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Dissecting the role of peroxisome proliferator-activated receptor- β/δ (PPAR β/δ) in colon, breast, and lung carcinogenesis

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Abstract

Peroxisome proliferator-activated receptor- β/δ (PPAR β/δ) is a promising drug target since its agonists increase serum high-density lipoprotein; decrease low-density lipoprotein, triglycerides, and insulin associated with metabolic syndrome; improve insulin sensitivity; and decrease high fat diet-induced obesity. PPAR β/δ agonists also promote terminal differentiation and elicit anti-inflammatory activities in many cell types. However, it remains to be determined whether PPAR β/δ agonists can be developed as therapeutics because there are reports showing either pro- or anti-carcinogenic effects of PPAR β/δ in cancer models. This review examines studies reporting the role of PPAR β/δ in colon, breast, and lung cancers. The prevailing evidence would suggest that targeting PPAR β/δ is not only safe but could have anti-carcinogenic protective effects.

Keywords

Peroxisome proliferator-activated receptor- β/δ ; PPAR β/δ ; Colon carcinogenesis; Breast carcinogenesis; Lung carcinogenesis

1 Introduction

Peroxisome proliferator-activated receptor- β/δ (PPAR β/δ , also referred to as PPAR β or PPAR δ) is one of three ligand-activated transcription factors in the peroxisome proliferator-activated receptor (PPAR) family. PPARs are metabolic regulators that have important roles in the regulation of glucose and lipid homeostasis. PPAR α has a central role in regulating expression of target genes involved in lipid transport and catabolism and is the target of the lipid-lowering fibrate drugs. PPAR γ functions to promote adipocyte terminal differentiation and lipid storage, and ligand activation of PPAR γ leads to increased insulin sensitivity in diabetic patients. PPAR α and PPAR γ also influence carcinogenesis in animal models [1, 2]. For example, prolonged activation of PPAR α causes liver cancer in rats and mice while

humans are resistant to the carcinogenic effects of PPAR α agonists [3]. PPAR γ expression and activation suppresses cancer in most instances [4], while it causes cell proliferation and survival in some cell culture models [5–7]. The role of PPAR β/δ in control of cell proliferation and tumorigenesis has also been widely studied in different systems.

There are at least three different mechanisms by which PPAR β/δ can regulate cellular function (Fig. 1). The first is the classic nuclear receptor-mediated transcription of responsive target genes. PPAR β/δ is found in both cytoplasm and the nucleus, but appears to be found more predominantly in the nucleus in most tissues [8]. In response to ligand binding, PPAR β/δ undergoes a conformational change causing release of co-repressors possessing histone deacetylase activity (HDAC), heterodimerization with RXR, and recruitment of transcriptional proteins including RNA polymerase and co-activators with histone acetyl transferase (HAT) activity. It is becoming increasingly clear in recent years that the expression patterns of HDACs and HATs likely represents a unique level of regulation that modulates expression patterns of target genes mediated by nuclear receptors due to their ability to selectively alter chromatin structure because of differences in their enzymatic activities and substrate specificities [9]. To date, this possible level of regulation has not been examined extensively for any of the PPARs. Once the ligand-activated receptor heterodimer remodels chromatin of target genes allowing for access to the coding sequence, transcription ensues (Fig. 1). Through this mechanism, PPAR β/δ , like other nuclear receptors, can regulate important biological functions. In recent years, it has also become clear that PPAR β/δ can interfere with other proteins and transcription factors through a “trans-repression” mechanism (Fig. 1). For example, PPAR β/δ can interact with the p65 subunit of the NF- κ B complex and prevent NF- κ B-dependent regulation of genes involved in pro-inflammatory responses [10–14]. PPAR β/δ may also repress the transcription of some target genes through binding to DNA response elements in association with co-repressors [15] (Fig. 1).

The activity of PPARs can be selectively targeted with the use of high-affinity agonists. For example, there are three well-characterized PPAR β/δ agonists with affinity for the human receptor in the low nanomolar range, including L165041, GW501516, and GW0742 [16]. While high-affinity PPAR β/δ ligands have been instrumental for elucidating the functional role of PPAR β/δ in normal physiology, it is important to note that, as with all small-molecule drugs, specificity can be limited, and off-target effects must be controlled for. Thus, coupling knockdown approaches, transgenic mouse models, or stable cell lines over-expressing PPAR β/δ with high-affinity agonists have led to significant advances in our understanding of PPAR β/δ function in recent years.

Table 1 lists functional roles of PPAR β/δ that have been discovered since 1999. Activating PPAR β/δ can increase serum high-density lipoprotein cholesterol (HDLc) in rats, non-human primates and humans [17–19]. Ligand activation of PPAR β/δ can also reduce serum triglycerides, ameliorate high fat diet-induced obesity, improve glucose intolerance, and improve metabolic syndrome [18, 20–22]. Some of these changes are due in part to regulation of genes encoding fatty acid-metabolizing enzymes in skeletal muscle [20, 21] and genes encoding lipogenic protein in the liver that collectively prevent insulin resistance [23]. These beneficial effects observed in pre-clinical models contributed to the body of evidence supporting the development of PPAR β/δ agonists as possible drugs to treat diabetes and/or metabolic syndrome. Interestingly, it was recently suggested that ligand activation of PPAR β/δ could be suitable for the prevention of Alzheimer’s disease due to modulation of glucose homeostasis [24, 25]. Similar interest for targeting PPAR β/δ for disease prevention and/or treatment is based on the ability of PPAR β/δ to inhibit hepatic inflammation resulting from genetic, dietary, or chemical stimuli [26–32]. Inhibition of pro-inflammatory signaling mediated by PPAR β/δ including repression of cytokine expression

(e.g., TNF α , IL1 β , IL6, etc.) has also been found in a wide variety of models including experimental autoimmune encephalomyelitis, septic shock, pleurisy, colitis, and many others [10, 11, 14, 33–54]. Lastly, PPAR β/δ was also shown to promote terminal differentiation in keratinocytes, intestinal epithelium, oligodendrocytes, and osteoblasts and in urothelial, breast, colon, and neuroblastoma cancer cell lines [46, 55–73]. Combined, there is a wide range of physiological roles for PPAR β/δ that have been elucidated in the past 10 years due in a large part to the availability of selective ligands and the use of transgenic and knockdown models. In contrast to these established roles of PPAR β/δ in normal physiology, the effect of PPAR β/δ in carcinogenesis remains controversial as there are opposing results in cell culture and animal models, and the mechanism of its effects in these systems has not been determined.

The reasons underlying the uncertainty of PPAR β/δ function in carcinogenesis center on the relative expression levels of PPAR β/δ and/or the molecular targets/mechanisms mediated by PPAR β/δ prior to, and during, tumor promotion and progression. The issues surrounding relative expression patterns of PPAR β/δ stem primarily from the original hypothesis that PPAR β/δ is upregulated by the APC/ β -CATENIN/TCF4 pathway during colon tumorigenesis [74]. Since it was originally postulated in this landmark paper, a larger body of evidence has been published that does not support this view (reviewed in [75, 76]). While several hypothetical mechanisms have been postulated suggesting that PPAR β/δ promotes cell survival and thereby promotes tumorigenesis (Table 2), most of these putative pathways are based primarily on the original observations made in cells resembling mouse primary keratinocytes, but that do not exhibit a primary keratinocyte phenotype based on morphology, lack of expression of keratin 6, and altered expression of differentiation markers [70, 77]. The original hypothesis based on data obtained from these cells is that ligand activation of PPAR β/δ increases expression of 3-phosphoinositide-dependent-protein kinase 1 (PDK1) and integrin-linked kinase (ILK) and decreases expression of phosphatase and tensin homolog deleted on chromosome Ten (PTEN) causing increased phosphorylation of protein kinase B (AKT) leading to anti-apoptotic signaling and enhanced cell survival (Tables 2 and 3). However, numerous studies do not support this view including data obtained from mouse primary keratinocytes and analysis of published microarray databases (Tables 2 and 3). These disparities are rarely acknowledged or addressed experimentally in studies examining these putative pathways in various cancer models. Since there is considerable overlap in the response elements of PPAR target genes (e.g., all three PPARs can activate *ANGPTL4*, *ADRP*, etc. following ligand activation), it is also difficult to reconcile the hypothesis that PPAR β/δ promotes tumorigenesis, since it is generally accepted that PPAR γ inhibits tumorigenesis. Given the uncertain role of PPAR β/δ in cancer, the purpose of this review is to compare and contrast the opposing views of PPAR β/δ function in colon, breast, and lung tumorigenesis with an emphasis on relative expression during carcinogenesis and the effect of ligand activation.

2 Role of PPAR β/δ in colon cancer

For more than 10 years, the role of PPAR β/δ in colon carcinogenesis has been widely disputed in the literature. Excellent reviews on this subject have been recently published, so this section will be limited to the most substantive issues. The reader is encouraged to refer to the following papers for a more extensive review on this subject [75, 76, 78].

2.1 Expression patterns

Until recently, data were limited to mRNA as quantitative expression patterns of PPAR β/δ in different tissues at the protein level were not available [79, 80]. Using a highly specific anti-PPAR β/δ antibody and quantitative Western blots, it was shown that expression of PPAR β/δ was highest in epithelium including intestine, colon, and keratinocytes in mice [8].

