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ELUCIDATING THE FUNCTIONAL ROLES OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR β/δ IN HUMAN COLON CANCER CELLS

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by

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Abstract

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) is an important regulator in various physiological processes, including lipid metabolism and glucose homeostasis. However, its role in cancer remains controversial. Although PPARβ/δ is highly expressed in the intestines of normal adults, it has been reported to be up- or down-regulated during colon tumorigenesis. Researchers have not reached a consensus for whether PPARβ/δ is beneficial, detrimental, or unrelated to colon cancer initiation, survival, growth, and metastasis, in mouse or and human cancer models.

One of the first mechanisms described that PPARβ/δ promotes carcinogenesis was the hypothesis that PPARβ/δ is a target gene of the oncogenic APC/β-CATENIN pathway, a major pathway that is activated by mutations in colon cancer. However, subsequent studies did not observe a correlation between PPARβ/δ expression and β-CATENIN activation, and questioned whether *PPARD* (gene coding for PPARβ/δ) is a bona fide APC/β-CATENIN target protein. Moreover, the functionality of PPARβ/δ as influenced by the APC/β-CATENIN pathway, has not been critically examined to date. Therefore, in the first part of this thesis, the hypothesis that PPARβ/δ is functionally regulated by the APC/β-CATENIN pathway as a tumor-promoting protein was tested. We first investigated whether mutations of the *APC*/*CTNNB1* (β-CATENIN) genes or overexpression of functional β-CATENIN modulate PPARβ/δ cellular retention and its response to ligand activation in human colon cancer cell lines. We further examined the effect of ligand activation of PPARβ/δ using a classic agonist, as well as selective repression of PPARβ/δ using ligands that stimulate its transcriptional repression activity, on the growth of colon cancer cells with wild-type or mutant *APC*/*CTNNB1*. We observed that cytosol and nuclear retention of

PPARβ/δ, with or without ligand activation, were not different between cell lines with wild-type or mutant *APC*/*CTNNB1* (gene coding for β-CATENIN). Second, target gene activation of PPARβ/δ following ligand activation occurred faster in cell lines with mutant *APC*/*CTNNB1* compared to a non-mutant cell line, although this difference was not observed with transient overexpression of β-CATENIN. Third, ligand activation and selective repression of PPARβ/δ inhibited growth in several *APC/CTNNB1* mutant cell lines but had no effect on the non-mutant cell line. These results suggest that cellular retention and transcriptional activity of PPARβ/δ are not directly regulated by the APC/β-CATENIN pathway. However, the results also suggest that PPARβ/δ may be enhanced by the presence of *APC/CTNNB1* mutations in human colon cancer cell lines.

The role of PPARβ/δ in colon cancer invasion and metastasis also remains elusive. In the second part of this thesis, the influence of PPARβ/δ activation on malignancy-related features of colon cancer was examined. We hypothesized that ligand activation or selective repression of PPARβ/δ would inhibit anchorage-independent growth, migration, invasion, epithelial to mesenchymal transition (EMT), and metalloprotease (MMP) activity. Results, some preliminary in nature, showed that selective repression of PPARβ/δ reduced anchorage-independent growth by inducing apoptosis, inhibited migration, and reduced EMT marker expression, but did not change TNFα/TGFβ-induced MMP activity. By contrast, ligand activation of PPARβ/δ reduced migration and TNFα/TGFβ-induced MMP activity, but did not affect anchorage-independent growth and EMT marker expression. These results suggest that both ligand activation and selective repression of PPARβ/δ reduce the malignant potential of colon cancer, although the underlying mechanisms could be different. Combined, results from this study indicate that PPARβ/δ is not functionally regulated by the APC/β-CATENIN pathway. Further, ligand

activation or selective repression of PPARβ/δ using synthetic ligands may modulate colon cancer growth and malignancy-related features, in particular in cells with *APC*/*CTNNB1* mutations.

