved in lipogenesis. Similarly, a unique set of coregulators could be involved in the ability of PPARγ to control epithelial cell proliferation. If this is the case, then selective PPARγ modulators could be developed that only lower blood glucose levels (without the associated increase in adipose mass) or only inhibit colorectal cancer cell growth (without effecting lipid or glucose homeostasis). A major focus in the PPARγ field in the future is likely to be the development of such PPARγ selective modulators.

REFERENCES

Altiok, S., Xu, M., and Spiegelman, B. M. (1997). PPARgamma induces cell cycle withdrawal: inhibition of E2F/DP DNA- binding activity via down-regulation of PP2A. Genes Dev *11*, 1987-98.

Auboeuf, D., Rieusset, J., Fajas, L., Vallier, P., Frering, V., Riou, J. P., Staels, B., Auwerx, J., Laville, M., and Vidal, H. (1997). Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans: no alteration in adipose tissue of obese and NIDDM patients. Diabetes *46*, 1319-27.

Avery, A., Paraskeva, C., Hall, P., Flanders, K. C., Sporn, M., and Moorghen, M. (1993). TGF-beta expression in the human colon: differential immunostaining along crypt epithelium. Br J Cancer *68*, 137-9.

Bach, S. P., Renehan, A. G., and Potten, C. S. (2000). Stem cells: the intestinal stem cell as a paradigm. Carcinogenesis *21*, 469-76.

Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., Koder, A., and Evans, R. M. (1999). PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell *4*, 585-95.

Bardot, O., Aldridge, T. C., Latruffe, N., and Green, S. (1993). PPAR-RXR heterodimer activates a peroxisome proliferator response element upstream of the bifunctional enzyme gene. Biochem Biophys Res Commun *192*, 37-45.

Barnard, J. A., Beauchamp, R. D., Coffey, R. J., and Moses, H. L. (1989). Regulation of intestinal epithelial cell growth by transforming growth factor type beta. Proc Natl Acad Sci U S A *86*, 1578-82.

Barroso, I., Gurnell, M., Crowley, V. E., Agostini, M., Schwabe, J. W., Soos, M. A., Maslen, G. L., Williams, T. D., Lewis, H., Schafer, A. J., Chatterjee, V. K., and O'Rahilly, S. (1999). Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. Nature *402*, 880-3.

Benchimol, S., Fuks, A., Jothy, S., Beauchemin, N., Shirota, K., and Stanners, C. P. (1989). Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. Cell *57*, 327-34.

Bernard-Perrone, F. R., Renaud, W. P., Guy-Crotte, O. M., Bernard, P., Figarella, C. G., Okamoto, H., Balas, D. C., and Senegas-Balas, F. O. (1999). Expression of REG protein during cell growth and differentiation of two human colon carcinoma cell lines. J Histochem Cytochem *47*, 863-70.

Bishop-Bailey, D., and Hla, T. (1999). Endothelial cell apoptosis induced by the peroxisome proliferator- activated receptor (PPAR) ligand 15-deoxy-Delta12, 14-prostaglandin J2. J Biol Chem *274*, 17042-8.

Blobe, G. C., Schiemann, W. P., and Lodish, H. F. (2000). Role of transforming growth factor beta in human disease. N Engl J Med *342*, 1350-8.

Bratt, T., Ohlson, S., and Borregaard, N. (1999). Interactions between neutrophil gelatinase-associated lipocalin and natural lipophilic ligands. Biochim Biophys Acta *1472*, 262-9.

Brown, K. K., Henke, B. R., Blanchard, S. G., Cobb, J. E., Mook, R., Kaldor, I., Kliewer, S. A., Lehmann, J. M., Lenhard, J. M., Harrington, W. W., Novak, P. J., Faison, W., Binz, J. G., Hashim, M. A., Oliver, W. O., Brown, H. R., Parks, D. J., Plunket, K. D., Tong, W. Q., Menius, J. A., Adkison, K., Noble, S. A., and Willson, T. M. (1999). A novel N-aryl tyrosine activator of peroxisome proliferator-activated receptor-gamma reverses the diabetic phenotype of the Zucker diabetic fatty rat. Diabetes *48*, 1415-24.

Brown, P. J., Stuart, L. W., Hurley, K. P., Lewis, M. C., Winegar, D. A., Wilson, J. G., Wilkinson, W. O., Ittoop, O. R., and Willson, T. M. (2001). Identification of a subtype selective human PPARs agonist through parallel-array synthesis. Bioorg. Med. Chem. Lett. *11*, 1225-1227.

Buckingham, R. E., Al-Barazanji, K. A., Toseland, C. D., Slaughter, M., Connor, S. C., West, A., Bond, B., Turner, N. C., and Clapham, J. C. (1998). Peroxisome proliferator-activated receptor-gamma agonist, rosiglitazone, protects against nephropathy and pancreatic islet abnormalities in Zucker fatty rats. Diabetes *47*, 1326-34.

Burant, C. F., Sreenan, S., Hirano, K., Tai, T. A., Lohmiller, J., Lukens, J., Davidson, N. O., Ross, S., and Graves, R. A. (1997). Troglitazone action is independent of adipose tissue. J Clin Invest *100*, 2900-8.

Calnek, D., and Quaroni, A. (1993). Differential localization by in situ hybridization of distinct keratin mRNA species during intestinal epithelial cell development and differentiation. Differentiation *53*, 95-104.

Candia, A. F., Hu, J., Crosby, J., Lalley, P. A., Noden, D., Nadeau, J. H., and Wright, C. V. (1992). Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. Development *116*, 1123-36.

Candia, A. F., and Wright, C. V. (1995). The expression pattern of Xenopus Mox-2 implies a role in initial mesodermal differentiation. Mech Dev *52*, 27-36.

Carrere, J., Guy-Crotte, O., and Figarella, C. (1998). Human pancreatic reg protein immunoenzymatic assay and molecular form in serum. Clin Chim Acta *273*, 185-94.

Castillo, G., Brun, R. P., Rosenfield, J. K., Hauser, S., Park, C. W., Troy, A. E., Wright, M. E., and Spiegelman, B. M. (1999). An adipogenic cofactor bound by the differentiation domain of PPARgamma. Embo J *18*, 3676-87.