PPAR β/δ was also found more exclusively in the nucleus in these tissues and could be co-immunoprecipitated with its heterodimerization partner, RXR [8]. These observations suggest that PPAR β/δ likely has an important constitutive function in epithelium, possibly due to the presence of an endogenous ligand. Antibody proteomic analysis also recently demonstrated that expression of PPAR β/δ is strong in human colon cells [81–83]. These observations are consistent with its known role in promoting terminal differentiation in epithelium (Table 1). Prior to our understanding that PPAR β/δ is expressed at high levels in normal colonic epithelium, it was hypothesized that PPAR β/δ is upregulated by the APC/ β -CATENIN/TCF4 pathway during colon tumorigenesis [74]. This hypothesis is now at odds with the normal expression patterns and is not supported by many reports showing reduced or unchanged expression of PPAR β/δ in colon cancer models (reviewed in [75, 76]). For example, expression of PPAR β/δ protein is markedly lower in both human and mouse colon tumors as compared with controls [81–84] (Table 4). While it is known that inflammatory signaling can increase the expression of PPAR β/δ and enhanced inflammation can be associated with colon tumorigenesis [85, 86], it remains possible that, in some instances, PPAR β/δ may be increased during colon cancer progression, but this increase is likely more related to the anti-inflammatory activities of PPAR β/δ . Collectively, the body of evidence showing that expression of PPAR β/δ is generally lower in colon tumors as compared with non-transformed colon tissue is greater than that suggesting expression of PPAR β/δ is higher in colon tumors [81–84], reviewed in [75, 76].

2.2 PPAR β/δ -dependent and ligand-induced effects

The first report to suggest a role for PPAR β/δ in colon cancer was based on the finding that expression of *PPAR β/δ* mRNA was higher in four colon tumors as compared with non-transformed tissue [74]. Given the fact that PPAR β/δ is expressed at high levels in normal human and mouse colon [8, 81–83], it is surprising to note that expression of *PPAR β/δ* mRNA was essentially absent in non-transformed colon tissue in this study [74]. Nevertheless, the observed increase in expression of *PPAR β/δ* mRNA in colon tumors was hypothesized at this time to be due to increased β -CATENIN/TCF4-mediated transcription, similar to *CYCLIN D1*, and that through yet-to-be identified target genes, PPAR β/δ increases cell proliferation, and thus serves as a tumor promoter in colon carcinogenesis [74]. These observations served as the foundation for many studies that have followed since, with some supporting this hypothesis while many others that do not (reviewed in [75, 76]).

Studies supporting the hypothesis that expression of PPAR β/δ is increased during colon carcinogenesis by β -CATENIN/TCF4-mediated transcription are based primarily on evidence from limited sample number showing higher expression of *PPAR β/δ* mRNA or protein using immunohisto-chemistry (reviewed in [75, 76]). However, there are more published studies to date showing that expression of PPAR β/δ is lower in colon cancer models (reviewed in [75, 76]). Most recently, an assessment of PPAR β/δ protein expression using quantitative Western blots revealed that expression of PPAR β/δ is lower in a panel of 19 human colon tumors and in a panel of nine colon tumors from *Apc^{+/Min}-FCCC* mice as compared with non-transformed colon tissue [84]. Lower expression of PPAR β/δ has also been confirmed in another cohort of human colorectal adenocarcinomas as compared with normal non-transformed colon tissue [81–83] (Table 4).

Four *in vivo* mouse studies support the hypothesis that activating PPAR β/δ will promote colon tumorigenesis. Administration of GW501516 caused an increase in the number of small intestine tumors in *Apc^{Min/+}* mice, but did not influence colon tumor multiplicity [87]. In a follow-up study from this group, tumor multiplicity in the small intestine and the colon was increased in *Apc^{Min/+}* mice by administration of GW501516, and this effect was not found in *Apc^{Min/+}* mice that lacked expression of PPAR β/δ [88]. These differences were associated with increased expression of VEGF, increased phosphorylation of AKT, and

increased cell survival in polyps in response to activating PPAR β/δ with GW501516 [88]. Using the azoxymethane-induced model, others showed that genetic disruption of PPAR β/δ in the colonocytes led to decreased colon tumor multiplicity, suggesting that PPAR β/δ promotes or enhances azoxymethane-induced colon cancer in mice [89]. The lower colon tumor multiplicity in the Ppar β/δ -null mice was associated with reduced expression of VEGF in colon polyps as compared with colon polyps from wild-type mice. Xenografts from HCT116 cells lacking PPAR β/δ expression are also markedly smaller as compared with xenografts that developed from control HCT116 colon cancer cells [90]. In contrast to these studies suggesting a tumor promoter role for PPAR β/δ in colon cancer, global disruption of PPAR β/δ in *Apc*^{Min/+} mice was shown to have no influence on intestinal tumorigenesis [91]. In contrast, another group also showed that colon tumorigenesis was exacerbated in *Apc*^{Min/+} mice lacking PPAR β/δ expression as well as in Ppar β/δ -null mice treated with azoxymethane as compared with wild-type mice [92]. While ligand activation of PPAR β/δ had no effect on intestinal tumorigenesis in *Apc*^{Min/+} mice, inhibition of azoxymethane-induced colon tumor multiplicity was found in wild-type mice but not in Ppar β/δ -null mice [63]. This effect was due in part to inhibition of cell proliferation and increased apoptotic signaling associated with the induction of terminal differentiation markers [63]. Additionally, in the latter study, ligand activation of PPAR β/δ had no effect on the expression of PDPK1, ILK, PTEN, or phosphorylation of AKT in mouse colon from the colon cancer bioassay [63]. Based on these reports, the *in vivo* evidence from animal models provides no consensus on the role of PPAR β/δ in mouse colon cancer models.

Analysis of human colon cancer cell lines has also yielded major discrepancies in the literature. Many studies using human colon cancer cell lines to delineate the possible mechanisms by which PPAR β/δ modulates cell growth have focused analysis on apoptosis/cell survival and are limited in scope. Most studies have also typically focused on mechanisms initially described in a keratinocyte-like cell that have inherent limitations (Table 2). Serum withdrawal-induced apoptosis is inhibited by GW501516 in HCT116 cells expressing PPAR β/δ but not in HCT116 cells where PPAR β/δ has been disrupted [87]. Similar inhibition of serum withdrawal-induced apoptosis has also been noted in LS-174 T colon cancer cells following treatment with GW501516, an effect attributed to a PPAR β/δ -dependent increase in expression of VEGF and increased phosphorylation of AKT causing enhanced cell survival [88]. These studies suggest that activation of PPAR β/δ promotes survival of human colon cancer cells but are limited because this phenotype requires the absence of culture medium serum that does not model normal physiology. In contrast to these studies, others found that serum withdrawal-induced apoptosis in human colon cancer cell lines is unaffected by ligand activation of PPAR β/δ [93]. Indeed, ligand activation of PPAR β/δ in human colon cancer cell lines has either no effect or inhibits cell proliferation [93, 94]. This is consistent with the lack of change in expression of VEGF or phosphorylation of AKT following ligand activation of PPAR β/δ using a broad concentration range of either GW501516 or GW0742 in the presence or absence of culture medium serum in HT29, HCT116, or LS174T human colon cancer cell lines [93]. More recently, it was shown that ligand activation of PPAR β/δ caused a dose-dependent decrease in the percentage of cells in the early stage of apoptosis in DLD1 human colon cancer cell in response to hydrogen peroxide-induced apoptosis [84]. However, this dose-dependent decrease in the percentage of cells in the early stage of apoptosis was not associated with an increase in viable cells, but rather, an increase in the percentage of cells in the late stages of apoptosis/necrosis [84]. This supports the notion that assessment of apoptosis in response to PPAR β/δ ligands should include comprehensive analysis. Silencing expression of PPAR β/δ in HCT116 cells was also reported to cause enhanced cell proliferation as compared with control HCT116 human colon cancer cells [95]. Similar to the findings from *in vivo* analysis, results from *in vitro* studies are collectively inconclusive and fail to provide definitive evidence of the precise role of PPAR β/δ in human colon cancer cell lines.