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Abbreviations

AF-1 (or 2)	Active function 1 (or 2)
AKT	Protein kinase B
AOM	Azoxymethane
APC	Adenomatous polyposis coli
AXIN 2	Axin-like protein 2
COX2	Cyclooxygenase 2
CPB	CREB-binding protein
CTNNB1	β-CATENIN; CATENIN, beta 1
DBD	DNA binding domain
DR-1	Direct repeat -1
DSS	Dextran sulfate sodium
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-Mesenchymal Transition
FAP	Familial adenomatous polyposis
GSK3β	Glycogen synthase kinase 3 beta
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDL	High-density lipoprotein
IHC	Immunohistochemistry
ILK	Integrin-linked kinase
LBD	Ligand binding domain
LDL	Low-density lipoprotein

LGR5	Leucine- rich repeat- containing G- protein-coupled receptor 5
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinases
NANOG	Homeobox protein NANOG
NCOR	Nuclear receptor co-repressor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NR	Nuclear receptor
OCT4	Octamer-binding transcription factor 4
PARP	Poly (ADP-ribose) polymerase
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PI3K	Phosphoinositide 3-kinase
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SMRT	Silencing mediator for retinoid and thyroid receptor
SOX2	SRY-box 2
TCF4	T cell factor 4
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
ZEB1/2	Zinc finger E-box binding homeobox 1/2

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Chapter 1. Literature Review

1.1. Nuclear receptors

1.1.1. History and classification

Nuclear receptors are a superfamily of intracellular proteins that are bound and activated by small biological molecules, such as hormones, fatty acids, and xenobiotics, and subsequently regulate gene expression as transcription factors. In the 1960s and 1970s, researchers identified various steroid hormone receptors and discovered their capabilities for nuclear translocation, DNA binding, and transcriptional regulation following ligand binding (Baxter et al., 1972; Jensen et al., 1968; Wira and Munck, 1970; Yamamoto and Alberts, 1972). It was revealed earlier that the glucocorticoid receptor, one of the first discovered nuclear receptors, contains DNA and ligand binding domains (Wrange and Gustafsson, 1978), However, it wasn't until the 1980s, when cDNA sequences of nuclear receptors became available, that scientists realized these domains are commonly shared among all nuclear receptors (Hollenberg et al., 1985; Walter et al., 1985; Giguère et al., 1986; Weinberger et al., 1985). Knowledge of nuclear receptors, their endogenous ligands, and target genes soon expanded. This gave rise to a clearer picture of the dynamic regulation between nuclear receptors and the metabolism pathways their ligands are involved in, as well as the considerable pharmaceutical potential of developing synthetic ligands for these receptors in order to treat diseases. To date, 48 nuclear receptors have been identified in humans; they participate in a wide range of physiological processes including development, endocrine regulation, and metabolism. They are organized into seven classes based on their

functions and ligands (Alexander et al., 2015). The classification of nuclear receptors is summarized in Table 1.

Class	Name (number of subtypes)
NR1	Thyroid Hormone Receptors (2), Retinoic Acid Receptors (RAR) (3), Peroxisome Proliferator-Activated Receptors (3), Reverse Erb Receptors (2), RAR-Related Orphan Receptors (3), Liver X Receptor-Like Receptors (3), Vitamin D Receptor-Like Receptors (3)
NR2	Hepatocyte Nuclear Factor 4 Receptors (2), Retinoid X Receptors (RXR) (3), Testicular Receptors (2), Tailless-Like Receptors (2), COUP-TF-Like Receptors (3)
NR3	Estrogen Receptors (ER) (2), Estrogen-Related Receptors (3). 3-Ketosteroid Receptors (4)
NR4	Nerve Growth Factor IB-Like Receptors (3)
NR5	Fushi Tarazu F1-Like Receptors (2)
NR6	Germ Cell Nuclear Factor Receptors (1)
NR0	DAX-Like Receptors (2)

Table 1. Nuclear receptor classification.

1.1.2. Structure and mechanisms of action

In order to regulate gene expression, a nuclear receptor must be in dimerized form, bind to a specific sequence named "response element" in the regulatory region of its target genes, recruit other transcriptional co-activators or co-repressors, and further regulate the expression of its target genes (Germain and Bourguet, 2013). Binding to a ligand can activate the function of a nuclear receptor in a number of ways, such as increasing nuclear localization (Htun et al., 1996), stabilizing active conformation (Bernardes et al., 2013; Johnson et al., 2000; Keidel et al., 1994);

facilitating receptor dimerization (Cheskis and Freedman, 1994; Depoix et al., 2001; Quack and Carlberg, 2000); inducing coregulator association (Jeyakumar et al., 1997; Oñate et al., 1995), and promoting DNA binding (Becker et al., 1986; Denis et al., 1988).