Chang, T. H., and Szabo, E. (2000). Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor gamma in non-small cell lung cancer. Cancer Res *60*, 1129-38.

Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., and Evans, R. M. (2001). PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med *7*, 48-52.

Chawla, A., Boisvert, W. A., Lee, C. H., Laffitte, B. A., Barak, Y., Joseph, S. B., Liao, D., Nagy, L., Edwards, P. A., Curtiss, L. K., Evans, R. M., and Tontonoz, P. (2001). A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol Cell *7*, 161-71.

Chen, H., Tini, M., and Evans, R. M. (2001). HATs on and beyond chromatin. Curr Opinion in Cell Biology *13*, 218-224.

Chen, J. D., and Evans, R. M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature *377*, 454-7.

Chinery, R., Coffey, R. J., Graves-Deal, R., Kirkland, S. C., Sanchez, S. C., Zackert, W. E., Oates, J. A., and Morrow, J. D. (1999). Prostaglandin J2 and 15-deoxy-delta12,14-prostaglandin J2 induce proliferation of cyclooxygenase-depleted colorectal cancer cells. Cancer Res *59*, 2739-46.

Chulada, P. C., Thompson, M. B., Mahler, J. F., Doyle, C. M., Gaul, B. W., Lee, C., Tiano, H. F., Morham, S. G., Smithies, O., and Langenbach, R. (2000). Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res *60*, 4705-8.

Chung, D. C. (2000). The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. Gastroenterology *119*, 854-65.

Cobb, J. E., Blanchard, S. G., Boswell, E. G., Brown, K. K., Charifson, P. S., Cooper, J. P., Collins, J. L., Dezube, M., Henke, B. R., Hull-Ryde, E. A., Lake, D. H., Lenhard, J. M., Oliver, W., Oplinger, J., Pentti, M., Parks, D. J., Plunket, K. D., and Tong, W. Q. (1998). N-(2-Benzoylphenyl)-L-tyrosine PPARgamma agonists. 3. Structure- activity relationship and optimization of the N-aryl substituent. J Med Chem *41*, 5055-69.

Cowland, J. B., and Borregaard, N. (1997). Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics *45*, 17-23.

Cui, W., Fowlis, D. J., Bryson, S., Duffie, E., Ireland, H., Balmain, A., and Akhurst, R. J. (1996). TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell *86*, 531-42.

Daniel, T. O., Liu, H., Morrow, J. D., Crews, B. C., and Marnett, L. J. (1999). Thromboxane A_2 is a mediator of cyclooxygenase-2-dependent endothelial migration and angiogenesis. Cancer Res. *59*, 4574-4577.

Datto, M. B., Yu, Y., and Wang, X. F. (1995). Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. J Biol Chem *270*, 28623-8.

De Vos, P., Lefebvre, A. M., Miller, S. G., Guerre-Millo, M., Wong, K., Saladin, R., Hamann, L. G., Staels, B., Briggs, M. R., and Auwerx, J. (1996). Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. J Clin Invest *98*, 1004-9.

Desvergne, B., and Wahli, W. (1999). Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev *20*, 649-88.

Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J., and Wahli, W. (1996). The PPARalpha-leukotriene B4 pathway to inflammation control. Nature *384*, 39-43.

DiRenzo, J., Soderstrom, M., Kurokawa, R., Ogliastro, M. H., Ricote, M., Ingrey, S., Horlein, A., Rosenfeld, M. G., and Glass, C. K. (1997). Peroxisome proliferator-activated receptors and retinoic acid receptors differentially control the interactions of retinoid X receptor heterodimers with ligands, coactivators, and corepressors. Mol Cell Biol *17*, 2166-76.

Dobens, L. L., Hsu, T., Twombly, V., Gelbart, W. M., Raftery, L. A., and Kafatos, F. C. (1997). The Drosophila bunched gene is a homologue of the growth factor stimulated mammalian TSC-22 sequence and is required during oogenesis. Mech Dev *65*, 197-208.

Dobens, L. L., Peterson, J. S., Treisman, J., and Raftery, L. A. (2000). Drosophila bunched integrates opposing DPP and EGF signals to set the operculum boundary. Development *127*, 745-54.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., and Wahli, W. (1992). Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. Cell *68*, 879-87.

DuBois, R. N., Abramson, S. B., Crofford, L., R.A., G., Simon, L. S., Van De Putte, L. B., and Lipsky, P. E. (1998). Cyclooxygenase in biology and disease. FASEB J. *12*, 1063-1073.

DuBois, R. N., Giardiello, F. M., and Smalley, W. E. (1996). Nonsteroidal anti-inflammatory drugs, eicosanoids and colorectal cancer prevention. Gastroenterology Clinics of North America *25*, 773-791.

Eidelman, F. J., Fuks, A., DeMarte, L., Taheri, M., and Stanners, C. P. (1993). Human carcinoembryonic antigen, an intercellular adhesion molecule, blocks fusion and differentiation of rat myoblasts. J Cell Biol *123*, 467-75.

Elbendary, A., Berchuck, A., Davis, P., Havrilesky, L., Bast, R. C., Iglehart, J. D., and Marks, J. R. (1994). Transforming growth factor beta 1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells. Cell Growth Differ *5*, 1301-7.

Emery, P., Zeidler, H., Kvien, T. K., Guslandi, M., Naudin, R., Stead, H., Verburg, K., M., Isakson, P. C., Hubbard, R. C., and Geis, G. S. (1999). Celecoxib versus diclofenac in long-term management of rheumatoid arthritis: randomised double-blind comparison. Lancet *354*, 2106-2111.

Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L., Thomsen, G. H., Wrana, J. L., and Attisano, L. (1996). MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell *86*, 543-52.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *2401*, 889-895.

Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R., Najib, J., Laville, M., Fruchart, J. C., Deeb, S., Vidal-Puig, A., Flier, J., Briggs, M. R., Staels, B.,

Vidal, H., and Auwerx, J. (1997). The organization, promoter analysis, and expression of the human PPARgamma gene. J Biol Chem *272*, 18779-89.