Three studies have examined the relationship between PPAR β/δ expression and colorectal cancer in humans. In one study that relied extensively on immunohistochemistry to estimate expression of PPAR β/δ , no relationship between PPAR β/δ expression and relative Ki-67 expression, a measure of cell growth, was observed [96]. However, this study provided correlative evidence indicating that colon tumors with greater immunohistochemical localization of PPAR β/δ exhibited a more malignant phenotype based on histopathology, as compared with colon tumors with lower immunohistochemical localization of PPAR β/δ , but this difference was not supported by statistical analysis [96]. In a cohort of 52 colorectal cancer patients, higher expression of PPAR β/δ and cyclooxygenase 2 (COX2) was negatively associated with overall survival [97]. While approximately 90% of patients with either low or absent PPAR β/δ and/or COX2 survived beyond 60 months after resection of primary colon tumors, less than 50% of patients that expressed both PPAR β/δ and COX2 survived beyond 60 months [97]. In this study, there was no direct analysis of PPAR β/δ expression; PPAR β/δ was estimated based solely on immunohistochemistry. This is problematic, in particular for colon cancer, because detection of PPAR β/δ by immunohistochemistry has led to misleading results due to the non-specific immunoreactivity associated with even highly specific anti-PPAR β/δ antibodies [8]. For example, it was previously suggested that expression of PPAR β/δ was high in the nuclei of *Apc*^{Min/+} mouse colon and intestinal tumors and that administration of nitric oxide (NO)-donating aspirin reduced the nuclear localization as revealed by immunohistochemical analysis of PPAR β/δ [98]. This led to the hypothesis that one mechanism by which NO-donating aspirin elicits colon cancer chemopreventive activities is through downregulation of PPAR β/δ [98]. However, subsequent re-analysis using samples obtained from this same study revealed that the expression of PPAR β/δ in colon was not different between wild-type and *Apc*^{Min/+} mice and that NO-donating aspirin had no effect on nuclear localization of PPAR β/δ based on quantitative Western blot analysis [84]. Because immunohistochemical analysis of PPAR β/δ is not optimal for quantitative assessment of PPAR β/δ expression and because no direct correlation between PPAR β/δ expression and survival was performed, the analysis suggesting a link between both PPAR β/δ and COX2 expression and survival of colorectal cancer patients could be considered inconclusive [97]. Moreover, both of these studies are inconsistent with the recent study showing that expression of PPAR β/δ is lower in a panel of 19 human colon tumors as compared with non-transformed colon tissue [84]. The most compelling clinical evidence to date examining the role of PPAR β/δ and colon cancer was recently obtained from retrospective analysis of 141 colorectal cancer patients [99]. This study used immunohistochemical analysis of PPAR β/δ expression and quantitative Western blots in resected colorectal tumors with levels of PPAR β/δ directly correlated with cell proliferation and apoptosis, and calculated survival rates over an approximate 15-year follow-up period. Relatively higher expression of PPAR β/δ in primary tumors was associated with a higher frequency of stage I cases, a lower frequency of stage II, stage III, and stage IV cases, and a lower rate of lymph node metastasis [99]. Similarly, relatively higher expression of PPAR β/δ was also associated with lower expression of Ki-67 in primary cancers, while expression of PPAR β/δ was unrelated to either COX2 or SURVIVIN expression in primary cancers [99]. The most striking observation from this analysis was the finding that disease-free survival was markedly greater for those patients with relatively high expression of PPAR β/δ in primary cancers as compared with those patients with relatively low expression [99]. In fact, colorectal cancer patients with relatively low expression of PPAR β/δ were 3.6 times more likely to die of colorectal cancer than those with relatively higher expression of PPAR β/δ in primary tumors [99]. Importantly, the immunohistochemical analysis of PPAR β/δ expression in this study was verified by quantitative Western blotting. Given the more precise quantification of PPAR β/δ , the large number of patients examined and the length of follow-up, this is the most compelling evidence published to date suggesting that PPAR β/δ has a protective role in human colorectal cancer.

2.3 Summary

The controversy of whether PPAR β/δ expression is either increased or decreased during colon carcinogenesis stems from the hypothesis that PPAR β/δ is upregulated by the APC/ β -CATENIN/TCF4 pathway during colon tumorigenesis [74]. Since this study was published, there is considerable evidence inconsistent with this hypothesis that outweighs the body of evidence supporting this hypothesis (reviewed in [75, 76]). Many of the studies supporting the view that PPAR β/δ is upregulated by the APC/ β -CATENIN/TCF4 pathway during colon tumorigenesis are based on studies with small sample size, coupled with analysis of PPAR β/δ expression largely determined by immunohistochemistry and not validated, and/or exclusive analysis of mRNA expression. In contrast, other studies examining expression using larger samples and microarrays of human or rodent colon cancer models indicate that expression of PPAR β/δ is lower during colon carcinogenesis (reviewed in [75, 76]). The most quantitative analysis to date also demonstrated unequivocally that expression of PPAR β/δ is markedly lower in both human and mouse colon tumors [84] that is consistent with proteomic analysis (Table 4). Why some studies report that one mechanism by which non-steroidal anti-inflammatory drugs (NSAIDs) mediate chemopreventive effects for colon cancer due to decreased expression of PPAR β/δ (Table 2) is also unclear. This is because more conclusive findings show that expression of PPAR β/δ is lower in human and mouse colon tumors, coupled with the observations that expression of PPAR β/δ is either unchanged or increased in human colon cancer cell lines treated with NSAIDs ([84], reviewed in [75, 76]). Since inflammatory signaling can increase expression of PPAR β/δ , it remains possible that the expression of PPAR β/δ may be higher in patients exhibiting relatively high inflammation. However, the potential effect of higher PPAR β/δ expression resulting from inflammation is unclear as it could be postulated to enhance or attenuate carcinogenesis. Given the potent anti-inflammatory effect of PPAR β/δ , it is possible that higher PPAR β/δ expression resulting from inflammation could function to help resolve inflammatory signaling, and indeed there is evidence supporting this view. However, further studies are needed to definitively test this hypothesis. Combined, there is strong evidence supported by recent clinical findings (see below) suggesting that low PPAR β/δ is not a good prognostic indicator of colorectal cancer and that strategies to increase expression or increase receptor activity may prevent colon carcinogenesis.

It is not possible at this time to determine why some *in vivo* and *in vitro* studies suggest that activating PPAR β/δ will potentiate colon carcinogenesis while others do not. Studies suggesting that ligand activation of PPAR β/δ will increase human colon cancer cell line survival are limited because they rely on serum starvation to exhibit this effect and have never demonstrated an increase in the number of viable cells. Indeed, recent studies indicate that while markers of early apoptosis can be dose-dependently decreased by ligand activation of PPAR β/δ in human colon cancer cells, this change was associated with an increase in late apoptotic/necrotic cells, not an increase in viable cells [84]. Past speculation to account for the reported disparities has included the idea that the null mouse models used may express mutant forms of PPAR β/δ capable of acting as dominant negative, the presence of modifier of min alleles, strain differences, and differences in ligand examined. Until these and other hypotheses to explain the observed differences can be critically examined experimentally, it is not feasible to suggest that the data obtained from *in vivo* animal models or *in vitro* models using human colon cancer cell lines support or refute a role for PPAR β/δ in promotion or attenuation of colon carcinogenesis. Notwithstanding, the most credible clinical evidence to date is the finding that survival of colorectal cancer patients is markedly greater in patients exhibiting relatively higher expression of PPAR β/δ as compared with patients exhibiting relatively lower expression of PPAR β/δ in primary tumors [99]. Additional studies like this, with accurate and definitive quantification of PPAR β/δ expression and function, would significantly advance the field.

3 Role of PPAR β/δ in breast cancer

Similar to the uncertainty surrounding the role of PPAR β/δ in colon cancer, the influence of PPAR β/δ in breast cancer is also unclear. There are only a limited number of studies published to date that have examined the effect of PPAR β/δ and its ligands in mouse breast cancer models and human breast cancer cell lines.

3.1 Expression patterns

Expression of PPAR β/δ in normal human breast tissue is strong, in particular in the glandular cells where it is localized in the nucleus (Table 4) [81–83]. This is similar to data showing that expression of both PPAR β/δ mRNA and protein is found at very high levels in epithelium such as intestine, colon, or keratinocytes as compared with other tissues [8, 80]. It is also known that PPAR β/δ mRNA expression is lower as compared with PPAR γ mRNA in mouse mammary tissue and that PPAR γ 2 mRNA is the most predominant PPAR isoform in this tissue [100]. Expression of PPAR β/δ protein is lacking or weak in 92% of human lobular and ductal carcinomas examined (Table 4) [81–83]. This is consistent with the finding that expression of PPAR β/δ protein is close to four times lower in the C20 mouse mammary gland cancer cell line as compared with mouse keratinocytes and more than two times lower in C20 cells as compared with human MCF10A cells [101]. Since the C20 cell is an aggressive pro-tumorigenic mouse mammary gland cancer cell line, this demonstrates that expression of PPAR β/δ is lower in this cell line as compared with normal mouse keratinocytes that are known to express relatively high levels of PPAR β/δ and as compared with the normal human breast epithelial cell line, MCF10A. Combined, there is good evidence that expression of PPAR β/δ in normal breast tissue is markedly higher in glandular cells as compared with breast tumors, where expression is absent or weak (Table 4).