Differences exist among the mechanisms of different receptors. Forms of dimerization and structures of response elements are two important ones. Steroid hormone receptors, such as glucocorticoid receptor and estrogen receptor, act as homodimers, and bind to response elements composed of two palindromic-ordered hexanucleotides separated by three random nucleotides (Beato, 1991). On the other hand, non-steroid receptors—such as peroxisome proliferator-activated receptor (PPAR) and retinoic acid receptors (RAR)—heterodimerize with retinoid X receptor (RXR) to be transcriptionally functional, and the same rule applies to RXR itself (Bugge et al., 1992; Kliewer et al., 1992; Leid et al., 1992; Marks et al., 1992; Yu et al., 1991; Zhang et al., 1992). The response elements for non-steroid receptor-RXR dimers are often tandem repeats of two hexanucleotides separated by one to five random base pairs, and the length of this random region is specific for each type of receptor (Koenig et al., 1987; Näär et al., 1991; Umesono et al., 1991). Each receptor in the dimer binds to one of the hexanucleotides in the response element (Chandra et al., 2008; Lou et al., 2014; Rastinejad et al., 1995).

1.2. Peroxisome proliferator-activated receptors (PPARs)

1.2.1. Overview of PPARs

The discovery of peroxisome proliferator-activated receptors, or PPARs, emerged from the study of peroxisome proliferators. Peroxisome proliferators are compounds, including several fibrates, that have been found to stimulate the proliferation of peroxisomes, the membrane-enclosed microbodies in which bioreactions such as fatty acid biosynthesis and antioxidant

metabolism occur (Reddy et al., 1980; Youssef and Badr, 2013). In searching for a mechanism of their physiological responses, researchers looked for a receptor protein that acts as an intermediate factor between the peroxisome proliferators and their physiological effects.

In 1990, the first PPAR cDNA was screened from a mouse liver cDNA library using oligonucleotide probes generated from the consensus region within the DNA binding domain of human glucocorticoid receptor, estrogen receptor, vitamin D receptor, thyroid hormone receptor, and retinoic acid receptor (Issemann and Green, 1990). Similar domains and sequences within the DNA- and ligand-binding domains were then found between this new protein and the other nuclear receptors, confirming it as a new form of nuclear receptor. Binding of this receptor to a number of peroxisome proliferators was also confirmed, using chimeric proteins containing a ligand-binding domain from this new receptor, and a DNA-binding domain from a better-studied receptor (Issemann and Green, 1990). With this finding, the name PPAR was given to this receptor, which later became PPARα. Soon after the discovery of PPARα, a total of three PPAR subtypes were found in *Xenopus*, and were named α, β, and γ (Dreyer et al., 1992). Similar isoforms were also identified in rat, human, and mouse (Göttlicher et al., 1992; Schmidt et al., 1992; Greene et al., 1995; Kliewer et al., 1994). The PPARδ (also referred to as NUC1 or FAAR) identified in human and mouse was thought at first to be a new PPAR isoform; however, researchers later determined that PPARδ is an ortholog of the *Xenopus* PPARβ. Therefore, today it is referred to as PPARβ/δ. Although nuclear receptors can be found throughout metazoan evolution, PPARs have been found only in vertebrates (Markov and Laudet, 2011; Owen and Zelent, 2000).

As introduced earlier, PPARs share a similar structure with other nuclear receptors. Each receptor is composed of four functional domains: from N- to C-terminal, the active function 1 (AF-1) ligand-independent activation domain; the DNA-binding domain (DBD), which contains

two zinc fingers; a hinge region; and the ligand-binding AF-2 domain (LBD) (Youssef and Badr, 2013) (Fig. 1). Between subtypes and species, the DBD and LBD of PPARs are most evolutionally conserved (>80% and >60%, respectively), whereas the AF-1 and hinge domains are less conserved (about 20% and 50%, respectively). This suggests that the DBD and LBD have functional importance, and that different PPAR isotypes may be more selective towards ligand binding than DNA binding (Kliewer et al., 1994; Schmidt et al., 1992).