Fajas, L., Fruchart, J. C., and Auwerx, J. (1998). PPARgamma3 mRNA: a distinct PPARgamma mRNA subtype transcribed from an independent promoter. FEBS Lett *438*, 55-60.

Forman, B. M., Chen, J., and Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A *94*, 4312-7.

Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995). 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell *83*, 803-12.

Frangsmyr, L., Baranov, V., and Hammarstrom, S. (1999). Four carcinoembryonic antigen subfamily members, CEA, NCA, BGP and CGM2, selectively expressed in the normal human colonic epithelium, are integral components of the fuzzy coat. Tumour Biol *20*, 277-92.

Fryer, D. J., and Archer, T. K. (1998). Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. Nature *393*, 88-91.

Fu, J. Y., Masferrer, J. L., Seibert, K., Raz, A., and Needleman, P. (1990). The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. J Biol Chem *265*, 16737-40.

Gampe, R. T., Montana, V. G., Lambert, M. H., Miller, A. B., Bledsoe, R. K., Milburn, M. V., Kliewer, S. A., Willson, T. M., and Xu, H. E. (2000). Asymmetry in the PPARgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. Mol Cell *5*, 545-55.

Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R., and Offerhaus, G. J. (1993). Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N. Engl. J. Med. *328*, 1313-1316.

Giovannucci, E., Egan, K. M., Hunter, D. J., Stampfer, M. J., Colditz, G. A., Willett, W. C., and Speizer, F. E. (1995). Aspirin and the risk of colorectal cancer in women. N. Eng. J. Med. *333*, 609-614.

Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., and Willett, W. C. (1994). Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. Ann. Intern. Med. *121*, 241-246.

Glass, C. K., and Rosenfeld, M. G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes and Dev. *14*, 121-141.

Glass, C. K., and Rosenfeld, M. G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev *14*, 121-41.

Gothe, S., Wang, Z., Ng, L., Kindblom, J. M., Barros, A. C., Ohlsson, C., Vennstrom, B., and Forrest, D. (1999). Mice devoid of all known thyroid hormone receptors are viable

but exhibit disorders of the pituitary-thyroid axis, growth, and bone maturation. Genes Dev *13*, 1329-41.

Gottlicher, M., Widmark, E., Li, Q., and Gustafsson, J. A. (1992). Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. Proc Natl Acad Sci U S A *89*, 4653-7.

Grady, W. M., Myeroff, L. L., Swinler, S. E., Rajput, A., Thiagalingam, S., Lutterbaugh, J. D., Neumann, A., Brattain, M. G., Chang, J., Kim, S. J., Kinzler, K. W., Vogelstein, B., Willson, J. K., and Markowitz, S. (1999). Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. Cancer Res *59*, 320-4.

Greene, M. E., Blumberg, B., McBride, O. W., Yi, H. F., Kronquist, K., Kwan, K., Hsieh, L., Greene, G., and Nimer, S. D. (1995). Isolation of the human peroxisome proliferator activated receptor gamma cDNA: expression in hematopoietic cells and chromosomal mapping. Gene Expr *4*, 281-99.

Gupta, R. A., Brockman, J. A., Sarraf, P., Willson, T. M., and DuBois, R. N. (2001). Target genes of peroxisome proliferator-activated receptor {gamma} in colorectal cancer cells. J Biol Chem *7*, 7.

Gupta, R. A., Tan, J., Krause, W. F., Geraci, M. W., Willson, T. M., Dey, S. K., and DuBois, R. N. (2000). Prostacyclin-mediated activation of peroxisome proliferator-activated receptor delta in colorectal cancer. Proc Natl Acad Sci U S A *97*, 13275-80.

He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. (1999). PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell *99*, 335-45.

Heid, H. W., Moll, R., Schwetlick, I., Rackwitz, H. R., and Keenan, T. W. (1998). Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. Cell Tissue Res *294*, 309-21.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature *387*, 43-8.

Henke, B. R., Blanchard, S. G., Brackeen, M. F., Brown, K. K., Cobb, J. E., Collins, J. L., Harrington, W. W., Hashim, M. A., Hull-Ryde, E. A., Kaldor, I., Kliewer, S. A., Lake, D. H., Leesnitzer, L. M., Lehmann, J. M., Lenhard, J. M., Orband-Miller, L. A., Miller, J. F., Mook, R. A., Noble, S. A., Oliver, W., Parks, D. J., Plunket, K. D., Szewczyk, J. R., and Willson, T. M. (1998). N-(2-Benzoylphenyl)-L-tyrosine PPARgamma agonists. 1. Discovery of a novel series of potent antihyperglycemic and antihyperlipidemic agents. J Med Chem *41*, 5020-36.

Herschman, H. R. (1996). Prostaglandin synthase 2. BBA *1299*, 125-140.

Herschman, H. R., Xie, W., and Reddy, S. (1995). Inflammation, reproduction, cancer and all that.... The regulation and role of the inducible prostaglandin synthase. Bioessays *17*, 1031-1037.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995). Ligand-independent

repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature *377*, 397-404.

Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996). Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. Science *274*, 2100-3.

Hu, X., and Lazar, M. A. (1999). The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature *402*, 93-6.

Hu, X., and Lazar, M. A. (2000). Transcriptional repression by nuclear hormone receptors. Trends Endocrinol Metab *11*, 6-10.

Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D., and Glass, C. K. (1999). Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase. Nature *400*, 378-82.

Hulin, B., McCarthy, P. A., and Gibbs, E. M. (1996). The glitazone family of antidiabetic agents. Curr. Phram. Des. *2*, 85-102.

Ikezoe, T., Miller, C. W., Kawano, S., Heaney, A., Williamson, E. A., Hisatake, J., Green, E., Hofmann, W., Taguchi, H., and Koeffler, H. P. (2001). Mutational analysis of peroxsiome proliferator-activated receptor-gamma gene in human malignancies. Cancer Res *61*, 5307-10.

Issemann, I., and Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature *347*, 645-50.

Jay, P., Ji, J. W., Marsollier, C., Taviaux, S., Berge-Lefranc, J. L., and Berta, P. (1996). Cloning of the human homologue of the TGF beta-stimulated clone 22 gene. Biochem Biophys Res Commun *222*, 821-6.