3.2 PPAR β/δ -dependent and ligand-induced effects

The first report to suggest that PPAR β/δ regulates breast cancer was the finding that proliferation of estrogen receptor (ER)-positive MCF7 and T47D human breast cancer cells is increased in response to PPAR β/δ ligands [94]. Doxycyclin-induced over-expression of PPAR β/δ in T47D cells also caused an increase in cell proliferation suggesting that PPAR β/δ facilitated this mitogenic effect induced by GW501516. Since the effect on proliferation was not found in PPAR β/δ -ligand-treated ER-negative human breast cancer cell lines, this suggests that the effect of ligand activation of PPAR β/δ by GW501516 on cell proliferation was dependent on ER. In contrast to these findings, expression of PPAR β/δ mRNA is transiently increased in MCF7 cells induced to differentiate by sodium butyrate [56]. As the induction of differentiation in MCF7 cells is known to be associated with a block in the G1 phase of the cell cycle [102], this observation suggests that the increase in PPAR β/δ expression induced by sodium butyrate could contribute to inhibition of cell cycle progression. This is consistent with inhibition of cell proliferation observed in MCF7 cells treated with either GW501516 or GW0742 [103]. Moreover, enhanced ligand-dependent inhibition of cell proliferation is found in MCF7 breast cancer cells constitutively over-expressing PPAR β/δ as compared with controls (unpublished, manuscript in preparation). Ligand activation of PPAR β/δ in C20 mouse mammary gland cancer cells inhibits proliferation due to an increase in apoptosis [101]. These changes are consistent with the observed dose-dependent inhibition of clonogenicity of C20 mouse mammary gland cancer cells following ligand activation of PPAR β/δ [101]. Interestingly, cell doubling time of MCF7 cells was also reported as unchanged following ligand activation of PPAR β/δ or treatment with the highly specific PPAR β/δ antagonist GSK3787 as assessed by real-time examination of cell growth using the xCELLigence System [104]. These combined results from human and mouse mammary cancer cell lines demonstrate why considerable confusion remains surrounding the role of PPAR β/δ in breast cancer. It is important to note that there

are differences in the approaches used to assess cell proliferation that may explain in part the reported differences. For example, Stephen and colleagues [94] used an enzyme-based assay to assess cell number, which depends on mitochondrial dehydrogenase activity to cleave substrate and estimate cell number based on this activity. Since expression of mitochondrial dehydrogenases can be increased by PPAR ligands [20, 105], the use of enzyme-based assays to estimate cell proliferation are likely unsuitable when examining the effect of PPAR ligands. In contrast, Coulter counting and flow cytometry was used to show fewer MCF7 and C20 mammary cancer cell lines following treatment with GW0742 and/or GW501516 [101, 103], while the use of the xCELLigence System that measures cell proliferation in real-time revealed no change by either a PPAR β/δ agonist or antagonist [104] but did detect dose-dependent inhibition of cell proliferation in MCF7 cells over-expressing PPAR β/δ (unpublished, manuscript in preparation). Thus, the published data examining the effect of PPAR β/δ in cultured mammary cancer cell lines provide no consensus and illustrate why more studies are needed to determine the functional role in breast cancer cells.

A number of studies have also examined the effect of PPAR β/δ using mouse models, but these studies are also limited. The incidence of mammary tumorigenesis is mitigated in COX2 transgenic mice on a Ppar β/δ -null background, suggesting that PPAR β/δ is required for mammary tumorigenesis in this model, but tumor multiplicity was not reported [106]. In FVB mice treated with medroxyprogesterone acetate and 7, 12-dimethylbenz[a] anthracene (DMBA), dietary administration of GW501516 at a concentration of 0.005% caused an increase in mammary tumorigenesis yet tumor multiplicity was not reported [107]. Using the same DMBA model, this group also found that dietary GW501516 accelerated mammary tumorigenesis in both wild-type and transgenic mice over-expressing PDPK1, and this was associated with a greater percentage of later-stage tumors [108]. However, no change in tumor multiplicity was observed in response to GW501516 [108]. It was suggested that this phenotype was mediated in part by interactions between PPAR β/δ and PDPK1, based in part on the reported increase in PDPK1 expression following treatment with GW501516 [107, 108]. While these studies suggest that activating PPAR β/δ may accelerate mammary tumorigenesis, there are significant limitations with this work. The small number of tumors per mouse (≤ 2) precludes rigorous statistical comparisons of the distribution of mammary tumors and tumor multiplicity. The use of a single concentration of GW501516 does not allow for dose-dependent analysis. This is important because GW501516 preferentially activates human PPAR β/δ with between 667–833 $\times$ greater affinity for this isoform as compared with PPAR α or PPAR γ , but this is not the case for mouse PPAR β/δ where this difference in affinity is only 33–62 $\times$ greater as compared with PPAR α or PPAR γ [109]. Thus, these studies do not demonstrate that PPAR β/δ mediates these effects because they do not control for receptor expression. It is also of great interest to note that, despite data showing increased expression of PDPK1 [108], analysis of microarray data from this same study revealed that GW501516 does not increase expression of PDPK1 in either wild-type or PDPK1 transgenic mice (Table 3). Additionally, microarray databases available in Gene Expression Omnibus from published peer-reviewed manuscripts reveal that PDPK1 is not responsive to ligand activation in muscle cells [110], human hepatoma cells over-expressing PPAR β/δ [111], or human monocytes [112]. This is consistent with microarray analysis from our studies showing no change in expression of PDPK1 following ligand activation of PPAR β/δ in mouse keratinocytes (Table 3). Coupled with observations showing that PDPK1 is not a PPAR β/δ target gene by direct examination of protein and/or mRNA expression [58, 59, 63] as suggested by some (Table 2) demonstrates why this mechanism of action remains a hypothesis that requires verification by others.

More recently, two ancillary hypotheses that PPAR β/δ mediates pro-tumorigenic effects in mammary cancer models have also been postulated. These hypotheses are based on the notion that a high intracellular ratio of fatty acid binding protein 5 (FABP5) to cellular

retinoic acid-binding protein II (CRABP-II) can divert all *trans* retinoic acid (atRA) from classic retinoic acid receptor (RAR) to PPAR β/δ , based on results from studies using a human keratinocyte cell line [113]. To examine this putative mechanism in a breast cancer model, two lines of MMTV-neu transgenic mice were generated with one lacking expression of CRABP-II and the other over-expressing CRABP-II [114]. Tumor volume was not different between MMTV-neu transgenic mice that lacked expression of CRABP-II as compared with control MMTV-neu transgenic mice [114]. The most striking result from this work was that tumor volume was decreased in MMTV-neu transgenic mice that over-expressed CRABP-II as compared with control MMTV-neu transgenic mice, and this correlated well with improved survival, as well as reduced markers of enhanced cell proliferation in the tumors examined [114]. No statistically significant differences in tumor multiplicity or tumor incidence were found between the different genotypes. Examination of tumors and cells derived from the tumors revealed that the ability of atRA to activate RAR target genes and genes associated with repressing cell proliferation was enhanced in MMTV-neu transgenic mice over-expressing CRABP-II as compared with controls. The authors provided *in vitro* evidence suggesting that the observed inhibition of mammary tumorigenesis was due to diversion of atRA away from PPAR β/δ towards RAR based primarily on analysis of expression of PDPK1 and the use of knockdown experiments. A related hypothesis was subsequently postulated that PPAR β/δ could modulate epidermal growth factor receptor (EGFR)-dependent proliferation of human breast cancer cells. This hypothesis is based primarily on data showing that HEREGULIN β 1 (HRG β 1) can upregulate FABP5 expression in MCF7 human breast cancer cells causing an increase in PPAR β/δ -dependent expression of *PDPK1* mRNA leading to enhanced proliferation [115]. This hypothesis is supported by previous work showing increased proliferation of MCF7 cells in response to PPAR β/δ ligands [94] but is in contrast to other studies showing inhibition of proliferation of MCF7 cells or mouse C20 cells by PPAR β/δ ligands [101, 103] or no change in proliferation of MCF7 cells following culture with either a PPAR β/δ agonist or a PPAR β/δ antagonist [104].

There are significant limitations with both studies described above [114, 115]. Since no dose-dependent analysis of atRA or PPAR β/δ ligand administration was performed for the *in vivo* analysis, it is not possible to conclude that this putative pathway between FABP5/CRABP-II and PPAR β/δ /RAR contributes to the mechanisms of the observed differences in tumorigenicity examined. However, it remains possible that the improved survival and reduced tumor volume are due CRABP-II-dependent modulation of pathways that are not influenced by PPAR β/δ . As the mice used for these studies were on a C57BL/6 and FVB mixed genetic background, this study is limited because the genetic background of mice can markedly influence the outcome of cancer bioassays [116–118]. The small number of animals examined in this work also significantly reduces the confidence levels. In most instances, the magnitude of the change in expression of mRNA encoding *PDPK1* in tumor cells with a high ratio of FABP5/CRABP-II (derived from MMTV neu transgenic X *Crabp-II*-null mice) was also very modest (less than twofold) as compared with the other tumor cells and was not confirmed at the protein level [114]. Surprisingly, a ~2-fold increase in expression of *PDPK1* mRNA was observed in both control MCF7 cells and MCF7 cells with FABP5 knocked down [115]. This is entirely inconsistent with the hypothesis that HRG β 1/EGFR-mediated induction of FABP5 expression in MCF7 cells increases cell survival/proliferation by diverting ligands from RAR to PPAR β/δ because reduced bromodeoxyuridine incorporation and cell proliferation was found in both MCF7 cells and MCF7 cells with FABP5 knocked down [115]. The absence of data demonstrating that these small changes in PDPK1 expression can differentially alter cell survival as compared with the control tumor cells and/or tumor cells with a higher FABP5/CRABP-II ratio, coupled with the lack of data showing differential effects by PPAR β/δ ligands are other limitations in these studies [114]. It is also uncertain how such a small difference in CRABP-II expression

can functionally outcompete FABP5 for PPAR β/δ ligands, since it was expressed at very low levels and markedly lower as compared with FABP5 [114]. This illustrates the need to demonstrate that the binding affinity of PPAR β/δ ligands for CRABP-II is substantively greater as compared with the binding affinity of PPAR β/δ ligands for FABP5. Without these data, support for the proposed hypothesis is not strong. Moreover, subsequent analysis demonstrated that the hypothetical mechanism by which the FABP5/CRABP-II ratio modulates the ability of atRA to activate either PPAR β/δ or RAR is not functional based on multiple levels of analysis from two independent laboratories [58, 119] (Table 2). This includes the facts that atRA does not compete with PPAR β/δ ligands in a ligand binding assay, atRA does not recruit co-activators to PPAR β/δ , atRA does not activate well-characterized PPAR β/δ target genes, and atRA or PPAR β/δ ligands do not increase expression of PDPK1 [58, 59, 63, 119]. Collectively, given these major limitations, these studies must be viewed with caution until they can be rigorously reexamined and confirmed by other independent laboratories.