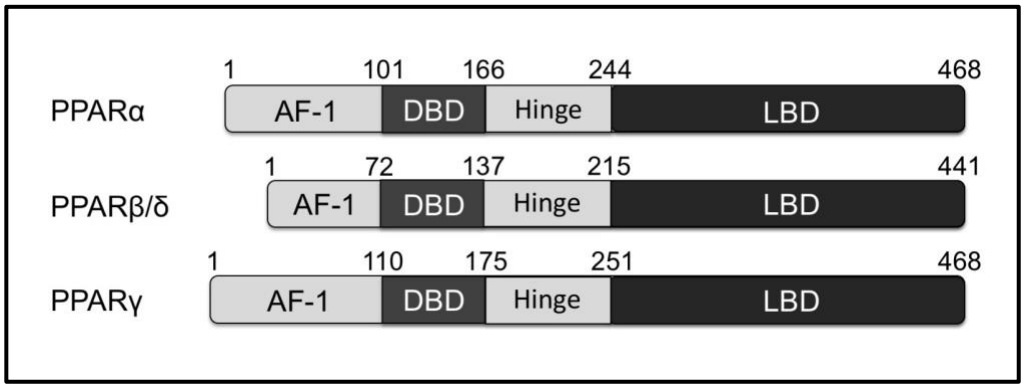


Figure 1. Sequence structure of PPARs.
The activation function 1 (AF1), DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD) for each PPAR subtype is shown. Numbers indicate amino acid positions from N- to C- terminal.

The three PPAR isoforms are expressed in all mammalian tissues, but at different levels. PPARα is most abundant in the liver, heart, kidney, and muscle; PPARγ is highly expressed in adipose tissue and the spleen; and PPARβ/δ has relatively strong expression in the small intestine, colon, liver, kidney, and skin (Issemann and Green, 1990; Schmidt et al., 1992; Kliewer et al., 1994; Braissant et al., 1996; Girroir et al., 2008; Uhlén et al., 2015; Escher et al., 2001).

PPARs are master regulators in fatty acid and glucose metabolism. They can be activated by a variety of fatty acids, and synthetic PPAR ligands have great pharmaceutical value in prevention and treatment of metabolic diseases. For example, synthetic PPARα ligands fibrates

help reduce triglyceride and cholesterol level in hyperglycemia (Reddy and Qureshi, 1979; Reddy et al., 1980; Marsman et al., 1988), while synthetic PPARγ ligands thiazolidinediones are used to treat type II diabetes by elevating insulin sensitivity (Ogihara et al., 1995; Taniguchi et al., 2001, 2001). PPARβ/δ ligands are in clinical trials, but studies have shown that activating this receptor may help to reduce obesity, lipid disorders, cardiovascular diseases, and inflammatory diseases (Bays et al., 2011; Jones et al., 2017; Risérus et al., 2008; Sprecher et al., 2006). Besides metabolism, PPARs participate in many other biological processes, including differentiation, inflammation, and carcinogenesis. The roles of PPARs in carcinogenesis, however, is controversial. While several reports of carcinogenic effects of PPAR ligands brought concerns for their clinical use, studies with opposite findings have given hope to the chemotherapeutic potentials of these compounds (reviewed in Peters et al., 2012, 2015a).

1.2.2. Introduction to PPARβ/δ

The human *PPARD* gene is located on chromosome 6p21.2 (Yoshikawa et al., 1996). This gene has nine exons, and the coding sequence is located from exon 4 to exon 9 (Skogsberg et al., 2000). PPARβ/δ protein is composed of 441 amino acids with a molecular weight of approximately 50 kDa (Schmidt et al., 1992).

PPARβ/δ is involved in many physiological processes. Earlier mouse studies found that ligand activation of PPARβ/δ increases high-density lipoprotein (HDL) cholesterol and regulates lipid metabolism (Leibowitz et al., 2000; Oliver et al., 2001). Studies with human subjects have shown that ligand activation of PPARβ/δ reduces low-density lipoprotein (LDL) cholesterol, reduces plasma triglyceride and free fatty acids, increases fatty acid oxidation, prevents obesity, promotes cardiovascular health, and alleviate liver inflammatory disease (Bays et al., 2011; Jones et al., 2017; Risérus et al., 2008; Sprecher et al., 2006). In contrast, the +294T/C

polymorphism, which affects the transcriptional activity of the *PPARD* gene, has been shown to associate with increased risk of cardiovascular diseases (Aberle et al., 2006; Skogsberg et al., 2003; Skogsberg Josefin et al., 2003). PPARβ/δ also regulates glucose metabolism and increases insulin sensitivity (Fan et al., 2017; Lee et al., 2006). PPARβ/δ promotes fatty acid oxidation in skeletal muscle and increases exercise endurance (Luquet et al., 2003; Narkar et al., 2008; Tanaka et al., 2003; Wang et al., 2003, 2004b). In additional to metabolic functions, strong evidence suggest the importance of PPARβ/δ for tissue/organ development, including brain, placenta, adipose tissue, and bone, and maintenance of terminal differentiation in many cell types, including epidermal cells and colonocytes (Barak et al., 2002; Peters et al., 2000; Nadra et al., 2006; Kim et al., 2005; Marin et al., 2006; Yang et al., 2010; Still et al., 2008; Varnat et al., 2006; Loreto et al., 2007; Westergaard et al., 2001; Schmuth et al., 2004). PPARβ/δ has been shown to reduce inflammation, partially through interfering with the NFκB pathway (reviewed in Section 1.2.3), in skin, liver, colon, lung, and many other models (Barish et al., 2008; Defaux et al., 2009; Di Paola et al., 2010; Ding et al., 2006; Hollingshead et al., 2007a; Kilgore and Billin, 2008; Lee et al., 2012; Man et al., 2008; Peters et al., 2000; Shan et al., 2008a). Despite some of these functions through which PPARβ/δ may inhibit cancer, its role in carcinogenesis has always been controversial (reviewed in Peters et al., 2008).