Jiang, C., Ting, A. T., and Seed, B. (1998). PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature *391*, 82-6.

Jones, M. K., Wang, H., Peskar, B. M., Levin, E., Itani, R. M., Sarfeh, I. J., and Tarnawski, A. S. (1999). Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: Insight into mechanisms and implications for cancer growth and ulcer healing. Nature Med. *5*, 1418-1423.

Juge-Aubry, C., Pernin, A., Favez, T., Burger, A. G., Wahli, W., Meier, C. A., and Desvergne, B. (1997). DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5'-flanking region. J Biol Chem *272*, 25252-9.

Kallen, C. B., and Lazar, M. A. (1996). Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes. Proc Natl Acad Sci U S A *93*, 5793-6.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell *85*, 403-414.

Kersten, S., Desvergne, B., and Wahli, W. (2000). Roles of PPARs in health and disease. Nature *405*, 421-4.

Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., and Wahli, W. (1999). Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. J Clin Invest *103*, 1489-98.

Kester, H. A., Blanchetot, C., den Hertog, J., van der Saag, P. T., and van der Burg, B. (1999). Transforming growth factor-beta-stimulated clone-22 is a member of a family of leucine zipper proteins that can homo- and heterodimerize and has transcriptional repressor activity. J Biol Chem *274*, 27439-47.

Kinzler, K. W., Nilbert, M. C., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hamilton, S. R., Hedge, P., Markham, A., and et al. (1991). Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. Science *251*, 1366-70.

Kinzler, K. W., and Vogelstein, B. (1998). Landscaping the cancer terrain. Science *280*, 1036-1037.

Kjeldsen, L., Cowland, J. B., and Borregaard, N. (2000). Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. Biochim Biophys Acta *1482*, 272-83.

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc Natl Acad Sci U S A *91*, 7355-9.

Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995). A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell *83*, 813-9.

Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A *94*, 4318-23.

Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1990). Convergence of 9-cis retinoic acid and peroxisome prolierator signaling pathways through heterodimer formation of their receptors. Nature *358*, 771-774.

Kliewer, S. A., and Willson, T. M. (1998). The nuclear receptor PPARgamma - bigger than fat. Curr Opin Genet Dev *8*, 576-81.

Kobayashi, S., Akiyama, T., Nata, K., Abe, M., Tajima, M., Shervani, N. J., Unno, M., Matsuno, S., Sasaki, H., Takasawa, S., and Okamoto, H. (2000). Identification of a receptor for reg (regenerating gene) protein, a pancreatic beta-cell regeneration factor. J Biol Chem *275*, 10723-6.

Komiya, T., Tanigawa, Y., and Hirohashi, S. (1999). Cloning of the gene gob-4, which is expressed in intestinal goblet cells in mice. Biochim Biophys Acta *1444*, 434-8.

Kubota, T., Koshizuka, K., Williamson, E. A., Asou, H., Said, J. W., Holden, S., Miyoshi, I., and Koeffler, H. P. (1998). Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. Cancer Res *58*, 3344-52.

Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., and Herschman, H. R. (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J. Biol. Chem. *266*, 12866-12872.

Kurokowa, M., Lynch, K., and Podolsky, D. K. (1987). Effects of growth factors on an intestinal epithelial cell line: transforming growth factor beta inhibits proliferation and stimulates differentiation. Biochem Biophys Res Commun *142*, 775-82.

Labayle, D., Fischer, D., Vielh, P., Drouhin, F., Pariente, A., Bories, C., Duhamel, O., Trousset, M., and Attali, P. (1991). Sulindac causes regression of rectal polyps in familial adenomatous polyposis. Gastroenterology *101*, 635-9.

Laine, L., Harper, S., Simon, T., Bath, R., Johanson, J., Schwartz, H., Stern, S., Quan, H., and Bolognese, J. (1999). A randomized trial comparing the effect of rofecoxib, a cyclooxygenase 2-specific inhibitor, with that of ibuprofen on the gastroduodenal mucosa of patients with osteoarthritis. Gastroenterology *117*, 776-783.

Leblanc, B. P., and Stunnenberg, H. G. (1995). 9-cis retinoic acid signaling: changing partners causes some excitement. Genes Dev *9*, 1811-6.

Lefebvre, A. M., Chen, I., Desreumaux, P., Najib, J., Fruchart, J. C., Geboes, K., Briggs, M., Heyman, R., and Auwerx, J. (1998). Activation of the peroxisome proliferator-activated receptor gamma promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. Nat Med *4*, 1053-7.

Lefebvre, M., Paulweber, B., Fajas, L., Woods, J., McCrary, C., Colombel, J. F., Najib, J., Fruchart, J. C., Datz, C., Vidal, H., Desreumaux, P., and Auwerx, J. (1999). Peroxisome proliferator-activated receptor gamma is induced during differentiation of colon epithelium cells. J Endocrinol *162*, 331-40.

Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem *270*, 12953-6.

Leone, T. C., Weinheimer, C. J., and Kelly, D. P. (1999). A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. Proc Natl Acad Sci U S A *96*, 7473-8.

Li, A. C., Brown, K. K., Silvestre, M. J., Willson, T. M., Palinski, W., and Glass, C. K. (2000). Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. J Clin Invest *106*, 523-31.

Li, J. M., Datto, M. B., Shen, X., Hu, P. P., Yu, Y., and Wang, X. F. (1998). Sp1, but not Sp3, functions to mediate promoter activation by TGF-beta through canonical Sp1 binding sites. Nucleic Acids Res *26*, 2449-56.

Li, M., Pascual, G., and Glass, C. K. (2000). Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. Mol Cell Biol *20*, 4699-707.

Lim, H., Gupta, R. A., Ma, W. G., Paria, B. C., Moller, D. E., Morrow, J. D., DuBois, R. N., Trzaskos, J. M., and Dey, S. K. (1999). Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. Genes Dev *13*, 1561-74.

Liu, C. H., Chang, S. H., Narko, K., Trifan, O. C., Wu, M. T., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. (2001). Overexpression of Cyclooxygenase-2 Is Sufficient to Induce Tumorigenesis in Transgenic Mice. J Biol Chem *276*, 18563-18569.