3.3 Summary

The review of the literature indicates that the role of PPAR β/δ in breast cancer is unclear. Analysis of human samples demonstrates that expression of PPAR β/δ expression is strong in glandular cells of human breast tissue and markedly lower in lobular and ductal carcinomas (Table 4). This is similar to PPAR β/δ expression in mouse and human colon and colon tumors [81–84]. Studies in breast cancer patients, similar to the compelling retrospective analysis of PPAR β/δ expression in primary tumors from colorectal cancer patients demonstrating that survival is markedly greater in patients with primary tumors exhibiting relatively higher expression of PPAR β/δ , as compared with patients with primary tumors with relatively lower expression of PPAR β/δ [99], would provide a major advance for the breast cancer field. Regardless of whether expression of PPAR β/δ is increased, decreased, or unchanged in breast cancer, the effect of ligand activation of the receptor on cell growth is also uncertain. Based on studies using human or mouse mammary cancer cell lines, some reports suggest that activating the receptor will increase cell proliferation; other studies suggest that activating the receptor will inhibit cell proliferation, and other studies suggest that activating the receptor or antagonizing the receptor has no effect on cell proliferation. The reason for these differences is unknown. Evidence from the limited *in vivo* studies generally suggests that PPAR β/δ may promote tumorigenesis. However, there are many limitations to these studies that preclude definitive conclusions including the marginal changes in tumor multiplicity, the lack of good dose-dependent analysis, and the lack of controls for receptor specificity given the relatively low selectivity of PPAR β/δ ligands for the mouse receptor as compared with the human isoform. Until the reason for these differences can be delineated, it is not possible to suggest that PPAR β/δ promotes, inhibits, or has no effect on breast cancer. The hypothesis that FABP5-dependent diversion of PPAR β/δ ligands to promote mammary tumorigenesis via induction of a single putative target, PDPK1, also has many inherent limitations that require significant re-examination. This is exemplified in part by the recent illustration that a single oncogenic target is unlikely the key to the mechanisms underlying any carcinogenesis model [120]. Coupled with the aforementioned uncertainty of the precise role of PPAR β/δ and its ligands in breast cancer, whether FABP5-dependent modulation of PPAR β/δ activity is indeed functional remains an unproven hypothesis.

4 Role of PPAR β/δ in lung cancer

As noted for the effect of PPAR β/δ in colon and breast cancer, the role of this receptor in lung cancer is also confusing. This section will summarize the literature to illustrate the opposing views. There are limited *in vitro* and *in vivo* studies that have focused on lung cancer models.

4.1 Expression patterns

As compared with tissues exhibiting high expression of PPAR β/δ such as colon, small intestine, and keratinocytes, expression of PPAR β/δ in lung is relatively low in rats and mice [8, 80]. Expression of PPAR β/δ protein in normal human lung as compared with either lung adenocarcinoma or lung squamous carcinoma is not significantly different based on Western blot analysis of four tumors [121]. These authors also performed extensive analysis of PPAR β/δ expression in a larger cohort of lung tumors using immunohistochemistry and suggested that expression of PPAR β/δ was higher in tumors as compared with non-transformed control lung tissue despite the Western blot analysis [121]. However, since immunohistochemical analysis of PPAR β/δ expression has been shown to incorrectly estimate expression due to high non-specific immunoreactivity of available antibodies [8, 122], this interpretation is inconclusive. Proteomic analysis of human tissues revealed that expression of PPAR β/δ is moderate in respiratory epithelial cells of the bronchus (Table 4) [81–83]. In contrast, expression of PPAR β/δ is lacking or weak in 91% of human lung adenocarcinomas and squamous cell carcinomas samples examined (Table 4) [81–83]. Combined, Western blot analysis of a small cohort of human lung tumors shows that expression of PPAR β/δ is not different from non-transformed control tissue. Immunohistochemical analysis suggests that expression of PPAR β/δ is higher in lung tumors as compared with non-transformed tissue, but this methodology has inherent limitations. In contrast, tissue microarray indicates that expression of PPAR β/δ is moderate in respiratory epithelial cells of the bronchus and lacking or weak in human lung adenocarcinomas and squamous cell carcinomas.

4.2 PPAR β/δ -dependent and ligand-induced effects

The first report to suggest a role for PPAR β/δ in a lung cancer model was based on data obtained from the human lung adenocarcinoma cell line A549. Proliferation of A549 cells was unchanged by the PPAR β/δ ligand L165041 (10 μ M) but inhibited with 20–40 μ M L165041 [123]. The observed inhibition of proliferation of A549 cells was associated with decreased expression of CYCLIN D1 and PCNA and growth arrest at the G1 phase of the cell cycle but without change in the percentage of apoptotic cells [123]. Interestingly, co-treatment of A549 cells with L165041 and a COX inhibitor, to reduce prostaglandins with known mitogenic effects, resulted in marked inhibition of cell proliferation due in part to the induction of apoptosis [123]. Enhanced induction of apoptosis by co-treatment with NSAIDs and PPAR β/δ ligands has also been observed in human colon cancer cell lines [84]. The former observations were the first to suggest that activation of PPAR β/δ in human lung adenocarcinoma cells could inhibit proliferation and if coupled with COX inhibitors, the observed inhibition could be markedly enhanced through increased apoptotic signaling. This is consistent with the finding that GW0742 and prostacyclin analogues can inhibit proliferation of mouse lung fibroblasts [124]. Since the effect of the prostacyclin analogue were not noted in mouse lung fibroblasts from *Ppar β/δ* -null mice, this shows that this effect requires a functional receptor to mediate the inhibition in lung fibroblasts.

In contrast to these observations, five publications from other laboratories suggest that activating PPAR β/δ in human lung cancer cell lines increases proliferation through a number of mechanisms. Culturing H157 cells in 1 μ M GW501516 caused an increase in 3 H-thymidine incorporation through a mechanism mediated by increased expression of prostaglandin E2 receptor subtype EP4 [125]. This increase in cell proliferation induced by GW501516 was also associated with increased phosphorylation of AKT and decreased expression of PTEN. These studies also provided evidence suggesting that PPAR β/δ was required for all of these effects including increased expression of EP4 and increased phosphorylation of AKT; however, this paper was recently retracted [125]. This group went on to show that increased proliferation of H1838 cells by GW501516 was due in large part

to PPAR β/δ -dependent repression of PTEN expression leading to increased phosphorylation of AKT and enhanced NF- κ B activity [126]. This hypothesis is supported by studies showing increased expression of PDPK1 and phosphorylation of AKT and decreased expression of PTEN in A549 cells treated with the PPAR β/δ ligand GW501516 [121]. These changes were associated with a small increase in proliferation of A549, H23, and H157 human lung cancer cells, inhibition of cisplatin-induced apoptosis in A549 and H23 human lung cancer cells, and an increase in anchorage-independent growth following treatment with GW501516 [121]. Consistent with these changes, repressed expression of PTEN by GW501516 was diminished in A549 cells expressing a dominant negative PPAR β/δ and proliferation of A549 cells was inhibited in cells expressing the dominant negative PPAR β/δ as compared with controls [121]. An ancillary mechanism whereby ligand activation of PPAR β/δ causes reduced activity of AMP kinase (AMPK) was also reported, but reduced AMPK activity resulting from GW501516 did not underlie the mitogenic effect caused by GW501516 treatment [127]. A more direct link between smoking, lung cancer, and PPAR β/δ was also suggested by induction of PPAR β/δ by nicotine in human lung cancer cells [128]. However, whether this change in expression of PPAR β/δ in human lung cancer cell lines resulting from nicotine exposure was causally related to an increase in cell proliferation or cell survival was not critically examined in this study.