1.2.3. Mechanisms of gene regulation by PPARβ/δ

As with the other PPAR isoforms, the transcriptional activation of PPARβ/δ involves ligand binding, receptor dimerization, binding to the regulatory region of a target gene, recruiting co-regulators, and regulation of target gene expression (Figure 2 A and B). PPARβ/δ ligands are classified into several categories based on the different modes of PPARβ/δ activity that they modulate. Agonist defines molecules that bind to PPARβ/δ and further up- or down-regulate the

expression of target genes. This is also referred to as ligand activation of PPARβ/δ. Selective repressive ligands, also known as inverse agonists, only repress target genes of PPARβ/δ. Antagonists inhibit the activity of PPARβ/δ and able to neutralize the effects of other PPARβ/δ ligands (Figure 2). Examples of these ligands, including natural and synthetic ligands, are summarized in Table 2. Currently known natural ligands of PPARβ/δ include mono- and poly-unsaturated fatty acids as well as a number of prostaglandins (summarized in Grygiel-Górniak, 2014; Peters et al., 2008). Several synthetic agonists for PPARβ/δ have been developed and used intensively in research, including L-165041, GW0742, and GW501516. These compounds are subtype-selective for PPARβ/δ and can induce PPARβ/δ activity within nanomolar concentration, although at higher concentrations they can activate PPARα and PPARγ non-specifically as well (Berger et al., 1999; Sznaidman et al., 2003). Additionally, MBX-8025 is a recently developed PPARβ/δ agonist that is structurally similar to GW0742 and GW501516. MBX-8025 is currently being evaluated in clinical trials for lipid metabolic and hepatic inflammatory diseases (Bays et al., 2011; Jones et al., 2017).

Category	Name	Example structure

Natural ligand	• Unsaturated fatty acids
	  • Oleic acid
	  • **Linoleic acid**
	  • etc.
	• Prostacyclin
		

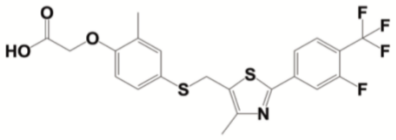
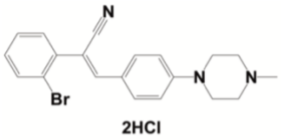
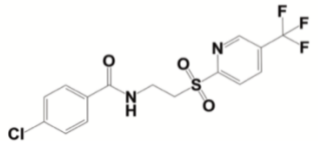
Synthetic agonist	• L-165041 • GW501516 • **GW0742** • MBX-8025	
Synthetic selective repressive ligand	• **DG172** • PTS264 • ST247	
Synthetic antagonist	• GSK0660 • **GSK3787**	

Table 2. Natural and synthetic ligands of PPARβ/δ.
Examples of natural and synthetic ligands of PPARβ/δ are shown. Structures correspond to the ligands in bold.

PPARβ/δ binds to DNA as a heterodimer complex with RXRα (Kliewer et al., 1992, 1994). Although there has been no evidence suggesting that ligand binding is essential for PPAR-RXRα dimerization, ligands of either PPARs or RXRα can stabilize the dimer conformation and promote their association with transcriptional coregulators (Feige et al., 2005; Tudor et al., 2007). The presence of a PPAR or RXRα ligand alone can activate the transcriptional activity of a PPAR-RXRα complex, however, synergistic effects can be achieved when ligands to both receptors are present (Kliewer et al., 1992).