Macadam, R. C., Sarela, A. I., Farmery, S. M., Robinson, P. A., Markham, A. F., and Guillou, P. J. (2000). Death from early colorectal cancer is predicted by the presence of transcripts of the REG gene family. Br J Cancer *83*, 188-95.

Majima, M., Hayashi, I., Muramatsu, M., Katada, J., Yamashina, S., and Katori, M. (2000). Cyclo-oxygenase-2 enhances basic fibroblast growth factor-induced angiogenesis through induction of vascular endothelial growth factor in rat sponge implants. Br J Pharmacol *130*, 641-9.

Majima, M., Isono, M., Ikeda, Y., Hayashi, I., Hatanaka, K., Harada, Y., Katsumata, O., Yamashina, S., Katori, M., and Yamamoto, S. (1997). Significant roles of inducible cyclooxygenase (COX)-2 in angiogenesis in rat sponge implants. Jpn J Pharmacol *75*, 105-14.

Mangelsdorf, D. J., and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. Cell *83*, 841-850.

Mangesldorf, D. J., Thummel, C., Beato, M., Herlich, P., Schultz, G., Umensono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995). Nuclear hormone receptors - the second decade. Cell *83*, 835-839.

Mansen, A., Guardiola-Diaz, H., Rafter, J., Branting, C., and Gustafsson, J. A. (1996). Expression of the peroxisome proliferator-activated receptor (PPAR) in the mouse colonic mucosa. Biochem Biophys Res Commun *222*, 844-51.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., and et al. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science *268*, 1336-8.

Marnett, L. J., and Kalgutkar, A. S. (1999). Cyclooxygenase 2 inhibitors: discovery, selectivity and the future. Trends Pharmacol Sci *20*, 465-9.

Masferrer, J. L., Leahy, K. M., Koki, A. T., Zweifel, B. S., Settle, S. L., Woerner, M. B., Edwards, D. A., Flickinger, A. G., Moore, R. J., and Seibert, K. (2000). Antiangiogenic and antitumor activities of cyclooxygenase-2 Inhibitors. Cancer Res. *60*, 1306-1311.

Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C., and Seibert, K. (1994). Selective inhibition of inducible cyclooxygenase-2 *in vivo* is antiinflammatory and nonulcerogenic. Proc. Natl. Acad. Sci. USA *91*, 3228-3232.

McKenna, N. J., Lanz, R. B., and O'Malley, B. M. (1999). Nuclear receptor coregulators: cellular and molecular biology. Endocrine Reviews *20*, 321-344.

Medhurst, A. D., Harrison, D. C., Read, S. J., Campbell, C. A., Robbins, M. J., and Pangalos, M. N. (2000). The use of TaqMan RT-PCR assays for semiquantitative analysis of gene expression in CNS tissues and disease models. J Neurosci Methods *98*, 9-20.

Minamikawa, J., Yamauchi, M., Inoue, d., and Koshiyama, H. (1998). J. Cllin. Endocrinolog. Metab. *83*, 1041-42.

Moll, R., Zimbelmann, R., Goldschmidt, M. D., Keith, M., Laufer, J., Kasper, M., Koch, P. J., and Franke, W. W. (1993). The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. Differentiation *53*, 75-93.

Moore, K. J., Rosen, E. D., Fitzgerald, M. L., Randow, F., Andersson, L. P., Altshuler, D., Milstone, D. S., Mortensen, R. M., Spiegelman, B. M., and Freeman, M. W. (2001). The role of PPAR-gamma in macrophage differentiation and cholesterol uptake. Nat Med *7*, 41-47.

Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M., Fletcher, C., Singer, S., and Spiegelman, B. M. (1998). Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell *1*, 465-70.

Mueller, E., Smith, M., Sarraf, P., Kroll, T., Aiyer, A., Kaufman, D. S., Oh, W., Demetri, G., Figg, W. D., Zhou, X. P., Eng, C., Spiegelman, B. M., and Kantoff, P. W. (2000). Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. Proc Natl Acad Sci U S A *97*, 10990-5.

Mukherjee, R., Jow, L., Croston, G. E., and Paterniti, J. R. (1997). Identification, characterization, and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPARgamma2 versus PPARgamma1 and activation with retinoid X receptor agonists and antagonists. J Biol Chem *272*, 8071-6.

Murata, T., He, S., Hangai, M., Ishibashi, T., Xi, X. P., Kim, S., Hsueh, W. A., Ryan, S. J., Law, R. E., and Hinton, D. R. (2000). Peroxisome proliferator-activated receptor-gamma ligands inhibit choroidal neovascularization. Invest Ophthalmol Vis Sci *41*, 2309-17.

Murchadt, C., and Yaniv, M. (1993). A human homologue of Saccharomyces cerevisiae SNF/SWI2 and Drosophila brm genes potentiates transcriptional activation by the glucocorticoid receptor. EMBO J. *12*, 4279-4290.

Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell *89*, 373-80.

Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H., and Evans, R. M. (1998). Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell *93*, 229-40.

Nakajima, A., Wada, K., Miki, H., Kubota, N., Nakajima, N., Terauchi, Y., Ohnishi, S., Saubermann, L. J., Kadowaki, T., Blumberg, R. S., Nagai, R., and Matsuhashi, N. (2001).

Endogenous PPAR gamma mediates anti-inflammatory activity in murine ischemia-reperfusion injury. Gastroenterology *120*, 460-9.

Nakashiro, K., Kawamata, H., Hino, S., Uchida, D., Miwa, Y., Hamano, H., Omotehara, F., Yoshida, H., and Sato, M. (1998). Down-regulation of TSC-22 (transforming growth factor beta-stimulated clone 22) markedly enhances the growth of a human salivary gland cancer cell line in vitro and in vivo. Cancer Res *58*, 549-55.

Neufang, G., Furstenberger, G., Heidt, M., Marks, F., and Muller-Decker, K. (2001). Abnormal differentiation of epidermis in transgenic mice constitutively expressing cyclooxygenase-2 in skin. Proc. Natl. Acad. Sci. USA *in press*.

Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998). Ligand binding and co-activator assembly of the peroxisome proliferator- activated receptor-gamma. Nature *395*, 137-43.

Nugent, K. P., Farmer, K. C., Spigelman, A. D., Williams, C. B., and Phillips, R. K. (1993). Randomized controlled trial of the effect of sulindac on duodenal and rectal polyposis and cell proliferation in patients with familial adenomatous polyposis. Brit. J. Surg. *80*, 1618-1619.

Obrink, B. (1997). CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. Curr Opin Cell Biol *9*, 616-26.

Okamoto, H. (1997). New aspects to the functioning and regeneration of pancreatic beta-cells. Cyclic ADP-ribose and Reg gene. Adv Exp Med Biol *426*, 301-11.

Oliver, W. R., Shenk, J. L., Snaith, M. R., Russell, C. S., Plunket, K. D., Bodkin, N. L., Lewis, M. C., Winegar, D. A., Sznaidman, M. L., Lambert, M. H., Xu, H. E., Sternbach, D. D., Kliewer, S. A., Hansen, B. C., and Willson, T. M. (2001). A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. Proc Natl Acad Sci U S A *98*.

Omotehara, F., Uchida, D., Hino, S., Begum, N. M., Yoshida, H., Sato, M., and Kawamata, H. (2000). In vivo enhancement of chemosensitivity of human salivary gland cancer cells by overexpression of TGF-beta stimulated clone-22. Oncol Rep *7*, 737-40.

Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996). Suppression of intestinal polyposis in $APC^{\Delta 716}$ knockout mice by inhibition of prostaglandin endoperoxide synthase-2 (COX-2). Cell *87*, 803-809.

Park, B. H., Vogelstein, B., and Kinzler, K. W. (2001). Genetic disruption of PPARdelta decreases the tumorigenicity of human colon cancer cells. Proc Natl Acad Sci U S A *98*, 2598-603.

Peters, J. M., Lee, S. S., Li, W., Ward, J. M., Gavrilova, O., Everett, C., Reitman, M. L., Hudson, L. D., and Gonzalez, F. J. (2000). Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). Mol Cell Biol *20*, 5119-28.

Poirier, H., Braissant, O., Niot, I., Wahli, W., and Besnard, P. (1997). 9-cis-retinoic acid enhances fatty acid-induced expression of the liver fatty acid-binding protein gene. FEBS Lett *412*, 480-4.

Poirier, H., Niot, I., Monnot, M. C., Braissant, O., Meunier-Durmort, C., Costet, P., Pineau, T., Wahli, W., Willson, T. M., and Besnard, P. (2001). Differential involvement of peroxisome-proliferator-activated receptors alpha and delta in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. Biochem J *355*, 481-488.

Puigserver, P., Adelmant, G., Wu, Z., Fan, M., Xu, J., O'Malley, B., and Spiegelman, B. M. (1999). Activation of PPARgamma coactivator-1 through transcription factor docking. Science *286*, 1368-71.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell *92*, 829-39.

Rachez, C., and Freedman, L. P. (2001). Mediator complexes and transcription. Curr Opinion in Cell Biology *13*, 274-280.

Raz, A., Wyche, A., Siegel, N., and Needleman, P. (1988). Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. J. Biol. Chem. *263*, 3022-3028.

Rechreche, H., Montalto, G., Mallo, G. V., Vasseur, S., Marasa, L., Soubeyran, P., Dagorn, J. C., and Iovanna, J. L. (1999). pap, reg Ialpha and reg Ibeta mRNAs are concomitantly up-regulated during human colorectal carcinogenesis. Int J Cancer *81*, 688-94.

Reddy, J. K., and Mannaerts, G. P. (1994). Peroxisomal lipid metabolism. Annu Rev Nutr *14*, 343-70.

Renaud, J. P., and Moras, D. (2000). Structural studies on nuclear receptors. Cell. Mol. Life Sci. *57*, 1748-1769.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. (1998). The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature *391*, 79-82.

Ristow, M., Muller-Wieland, D., Pfeiffer, A., Krone, W., and Kahn, C. R. (1998). Obesity associated with a mutation in a genetic regulator of adipocyte differentiation. N Engl J Med *339*, 953-9.

Roeder, R. G. (1996). The role of general initiation factors in transcription by RNA polymerase II. TIBS *21*, 327-336.

Rojas, M., Fuks, A., and Stanners, C. P. (1990). Biliary glycoprotein, a member of the immunoglobulin supergene family, functions in vitro as a Ca2(+)-dependent intercellular adhesion molecule. Cell Growth Differ *1*, 527-33.

Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., Spiegelman, B. M., and Mortensen, R. M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell *4*, 611-7.

Saez, E., Tontonoz, P., Nelson, M. C., Alvarez, J. G., Ming, U. T., Baird, S. M., Thomazy, V. A., and Evans, R. M. (1998). Activators of the nuclear receptor PPARgamma enhance colon polyp formation. Nat Med *4*, 1058-61.

Sarraf, P., Mueller, E., Jones, D., King, F. J., DeAngelo, D. J., Partridge, J. B., Holden, S. A., Chen, L. B., Singer, S., Fletcher, C., and Spiegelman, B. M. (1998). Differentiation and reversal of malignant changes in colon cancer through PPARgamma. Nat Med *4*, 1046-52.

Sarraf, P., Mueller, E., Smith, W. M., Wright, H. M., Kum, J. B., Aaltonen, L. A., de la Chapelle, A., Spiegelman, B. M., and Eng, C. (1999). Loss-of-function mutations in PPAR gamma associated with human colon cancer. Mol Cell *3*, 799-804.

Schmidt, A., Endo, N., Rutledge, S. J., Vogel, R., Shinar, D., and Rodan, G. A. (1992). Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. Mol Endocrinol *6*, 1634-41.

Schulman, I. G., Shao, G., and Heyman, R. A. (1998). Transactivation by retinoid X receptor-peroxisome proliferator- activated receptor gamma (PPARgamma) heterodimers: intermolecular synergy requires only the PPARgamma hormone-dependent activation function. Mol Cell Biol *18*, 3483-94.