While some studies using human lung cancer cell lines suggest that activating PPAR β/δ inhibits proliferation and others suggest that activating PPAR β/δ promotes cell proliferation and survival through anti-apoptotic activities, one study reported that activating PPAR β/δ has no effect of human lung cancer cell line cell proliferation. Using H1838 and A549 human lung cancer cell lines, it was found that cell proliferation was unaffected by activating PPAR β/δ with 0.1 through 10 μ M GW501516 or GW0742 [129]. These data were examined over a 4-day culture period, and cell number was quantified with a Coulter counter, which is more quantitative than enzyme-based assays to assess cell proliferation as used by others [121, 125, 126]. Furthermore, examination of cell cycle progression by flow cytometry after staining with propidium iodide were consistent with the data obtained from assessment of cell number by Coulter counting, as no changes in the percentage of cells in any phase of the cell cycle were observed following treatment with 0.1 through 10 μ M GW501516 or GW0742 in either H1838 or A549 cells [129]. Moreover, no change in expression of PTEN, PDPK1, or phosphorylation of AKT was observed in either H1838 or A549 cells following treatment with 0.1 through 10 μ M GW501516 or GW0742 [129]. Examination of real-time cell proliferation of A549 cells also revealed no difference following ligand activation of PPAR β/δ with GW0742 or treatment with the PPAR β/δ antagonist GSK3787 [104]. Combined, these data show that activating PPAR β/δ in H1838 or A549 does not influence cell proliferation or alter expression of putative targets that could modulate cell proliferation or survival.

Several studies have directly and indirectly examined the effect of PPAR β/δ or PPAR β/δ ligands in lung cancer models *in vivo*. Prostacyclin and stable prostacyclin analogues can activate PPAR β/δ as based primarily on *in vitro* reporter assays [130]. Pulmonary over-expression of prostacyclin synthase increases production of prostacyclin and markedly inhibits lung cancer resulting from exposure to urethane, 3-methylcholanthrene, or tobacco smoke in mouse models [131, 132]. Since prostacyclin may activate PPAR β/δ , these observations suggest that PPAR β/δ could protect against chemically induced lung carcinogenesis. However, there is evidence suggesting that the chemopreventive effects of a stable prostacyclin analogue observed in these models could be mediated by PPAR γ [133]. Additionally, since it was also shown that increasing prostacyclin synthesis does not increase activation of PPAR β/δ in a number of cell lines [134], this also suggests that the effects observed in the lung cancer models may not be directly mediated by PPAR β/δ . However, the hypothesis that PPAR β/δ is responsible for the chemopreventive effects of

prostacyclin in lung cancer models has not been directly examined to date using transgenic mouse models. Lung tumor multiplicity was more than two times higher in C-RAF transgenic mice crossed with *Pparβ/δ*-null mice as compared with lung tumor multiplicity of control C-RAF transgenic mice [135]. This observation suggests that PPARβ/δ protects against C-RAF-mediated lung tumorigenesis in a mouse model. Evaluation of genetic variants in human lung cancer patients revealed associations between inflammation and polymorphisms in interleukin 1A (*IL1A*), *IL1B*, and *PPARβ/δ*, although the false-positive report probability was high for *PPARβ/δ* [136]. This is of interest to note because PPARβ/δ and PPARβ/δ agonists are known to have potent anti-inflammatory activities [137], which might in part explain this association between inflammation and lung cancer in humans. This is consistent with repression of pro-inflammatory cytokines, IL6, IL1β, and TNFα observed following LPS-induced pulmonary inflammation in response to the PPARβ/δ ligand GW0742 [41]. Similarly, carrageenan-induced pleurisy is also significantly inhibited following ligand activation of PPARβ/δ with GW0742 as shown by reduced infiltration of polymorphonuclear neutrophils, reduced lipid peroxidation, and reduced expression of TNFα and IL1β in mice [35]. Since pulmonary inflammation is known to contribute to the mechanisms causing lung cancer [138], these observations support the hypothesis that the anti-inflammatory activities of PPARβ/δ agonists could be of use for the prevention of lung cancer. Combined, the available *in vivo* data shows that PPARβ/δ may inhibit lung tumorigenesis in mouse and human models, but provides no evidence that activating PPARβ/δ promotes lung tumorigenesis.

Similar to many of the studies reported describing the effect of PPARβ/δ in breast cancer models, there are many limitations to studies describing the effect of PPARβ/δ in lung cancer models. It is not possible to reconcile why some studies report increases in cell proliferation following treatment with PPARβ/δ agonists in cultured human lung cancer cell lines [121, 125, 126], while others show inhibition [123] or no change in cell proliferation [104, 129]. However, it should be noted again that the studies demonstrating an increase in cell proliferation in human lung cancer cell lines typically rely on enzyme-based assays that may be unsuitable because PPARβ/δ ligands can increase expression of mitochondrial dehydrogenases [20] required for cleavage of tetrazolium salts used to estimate cell number leading to spurious results. The rather unique mechanism suggesting that the p65 subunit of NF-κB mediates the enhanced cell proliferation observed in human lung cancer cell lines [126] is at odds with numerous reports showing that PPARβ/δ inhibits inflammatory signaling by interfering with p65 [10–14] but not activating p65. The data indicating that the PPARβ/δ ligand inhibits AMPK activity in a human lung cancer cell line [127] are also inconsistent with a number of reports showing that PPARβ/δ activates AMPK [11, 28, 110, 139, 140]. This is important to note because activated AMPK has been shown to increase apoptosis and/or inhibit proliferation in human cancer cell lines [141–144], including lung cancer cell lines [145]. Lastly, there is a lack of dose-dependent analysis for the increase in PDPK1 expression, decrease in PTEN expression, and/or increase in phosphorylation of AKT caused by GW501516 in human lung cancer cell lines reported by some studies [121, 125, 126], whereas dose-dependent analysis using two different PPARβ/δ ligands, including GW501516, in similar human lung cancer cell lines demonstrates no change in expression of PDPK1, PTEN, or phosphorylation of AKT [129]. Additionally, published microarray datasets from other cells shows clear increases in expression of PPARβ/δ target genes but not in expression of putative targets such as PDPK1 or PTEN [108, 110–112] (Table 3). This re-emphasizes why the putative mechanistic link between PPARβ/δ, PDPK1, PTEN, and AKT requires considerable research before it can be accepted or refuted as being functional in lung cancer.

4.3 Summary

Review of the literature describing effects of PPAR β/δ and its ligands in lung cancer models are inconclusive in establishing whether this receptor influences this disease. Western blot analysis indicates no difference in expression between lung tumors and normal lung tissue, while immunohistochemical analysis suggests higher expression of PPAR β/δ in lung tumors as compared with control tissue. The latter interpretation must be viewed cautiously because of the known problems with using immunohistochemistry to quantify PPAR β/δ expression due to non-specific immunoreactivity associated with anti-PPAR β/δ antibodies proven to result in misleading interpretations [98, 122] and the fact that Western blot analysis does not support the notion that expression of PPAR β/δ is increased. Tissue microarray analysis indicates that expression of PPAR β/δ is moderate in respiratory epithelial cells of the bronchus and lacking or weak in human lung adenocarcinomas and squamous cell carcinomas. Because of these differences in the reported studies, there remains a need to critically examine the expression of PPAR β/δ in lung cancer, as the available literature does not allow for any definitive conclusion. The data obtained from *in vitro* models using human lung cancer cell lines is also very confusing and inconclusive. The reason why some studies suggest that activating PPAR β/δ promotes proliferation of human lung cancer cell lines, while other studies indicate that PPAR β/δ agonists or antagonists either inhibit proliferation or have no effect on proliferation of human lung cancer cell lines remains unclear. Some of the differences could be due to the use of enzyme-based assays to assess cell proliferation. More quantitative, dose-dependent analysis of cell proliferation and cell cycle progression, such as that used to show no change in cell cycle progression of H1838 or A549 cells following treatment with either GW501516 or GW0742 [129] would improve the available database. The lack of concordance in putative mechanistic changes in signaling pathways observed following activation of PPAR β/δ in human lung cancer cell lines should also be examined with more quantitative, dose-dependent approaches. For example, the reasons why some report that activating PPAR β/δ increases expression of PDPK1 and decreases expression of PTEN, while others show that these changes do not occur, cannot be explained at this point but illustrates another level of uncertainty regarding the effect of PPAR β/δ in lung cancer. In contrast to the confusing literature reported using human lung cancer cell lines, the evidence to date from *in vivo* models of lung cancer points to a preventive role for PPAR β/δ . Results from mouse models to date indicate that PPAR β/δ protects against lung tumorigenesis, but whether PPAR β/δ mediates prostacyclin-dependent chemoprevention found in chemically induced lung cancer models in mice has not been critically examined to date. It is also not clear whether the protective effect of PPAR β/δ is due to its expression in lung epithelia or expression in immune cells such as macrophages. PPAR β/δ has anti-inflammatory effects, and inflammation has a major role in carcinogenesis. The finding that a PPAR β/δ ligand can prevent pulmonary inflammation, which can promote lung cancer, is consistent with a weak link between a polymorphism in PPAR β/δ and enhanced inflammation in human lung cancer patients.

5 Conclusions

Review of the literature indicates that there is no consensus regarding the role of PPAR β/δ in colon, breast, or lung cancers. There are four central issues surrounding the controversial nature of PPAR β/δ in cancer: (1) whether expression of the receptor is altered during tumorigenesis, (2) the nature of any endogenous ligand(s) that activate the receptor during carcinogenesis, (3) what the outcome of activating PPAR β/δ in these cancer models is, and (4) the molecular targets of PPAR β/δ .