The PPARβ/δ-RXRα complex binds to a sequence termed PPAR response element (PPRE), located in the promoter or enhancer region of a target gene (Fig. 2). A PPRE contains two identical or similar hexanucleotides separated by one nucleotide. A sequence found in the acyl-CoA oxidase (*ACO*) gene promoter (AGGACA-A-AGGTCA), as well as Direct Repeat-1

(DR-1) sequence (AGGTCA-N-AGGTCA), were two of the first discovered PPREs (Kliewer et al., 1992; Palmer et al., 1995). Structural analyses have revealed that PPAR binds to the upstream half-site of PPRE, while RXRα binds to the downstream half (Chandra et al., 2008). More recently, two studies discovered similar consensus PPRE sequences using ChIP-seq technique, and demonstrated a degree of flexibility of PPREs. The first consensus PPRE sequence is AGGGGA-A-AGGTCA, discovered in a human prostate cancer cell line (Adhikary et al., 2011), while the other one is (G/A)GGNCA-N-AGGTCA, discovered in primary mouse keratinocytes (Khozoie et al., 2012).

Heterodimerized PPARβ/δ-RXRα can either associate with co-repressors to suppress target gene expression, or associate with co-activators, to promote the access of the transcription complex to the promoter of a target gene and induce its expression (Fig 2. A). Common co-activators that associate with PPARβ/δ include CREB-binding protein (CBP) /p300 and histone acetyltransferases (HAT), and co-repressors of PPARβ/δ include nuclear receptor co-repressor 1/2 (NCOR/SMRT) and histone deacetylase (HDAC) (Krogsdam et al., 2002; Shi et al., 2002). It is traditionally believed that while PPARβ/δ-RXRα associates with co-repressors in the absence of ligand, ligand activation of PPARβ/δ switches it to a conformation that favors interaction with co-activators (Peters et al., 2008; Viswakarma et al., 2010). While this may remain true in some cases, results from recent studies have challenged this theory. One study identified 203 direct PPARβ/δ target genes using a combination of microarray and Chip-seq techniques, and summarized the mode of their regulation. In this study, PPARβ/δ-knockout (*Ppard*$^{-/-}$) mouse keratinocytes were compared against wild-type keratinocytes, with or without the presence of an exogenous agonist. Results from this study revealed that the majority of PPARβ/δ target genes do not respond to both knockout of endogenous PPARβ/δ and activation of PPARβ/δ with an exogenous ligand. furthermore, among the target genes that are regulated

both endogenously and by exogenous ligand activation, about 60% are being activated by PPARβ/δ, while 40% are being repressed (Khozoie et al., 2012). Similar patterns were demonstrated by another study in the WPMY-1 human prostate epithelial cell line (Adhikary et al., 2011). These findings suggest that activation of PPARβ/δ by an agonist can in fact promote both activation and repression of target genes (Figure 2 A). The dynamic regulation of PPARβ/δ is not completely understood. It is possible that endogenous PPARβ/δ ligands played a role in these observations. Similar dynamic gene regulation and interaction with co-regulators have been observed with other nuclear receptors, including estrogen receptor, glucocorticoid receptor, and thyroid hormone receptor (Carroll et al., 2006; Chi-Yi et al., 2010; Grøntved et al., 2015).

Different from synthetic agonists, another group of selective repressive ligands have been recently synthesized, that include DG172, ST247, and PTS264 (Table 2). Unlike PPARβ/δ agonists, these ligands only enhance association of PPARβ/δ with corepressor proteins, therefore promote only its repressive function (Lieber et al., 2015; Naruhn et al., 2011; Toth et al., 2016) (Figure 2 B). These selective repressive ligands are sometimes mistermed as antagonists. Theoretically, an antagonist, such as GSK3787 or GSK0660, competes with ligands including agonist and selective repressive ligands for the same binding site, and block further activity of the receptor (Figure 2 C). It is important to notice that ligand activation and selective repression of PPARβ/δ are not simply activating or suppressing PPARβ/δ. This is because that PPARβ/δ naturally exhibits both activation and repression on the expression of its target genes, and some of these genes are oppositely regulated by exogenous ligands. The *ANGPTL4* gene, for example, is repressed by PPARβ/δ without an exogenous ligand (i.e., up-regulated by PPARβ/δ-knockout or knockdown), is activated in the presence of synthetic PPARβ/δ agonist, and is repressed in the presence of selective repressive PPARβ/δ ligand (Adhikary et al., 2011, 2013; Khozoie et al.,

2012). Therefore, both ligand activation and selective repression promote the function of PPARβ/δ in different ways.