Seidel, G., Adermann, K., Schindler, T., Ejchart, A., Jaenicke, R., Forssmann, W. G., and Rosch, P. (1997). Solution structure of porcine delta sleep-inducing peptide immunoreactive peptide A homolog of the shortsighted gene product. J Biol Chem *272*, 30918-27.

Shao, D., Rangwala, S. M., Bailey, S. T., Krakow, S. L., Reginato, M. J., and Lazar, M. A. (1998). Interdomain communication regulating ligand binding by PPAR-gamma. Nature *396*, 377-80.

Sheng, H., Shao, J., Kirkland, S. C., Isakson, P., Coffey, R., Morrow, J., Beauchamp, R. D., and DuBois, R. N. (1997). Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J. Clin. Invest. *99*, 2254-2259.

Shibanuma, M., Kuroki, T., and Nose, K. (1992). Isolation of a gene encoding a putative leucine zipper structure that is induced by transforming growth factor beta 1 and other growth factors. J Biol Chem *267*, 10219-24.

Shinozaki, S., Nakamura, T., Iimura, M., Kato, Y., Iizuka, B., Kobayashi, M., and Hayashi, N. (2001). Upregulation of Reg 1alpha and GW112 in the epithelium of inflamed colonic mucosa. Gut *48*, 623-629.

Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem *69*, 145-82.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., and Fruchart, J. C. (1998). Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation *98*, 2088-93.

Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I. P., Delerive, P., Fadel, A., Chinetti, G., Fruchart, J. C., Najib, J., Maclouf, J., and Tedgui, A. (1998). Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators. Nature *393*, 790-3.

Stappenbeck, T. S., Wong, M. H., Saam, J. R., Mysorekar, I. U., and Gordon, J. I. (1998). Notes from some crypt watchers: regulation of renewal in the mouse intestinal epithelium. Curr Opin Cell Biol *10*, 702-9.

Steinbach, G., Lynch, P. M., Phillips, R. K. S., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., Levin, B., Godio, L., Patterson, S., Rodriguez-Bigas, M. A., Jester, S. L., King, K. L., Schumacher, M., Abbruzzese, J., DuBois, R. N., Hittelman, W. N., Zimmerman, S., Sherman, J. W., and Kelloff, G. (2000). The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N. Engl. J. Med. *342*, 1946-1952.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A. (2001). The hormone resistin links obesity to diabetes. Nature *409*, 307-12.

Su, C. G., Wen, X., Bailey, S. T., Jiang, W., Rangwala, S. M., Keilbaugh, S. A., Flanigan, A., Murthy, S., Lazar, M. A., and Wu, G. D. (1999). A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. J Clin Invest *104*, 383-9.

Su, J. L., Simmons, C. J., Wisely, B., Ellis, B., and Winegar, D. A. (1998). Monitoring of PPAR alpha protein expression in human tissue by the use of PPAR alpha-specific MAbs. Hybridoma *17*, 47-53.

Suh, N., Wang, Y., Williams, C. R., Risingsong, R., Gilmer, T., Willson, T. M., and Sporn, M. B. (1999). A new ligand for the peroxisome proliferator-activated receptor-gamma (PPAR-gamma), GW7845, inhibits rat mammary carcinogenesis. Cancer Res *59*, 5671-3.

Takagi, Y., Kohmura, H., Futamura, M., Kida, H., Tanemura, H., Shimokawa, K., and Saji, S. (1996). Somatic alterations of the DPC4 gene in human colorectal cancers in vivo. Gastroenterology *111*, 1369-72.

Takahashi, N., Okumura, T., Motomura, W., Fujimoto, Y., Kawabata, I., and Kohgo, Y. (1999). Activation of PPARgamma inhibits cell growth and induces apoptosis in human gastric cancer cells. FEBS Lett *455*, 135-9.

Tanaka, T., Kohno, H., Yoshitani, S., Takashima, S., Okumura, A., Murakami, A., and Hosokawa, M. (2001). Ligands for Peroxisome Proliferator-activated Receptors alpha and gamma Inhibit Chemically Induced Colitis and Formation of Aberrant Crypt Foci in Rats. Cancer Res *61*, 2424-2428.

Terazono, K., Yamamoto, H., Takasawa, S., Shiga, K., Yonemura, Y., Tochino, Y., and Okamoto, H. (1988). A novel gene activated in regenerating islets. J Biol Chem *263*, 2111-4.

Thun, M. J., Namboodiri, M. M., and Heath, C. W. J. (1991). Aspirin use and reduced risk of fatal colon cancer. N. Engl. J. Med. *325*, 1593-1596.

Tian, J. Q., and Quaroni, A. (1999). Dissociation between growth arrest and differentiation in Caco-2 subclone expressing high levels of sucrase. Am J Physiol *276*, G1094-104.

Tian, J. Q., and Quaroni, A. (1999). Involvement of p21(WAF1/Cip1) and p27(Kip1) in intestinal epithelial cell differentiation. Am J Physiol *276*, C1245-58.

Tontonoz, P., Graves, R. A., Budavari, A. I., Erdjument-Bromage, H., Lui, M., Hu, E., Tempst, P., and Spiegelman, B. M. (1994). Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPAR gamma and RXR alpha. Nucleic Acids Res *22*, 5628-34.

Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid- activated transcription factor. Cell *79*, 1147-56.

Tontonoz, P., Nagy, L., Alvarez, J. G., Thomazy, V. A., and Evans, R. M. (1998). PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell *93*, 241-52.

Tontonoz, P., Singer, S., Forman, B. M., Sarraf, P., Fletcher, J. A., Fletcher, C. D., Brun, R. P., Mueller, E., Altiok, S., Oppenheim, H., Evans, R. M., and Spiegelman, B. M. (1997). Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. Proc Natl Acad Sci U S A *94*, 237-41.

Treisman, J. E., Lai, Z. C., and Rubin, G. M. (1995). Shortsighted acts in the decapentaplegic pathway in Drosophila eye development and has homology to a mouse TGF-beta-responsive gene. Development *121*, 2835-45.

Tsujii, M., and DuBois, R. N. (1995). Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. Cell *83*, 493-501.

Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. (1998). Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell *93*, 705-716.