It is difficult to reconcile why expression of PPAR β/δ , which is already expressed at relatively high levels in human and mouse colon [8, 81, 82], would be increased during colon tumorigenesis and whether this increase could influence gene expression and

tumorigenesis. The relatively high expression of PPAR β/δ found in normal colon and the recent demonstration that expression of PPAR β/δ is markedly lower in human and mouse colon tumors [81–84] are more consistent with findings suggesting that PPAR β/δ protects against colon tumorigenesis, possibly due in part to PPAR β/δ -dependent regulation of terminal differentiation (Fig. 2). Comparable evidence also suggests that PPAR β/δ is moderate to strong and localized in the nucleus of respiratory epithelial cells of the bronchus nucleus and breast glandular cells (Table 4), consistent with the hypothesis that constitutive expression of PPAR β/δ serves an important function in these cells. In contrast, expression of PPAR β/δ is lacking or weak in ~90% of breast and lung tumors (Table 4). This supports the hypothesis that PPAR β/δ expression may protect against breast and lung cancers. It is also worth noting that the relatively negligible expression pattern of PPAR β/δ is found in many human tumors types, strong expression of PPAR β/δ is only rarely found in human tumors, and malignant cells in general were negative for PPAR β/δ expression (Table 4). To yield convincing evidence and to move the field forward, the use of indirect methods such as immunohistochemistry must be coupled with quantitative Western blots for more precise assessment of PPAR β/δ expression levels because of the non-specific nature of available PPAR β/δ antibodies, especially for immunohistochemistry [8, 122]. Indeed, the use of immunohistochemical analysis has led to misinterpretation of PPAR β/δ expression in colon [122]. Additionally, examination of PPAR β/δ expression in clinical samples from lung and breast cancer patients for correlation with overall survival would also be highly useful, similar to the recent study published for PPAR β/δ in colorectal cancer patients [99]. Based on the recent study by Yang and colleagues, which has considerably greater strength due to the number of colorectal cancer patients examined and the length of the follow-up, it may be of interest to determine whether there are suitable approaches to increase expression of PPAR β/δ for the prevention of colorectal cancer. Since targeting a single molecular target to prevent or treat cancer is likely unsuitable [120], whether it is feasible to combine increased expression, and/or activation of PPAR β/δ , with other molecular targets to improve the efficacy of chemoprevention or chemotherapy for colorectal cancer as compared with existing approaches might also be considered. In the absence of more compelling evidence from clinical studies in humans, this approach is not feasible for consideration for the prevention or treatment of breast or lung cancer due to the uncertainty of the role of PPAR β/δ in these diseases.

No consensus has been established to conclusively demonstrate the effect of activating PPAR β/δ in colon, breast, or lung cancer models. This is due in large part to conflicting evidence describing various mechanisms of action. All of the molecular mechanisms described that putatively underlie the tumor promoting effects of PPAR β/δ and its ligands remain unconfirmed due in large part to published studies that refute the reported results (Table 3). Interestingly, most of the mechanisms describing tumor-promoting effects of PPAR β/δ and its ligands are based on molecular targets originally described to be modulated in cells resembling keratinocytes [77] (Table 2). However, the concordance of data supporting the notion that proteins such as PDPK1 or ILK are bona fide molecular targets of PPAR β/δ in somatic cells or cancer cells is not strong (Tables 2 and 3). The majority of studies showing tumor-promoting effects of PPAR β/δ ligands typically lack large sample sizes and rarely address the inconsistencies in their findings with reports not supporting these studies (reviewed in [75, 76]). In contrast to the putative mechanisms postulated to explain the tumor promoting effects of PPAR β/δ and its ligands in colon cancer, the putative mechanisms underlying the preventive effects of ligand activation of PPAR β/δ in colon cancer center on the ability to induce terminal differentiation and/or inhibit pro-inflammatory signaling. The ability of ligand activation of PPAR β/δ to induce terminal differentiation and/or inhibit pro-inflammatory signaling are two effects that are not disputed (Table 1), however, the identity of the direct target genes mediating these effects remains unknown. Interestingly, it is also unquestionable that ligand activation of PPAR β/δ inhibits

chemically induced skin carcinogenesis through mechanisms that include induction of terminal differentiation and inhibition of pro-inflammatory signaling through receptor-dependent and receptor-independent mechanisms [62, 146–152]. Some of the uncertainty regarding the effect of ligand activation of PPAR β/δ on colon, breast, or lung carcinogenesis could also be influenced by interactions with other receptors that are known to modulate these diseases (e.g., ER, AR). The effect of ligand activation of PPAR β/δ on terminal differentiation and pro-inflammatory signaling should also be examined in all of these models given the strong functional relationship between differentiation and inflammation with PPAR β/δ (Table 1). This possibility is also supported based on the observations that ligand activation of PPAR β/δ can inhibit or prevent metabolic syndrome, obesity, dyslipidemias, glucose intolerance, and chronic inflammation based on preclinical and clinical studies since these diseases are negatively associated with cancer [86, 153–156].

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Fig. 1.

Mechanisms of PPARβ/δ transcriptional regulation. *Top panel*, upregulation of gene expression mediated by ligand activation of the receptor causing recruitment of transcriptional machinery including RNA polymerase and co-activators with HAT activity that remodel chromatin allowing for expression of mRNAs. *Middle panel*, PPARβ/δ can interact with other transcription factors such as the p65 subunit of NF-κB, preventing NF-κB-dependent transcription including pro-inflammatory signaling molecules. *Bottom panel*, repression of gene expression by PPARβ/δ

Fig. 2.

Expression of PPARβ/δ in colon and colon tumorigenesis. Expression of PPARβ/δ is high in human and mouse colon, and found primarily in the nucleus. Nuclear PPARβ/δ can be co-immunoprecipitated with its heterodimerization partner, RXR. These observations suggest that PPARβ/δ may have an important constitutive function in the colon, likely mediated by the presence of an endogenous ligand. Based on multiple studies, it is likely that PPARβ/δ promotes terminal differentiation of colonocytes as they cycle from the base of the crypt towards the lumen. Following either genetically induced or chemical exposure and mutation(s), expression of PPARβ/δ is reduced in transformed colon tumors. Reduced expression of PPARβ/δ could prevent cell cycle withdrawal associated with the induction of terminal differentiation leading to dysregulation of cell proliferation. Recent evidence showing that colorectal cancer patients with relatively low levels of PPARβ/δ are ~4× as likely to die from the disease as compared with patients expressing relatively higher levels of PPARβ/δ in primary tumors strongly support this proposed mode of action

Table 1Confirmed functional roles of PPAR β/δ

Function	Reference(s)
Increases serum HDLc	[17, 18]
Increases expression of proteins that catabolize fatty acids in skeletal muscle	[20, 21]
Improves genetic and high fat diet-induced metabolic syndrome	[18, 20–22]
Improves insulin sensitivity	[23]
Inhibits hepatic inflammation	[26–32]
Inhibits pro-inflammatory signaling	[10, 11, 14, 33–54]
Promotes terminal differentiation	[46, 55–65, 67–73, 157, 158]