Non-transcriptional regulation by PPARβ/δ has also been discovered. The best-known example is its interaction with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Figure 2 A). NF-κB is a core transcription factor regulating inflammation signaling pathways that is composed of two subunits, p65 and p50. Studies have shown that ligand activation of PPARβ/δ can induce association of PPARβ/δ with the p65 subunit of NF-κB, thereby preventing its binding with the p50 subunit and regulation of pro-inflammatory signaling genes (Álvarez-Guardia et al., 2011; Barroso et al., 2011; Coll et al., 2010; Planavila et al., 2005; Rodríguez-Calvo et al., 2008; Schnegg et al., 2012; Shan et al., 2008b, 2008a). This effect can also be inhibited by an antagonist, while the role of selective repressive ligands in this mechanism is currently unknown. This mechanism plays an important role in the anti-inflammatory function of PPARβ/δ observed in many models (reviewed in Peters et al., 2011a, 2015b).

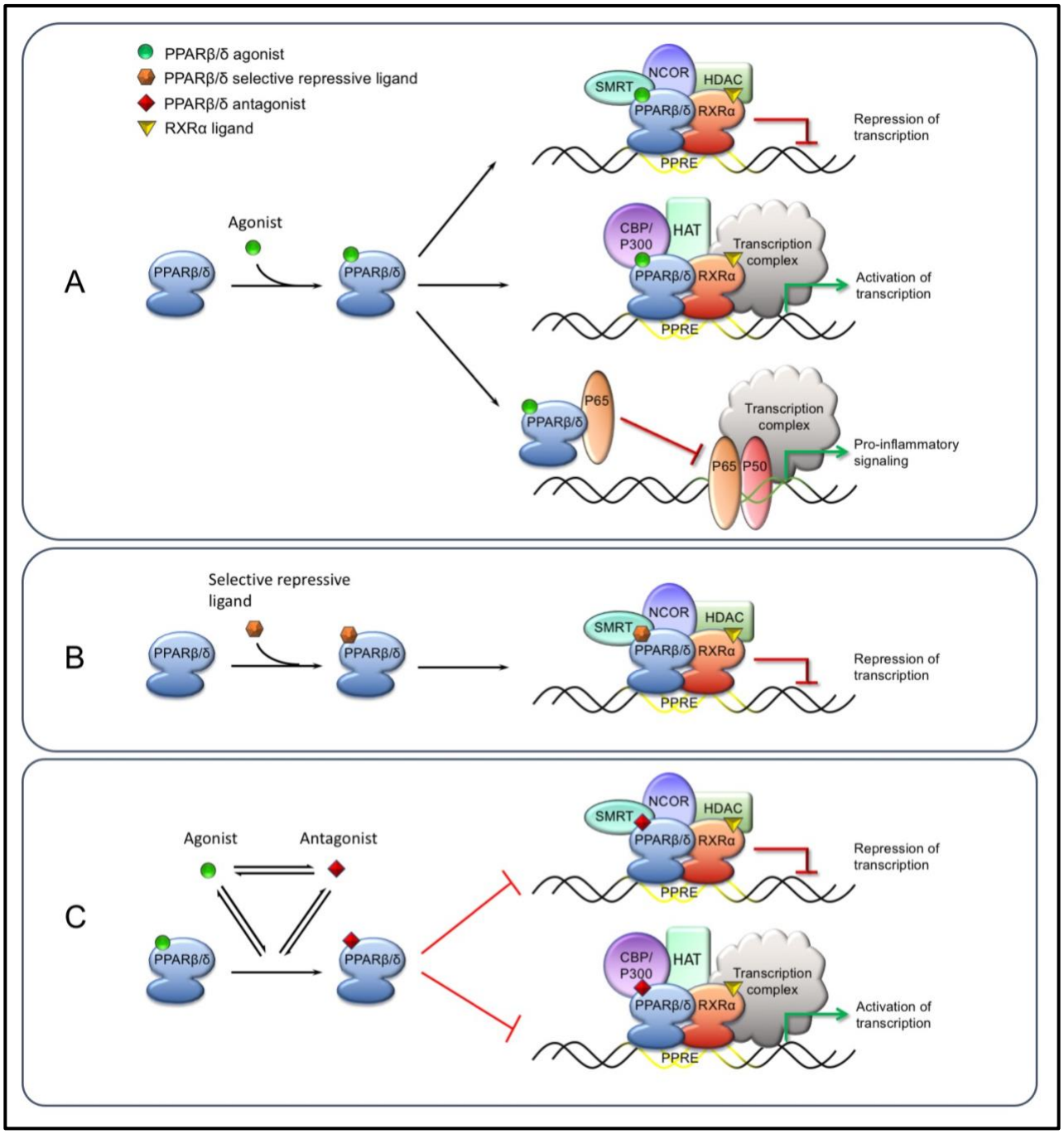


Figure 2. Mechanisms of regulation by PPARβ/δ.
Three forms of transcriptional and non-transcriptional regulation by PPARβ/δ are shown. A, Ligand activation of PPARβ/δ can activate or repress transcription by associating with co-activators or co-repressors. Ligand activation of PPARβ/δ also inhibits NF-κB activity by sequestering the P65 subunit. B, selective repression of PPARβ/δ only represses transcription by recruiting co-repressors. C, Antagonists of PPARβ/δ compete with agonists and inhibits transcriptional up- and down-regulation of target genes by the agonists. Abbreviations: RXR, Retinoid X receptor; SMRT, Silencing mediator for retinoid and thyroid receptor; NCOR, Nuclear receptor co-repressor; HDAC, Histone deacetylase; CPB, CREB-binding protein; HAT, histone Acetyltransferase; PPRE, PPAR response element.

1.3. Colon cancer and PPARβ/δ

1.3.1. Development of colon cancer

Colon cancer is the second leading cause of death from cancer in the United States, and the fifth worldwide. About one in 24 people develop colon cancer in their lifetime (Siegel et al., 2019). The majority of colon tumors are adenocarcinomas that originate from epithelial cells. The colon epithelium is folded into millions of crypts. Stem cells are located at the bottom of each crypt, and as they proliferate and move upwards, these cells gradually differentiate and eventually undergo apoptosis at the top. The lifespan of a normal epithelial cell is usually three to four days. Although some debates exist, colon cancer likely originates in the stem cells or the partially differentiated cells at the lower side of crypts (Huels and Sansom, 2015).