Tsujii, M., Kuwano, S., and DuBois, R. N. (1997). Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc. Natl. Acad. Sci. USA *94*, 3336-3340.

Valverde, A. M., Teruel, T., Navarro, P., Benito, M., and Lorenzo, M. (1998). Tumor necrosis factor-alpha causes insulin receptor substrate-2- mediated insulin resistance and inhibits insulin-induced adipogenesis in fetal brown adipocytes. Endocrinology *139*, 1229-38.

Vu-Dac, N., Schoonjans, K., Kosykh, V., Dallongeville, J., Fruchart, J. C., Staels, B., and Auwerx, J. (1995). Fibrates increase human apolipoprotein A-II expression through activation of the peroxisome proliferator-activated receptor. J Clin Invest *96*, 741-50.

Vu-Dac, N., Schoonjans, K., Laine, B., Fruchart, J. C., Auwerx, J., and Staels, B. (1994). Negative regulation of the human apolipoprotein A-I promoter by fibrates can be attenuated by the interaction of the peroxisome proliferator-activated receptor with its response element. J Biol Chem *269*, 31012-8.

Waddell, W. R., and Loughry, R. W. (1983). Sulindac for polyposis of the colon. J. Surg. Oncol. *24*, 83-87.

Way, J. M., Harrington, W. W., Brown, K. K., Gottschalk, W. K., Sundseth, S. S., Mansfield, T. A., Ramachandran, R. K., Willson, T. M., and Kliewer, S. A. (2001).

Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. Endocrinology *142*, 1269-77.

Williams, C. S., Tsujii, M., Reese, J., Dey, S. K., and DuBois, R. N. (2000). Host cyclooxygenase-2 modulates carcinoma growth. J. Clin. Invest. *105*, 1589-1594.

Willson, T. M., Brown, P. J., Sternbach, D. D., and Henke, B. R. (2000). The PPARs: from orphan receptors to drug discovery. J Med Chem *43*, 527-50.

Willson, T. M., Cobb, J. E., Cowan, D. J., Wiethe, R. W., Correa, I. D., Prakash, S. R., Beck, K. D., Moore, L. B., Kliewer, S. A., and Lehmann, J. M. (1996). The structure-activity relationship between peroxisome proliferator- activated receptor gamma agonism and the antihyperglycemic activity of thiazolidinediones. J Med Chem *39*, 665-8.

Willson, T. M., Lambert, M. H., and Kliewer, S. A. (2001). Peroxisome proliferator-activated receptor-gamma and metabolic disease. Annu. Rev. Biochem. *70*, 341-367.

Xie, W., Chipman, J., Robertson, D., Erikson, R., and Simmons, D. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc. Natl. Acad. Sci. USA *88*, 2692-2696.

Xin, X., Yang, S., Kowalski, J., and Gerritsen, M. E. (1999). Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo. J Biol Chem *274*, 9116-21.

Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., Sternbach, D. D., Lehmann, J. M., Wisely, G. B., Willson, T. M., Kliewer, S. A., and Milburn, M. V. (1999). Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell *3*, 397-403.

Yamaoka, T., Yoshino, K., Yamada, T., Idehara, C., Hoque, M. O., Moritani, M., Yoshimoto, K., Hata, J., and Itakura, M. (2000). Diabetes and tumor formation in transgenic mice expressing Reg I. Biochem Biophys Res Commun *278*, 368-76.

Yan, Z., Deng, X., Chen, M., Xu, Y., Ahram, M., Sloane, B. F., and Friedman, E. (1997). Oncogenic c-Ki-ras but not oncogenic c-Ha-ras up-regulates CEA expression and disrupts basolateral polarity in colon epithelial cells. J Biol Chem *272*, 27902-7.

Yen, C. J., Beamer, B. A., Negri, C., Silver, K., Brown, K. A., Yarnall, D. P., Burns, D. K., Roth, J., and Shuldiner, A. R. (1997). Molecular scanning of the human peroxisome proliferator activated receptor gamma (hPPAR gamma) gene in diabetic Caucasians: identification of a Pro12Ala PPAR gamma 2 missense mutation. Biochem Biophys Res Commun *241*, 270-4.

Yoh, S. M., Chatterjee, V. K., and Privalsky, M. L. (1997). Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T3 receptors and transcriptional corepressors. Mol Endocrinol *11*, 470-80.

Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M., and Lazar, M. A. (1995). Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J Biol Chem *270*, 23975-83.

Zamir, I., Zhang, J., and Lazar, M. A. (1997). Stoichiometric and steric principles governing repression by nuclear hormone receptors. Genes Dev *11*, 835-46.

Zhou, G., Cummings, R., Li, Y., Mitra, S., Wilkinson, H. A., Elbrecht, A., Hermes, J. D., Schaeffer, J. M., Smith, R. G., and Moller, D. E. (1998). Nuclear receptors have distinct affinities for coactivators: characterization by fluorescence resonance energy transfer. Mol Endocrinol *12*, 1594-604.

Zhou, H., Fuks, A., and Stanners, C. P. (1990). Specificity of intercellular adhesion mediated by various members of the immunoglobulin supergene family. Cell Growth Differ *1*, 209-15.

Zhou, X. P., Smith, W. M., Gimm, O., Mueller, E., Gao, X., Sarraf, P., Prior, T. W., Plass, C., von Deimling, A., Black, P. M., Yates, A. J., and Eng, C. (2000). Over-representation of PPARgamma sequence variants in sporadic cases of glioblastoma multiforme: preliminary evidence for common low penetrance modifiers for brain tumour risk in the general population. J Med Genet *37*, 410-4.

Zhu, Y., Alvares, K., Huang, Q., Rao, M. S., and Reddy, J. K. (1993). Cloning of a new member of the peroxisome proliferator-activated receptor gene family from mouse liver. J Biol Chem *268*, 26817-20.

Zhu, Y., Qi, C., Calandra, C., Rao, M. S., and Reddy, J. K. (1996). Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor gamma. Gene Expr *6*, 185-95.

Zhu, Y., Qi, C., Jain, S., Rao, M. S., and Reddy, J. K. (1997). Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. J Biol Chem *272*, 25500-6.