Table 2

Four putative molecular mechanisms of PPAR β/δ action to promote cell survival

Target(s)	Basis	Limitations and inconsistencies
PDPK1, ILK, PTEN, AKT	Based on analysis in mouse primary keratinocytes and human HaCaT keratinocytes, including promoter analysis of PDPK1 and ILK suggesting that PDPK1 and ILK are direct PPAR β/δ target genes. Ligand activation of PPAR β/δ by L165041 increases expression of PDPK1 and ILK and decreases expression of PTEN. These changes collectively cause increased phosphorylation of AKT, which promotes cell survival due to anti-apoptotic activities [77].	The cells used to suggest that the PPAR β/δ ligand L165041 increases PDPK1 and ILK expression do not exhibit a phenotype consistent with a primary keratinocyte based on morphology, lack of keratin 6 (K6) expression and differences in expression of other differentiation markers [70, 77]. Expression of PTEN is not decreased, expression of PDPK1 and ILK is not increased, and phosphorylation of AKT is not increased by ligand activation of PPAR β/δ in mouse primary keratinocytes that express K6 and normal patterns of differentiation markers, or in N/TERT-1 human keratinocytes, despite clear upregulation of the known PPAR β/δ target gene <i>ADRP</i> [59]. Ligand activation of PPAR β/δ increases expression of known PPAR β/δ target gene <i>ANGPTL4</i> but does not alter expression of PDPK1 in human HaCaT keratinocytes [58]. Ligand activation of PPAR β/δ inhibits proliferation of mouse keratinocytes and N/TERT-1 human keratinocytes [59]. Expression of <i>PDPK1</i> , <i>ILK</i> , and <i>PTEN</i> mRNA is unaffected by ligand activation of PPAR β/δ in mouse primary keratinocytes, mouse skeletal muscle, mouse mammary tissue, or human dendritic cells (Table 3). Ligand activation of PPAR β/δ has no effect on expression of PDPK1, ILK, PTEN, or phosphorylation of AKT in mouse colon from a colon cancer bioassay [63]. Analysis of this pathway in cancer models is typically limited to a single protein and increased promoter occupancy of PPAR β/δ on <i>PDPK1</i> , <i>ILK</i> , or <i>PTEN</i> genes is never confirmed.
VEGF, AKT	Based on analysis of human colon cancer cell lines and <i>Apc^{min/+}</i> mice. Ligand activation of PPAR β/δ increases expression of VEGF through a PPAR β/δ -dependent mechanism causing increased phosphorylation of AKT, which promotes cell survival in colon tumor cells due to anti-apoptotic activities [88].	Ligand activation of PPAR β/δ in human colon cancer cell lines, <i>Apc^{min/+}</i> mouse colon, or wild-type mouse colon does not increase expression of VEGF or increase phosphorylation of AKT [93]. Ligand activation of PPAR β/δ inhibits proliferation of human colon cancer cell lines [93]. Expression of <i>VEGF</i> mRNA is unaffected by ligand activation of PPAR β/δ in mouse primary keratinocytes, mouse skeletal muscle, mouse mammary tissue, or human dendritic cells (Table 3).
PDPK1	Based on original analysis in human HaCaT keratinocytes, high ratio of intracellular FAB5 to CRABP-II diverts atRA or PPAR β/δ ligands to PPAR β/δ rather than RAR causing increased expression of PDPK1 leading to anti-apoptotic activities and increased cell survival [113]. Contingent on the hypothesis that atRA can activate PPAR β/δ after delivery to receptor by FABP5 due to high ratio of FABP5 to CRABP-II found in human HaCaT keratinocytes.	Ancillary to putative pathways described above for PDPK1/ILK/PTEN/AKT signaling in mouse primary keratinocytes and human HaCaT keratinocytes; inherent limitations noted above exist for this postulated mechanism as well. Despite clear upregulation of RAR target gene <i>CYP26</i> by atRA, atRA does not increase expression of the PPAR β/δ target genes <i>ADRP</i> or <i>ANGPTL4</i> in human HaCaT keratinocytes [58]. atRA does not compete with PPAR β/δ binding in a ligand-binding assay [119]. atRA does not cause interaction of PPAR β/δ with co-activator peptides in a time-resolved fluorescence resonance energy transfer assay [119]. atRA inhibits cell proliferation, but does not cause increased cell survival, in human HaCaT keratinocytes and in wild-type and <i>Pparβ/δ</i> -null mouse keratinocytes [58]. atRA does not increase phosphorylation of AKT in human HaCaT keratinocytes [58]. Human HaCaT keratinocytes exhibit enhanced apoptosis, but no inhibition of apoptosis, in response to ligand activation of PPAR β/δ or treatment with atRA is found [58]. Analysis to date suggesting that activating PPAR β/δ in cancer models via delivery of ligands by FABP5 will increase cell survival is limited to verification of modest changes in PDPK1 expression but does not account for observations that PDPK1 is not a PPAR β/δ target gene or the fact that expression of a single protein is unlikely to underlie pro-survival signaling [114, 115]. No evidence to date demonstrating that CRABP-II expression can functionally outcompete FABP5 for PPAR β/δ ligands for delivery to RAR, since it is expressed at very low levels and markedly lower as compared with FABP5 [114] or that the binding affinity of PPAR β/δ ligands for CRABP-II is substantially lower as compared to the binding affinity of PPAR β/δ ligands for FABP5.
14-3-3 ϵ , BAD	Based on analysis of human colon cancer cell lines. PPAR β/δ prevents apoptosis by increasing expression of 14-3-3 ϵ whose elevated levels enhance sequestration of BAD, a pro-apoptotic member of the B-cell CLL/lymphoma 2 (BCL-2) family [159, 160]. NSAIDs increase apoptosis by decreasing	Expression of PPAR β/δ is unchanged or increased by NSAIDs in human colon cancer cell lines [84, 122]. Expression of PPAR β/δ is lower in human and mouse colon tumors as compared with non-transformed tissue [82, 84]. Ligand activation of PPAR β/δ in some cases enhances, but does not inhibit, NSAID-induced apoptosis [84]. Expression of 14-3-3 ϵ is unaffected by NSAIDs or ligand activation of PPAR β/δ in human colon cancer cell lines [84].

Target(s)	Basis	Limitations and inconsistencies
	expression of PPAR β/δ , which allows BAD to function as a pro-apoptotic protein. Contingent on hypothesis that NSAIDs inhibit expression of PPAR β/δ .	

Table 3
Comparison of four published and one unpublished microarray analysis of select PPAR β/δ target genes and putative target genes

Reference	Gene	Tachibana ^a		Szeles ^b	Narkar ^c	Pollock ^d	Unpublished ^e	
		GW/DMSO Fold Δ	GW/DMSO Fold Δ				(+/+) GW/DMSO Fold Δ	(-/-) GW/DMSO Fold Δ
	<i>ANGPTL4</i>	8.56	1.28	6.30	1.63	1.51	13.45	1.02
	<i>ADRP</i>	13.02	4.54	1.58	1.43	1.21	2.79	1.09
	<i>CPT2</i>	2.36	1.01	1.04	1.42	1.31	2.53	0.66
	<i>PDK4</i>	0.88	34.50	2.87	2.60	1.19	1.19	0.87
	<i>PDPK1</i>	0.89	0.99	0.99	1.03	1.09	1.15	1.06
	<i>ILK</i>	0.85	0.97	1.01	0.91	1.08	1.13	1.19
	<i>PTEN</i>	1.25	0.99	1.01	1.21	1.00	1.19	1.32
	<i>VEGF</i>	1.15	1.00	0.85	1.07	0.99	1.03	1.03

Datasets obtained from the Gene Expression Omnibus (GEO) for the below referenced manuscripts were queried for the genes of interest. The native normalization for the microarrays was maintained for all datasets except that from the Szeles reference where the log₂ transformation was reversed to more closely resemble the other datasets. Values are presented as the ratio of relative expression in GW501516 (or GW0742)-treated sample(s) compared with relative expression in the control sample(s). If more than one probe set was present for each gene and/or more than one replicate per treatment, the mean of the mean values are shown.

^a RNA isolated from HepG2 cells over-expressing PPAR β/δ were cultured with 0.1 μ M GW501516 for 24 h and compared with RNA isolated from control cells treated with DMSO [111].

^b RNA isolated from human monocyte-derived dendritic cells cultured with 1.0 μ M GW501516 for 12 h and compared with RNA isolated from control cells treated with DMSO [112].

^c RNA isolated from skeletal muscle of C57BL/6 J mice treated with GW501516 (oral gavage (5 mg/kg) for 6 days) and compared with RNA isolated from skeletal muscle of control C57BL/6 J mice [110].

^d RNA isolated from mammary gland of wild-type mice treated with GW501516 (fed diet containing 0.005% GW501516 for 1 week) and compared with RNA-isolated wild-type mice fed a control diet; or RNA isolated from mammary gland of PDPK1 transgenic mice treated with GW501516 (fed diet containing 0.005% GW501516 for 1 week) and compared with RNA isolated PDPK1 transgenic mice fed a control diet [108].

^e Mouse primary keratinocytes from wild-type (+/+) and Ppar β/δ -null mice (-/-) were treated with 0.2 μ M GW0742 for 8 h; RNA was isolated and used for microarray analysis using the Agilent platform. RNA from GW0742-treated cells was compared with RNA from DMSO-control cells. Note lack of change of induced genes in the (-/-) samples demonstrating requirement of the PPAR β/δ for this change [unpublished data]

Table 4

Relative expression of PPARβ/δ in normal and cancerous tissue in humans

Tissue ^d	Cell type	Relative expression	Distribution
Colon	Glandular cells	Strong	Nuclear
Rectum	Glandular cells	Strong	Nuclear
Breast	Glandular cells	Strong	Nuclear
Bronchus	Respiratory epithelial cells	Moderate	Nuclear
Tumor type ^b	Negative ^c	Weak	Distribution
Colorectal	37%	45%	0%
Breast	67%	25%	0%
Lung	82%	9%	0%
All ^d	63%	23%	2%

^aFrom the Protein Atlas version 8.0–2011.05.16 [82, 83] (see <http://www.proteinatlas.org>)

^bFor colorectal tumors, 11 adenocarcinomas were examined; for breast tumors, four lobular carcinomas and eight ductal carcinomas were examined; for lung tumors, six adenocarcinomas and five squamous cell carcinomas were examined

^cThe percentage of tumors samples exhibiting negative, weak, moderate, or strong expression of PPARβ/δ are shown

^dIncludes analysis of the above-mentioned colorectal tumors, breast tumors, lung tumors, and cervical tumors (*n*=11), endometrial tumors (*n*=12), head and neck tumors (*n*=4), liver tumors (*n*=10), malignant carcinoid (*n*=4), malignant glioma (*n*=11), malignant lymphoma (*n*=12), malignant melanoma (*n*=10), ovarian tumors (*n*=12), pancreatic tumors (*n*=10), prostate tumors (*n*=12), renal tumors (*n*=10), skin tumors (*n*=11), stomach tumors (*n*=9), testis tumors (*n*=4), thyroid tumors (*n*=10), and urothelial tumors (*n*=10). The majority of malignancies showed positivity in fractions of cells, but malignant cells in general were negative