The development of any cancer needs to achieve several hallmarks: self-sufficient proliferation signaling, resistance to cell death, sustained replication, building angiogenesis, and spreading through metastasis (Hanahan and Weinberg, 2011). This applies to the stages of colon cancer development as well. In the early stages of colon cancer, genetic mutations—especially in the APC/β-CATENIN pathway—allow epithelial cells to proliferate uncontrollably and form polyps. As the tumor mass becomes larger, angiogenesis becomes necessary to supply oxygen and nutrients to the cells inside the tumor. Inflammation is also a contributor to colon cancer development, since the production of cytokines during this process can cause more mutations and induce metastasis (Terzić et al., 2010). Epithelial to mesenchymal transition (EMT) and stem cell properties are two other important factors involved in invasion and metastasis (Polyak and Weinberg, 2009).

1.3.2. Molecular features of colon cancer

Like other types of cancer, colon cancer has a unique pattern of genetic mutations. Mutations in the adenomatous polyposis coli (*APC*) gene of the APC/β-CATENIN pathway, the *KRAS* gene of the mitogen-activated protein kinases (MAPK) pathway, the *PIK3CA* gene of the phosphoinositide 3-kinase (*PI3K*) pathway, and the *TP53* tumor suppressor are most frequently identified in human colorectal tumors (Carethers and Jung, 2015; Kandoth et al., 2013; Schell et al., 2016).

The APC/β-CATENIN pathway is the most frequently mutated pathway in colon cancer. About 80% of human colorectal tumors have mutant *APC*, while another 5% have mutant *CTNNB1* (gene coding for β-CATENIN) (Kandoth et al., 2013). In a normal colon epithelial cell, a protein complex containing APC constantly phosphorylates β-CATENIN and sends it for proteasomal degradation, thus to maintain low β-CATENIN activity (MacDonald et al., 2009). In colon cancer cells where mutations disrupt APC or constitutively activate β-CATENIN, β-CATENIN can evade the degradation, subsequently accumulate in the nucleus, bind to T cell factor 4 (TCF4), and activate a number of tumor-promoting genes. These include CYCLIN D1 and c-MYC, which govern cell cycle progression; vascular endothelial growth factor (VEGF), which is essential for angiogenesis; and matrix metalloproteinases (MMPs), which helps cancer cell invasion (reviewed in MacDonald et al., 2009). Mutations of *APC* often initiate colon cancer. Hereditary mutations of *APC* are the major cause of familial adenomatous polyposis, which have an almost 100% chance of developing colon cancer if left untreated (Markowitz and Bertagnolli, 2009). Because of its unique importance in colon cancer, *Apc*-mutant mouse strains, such as $Apc^{+/min}$ and $Apc^{+/min\text{-}FCCC}$ mice, are also commonly used as colon tumor models.

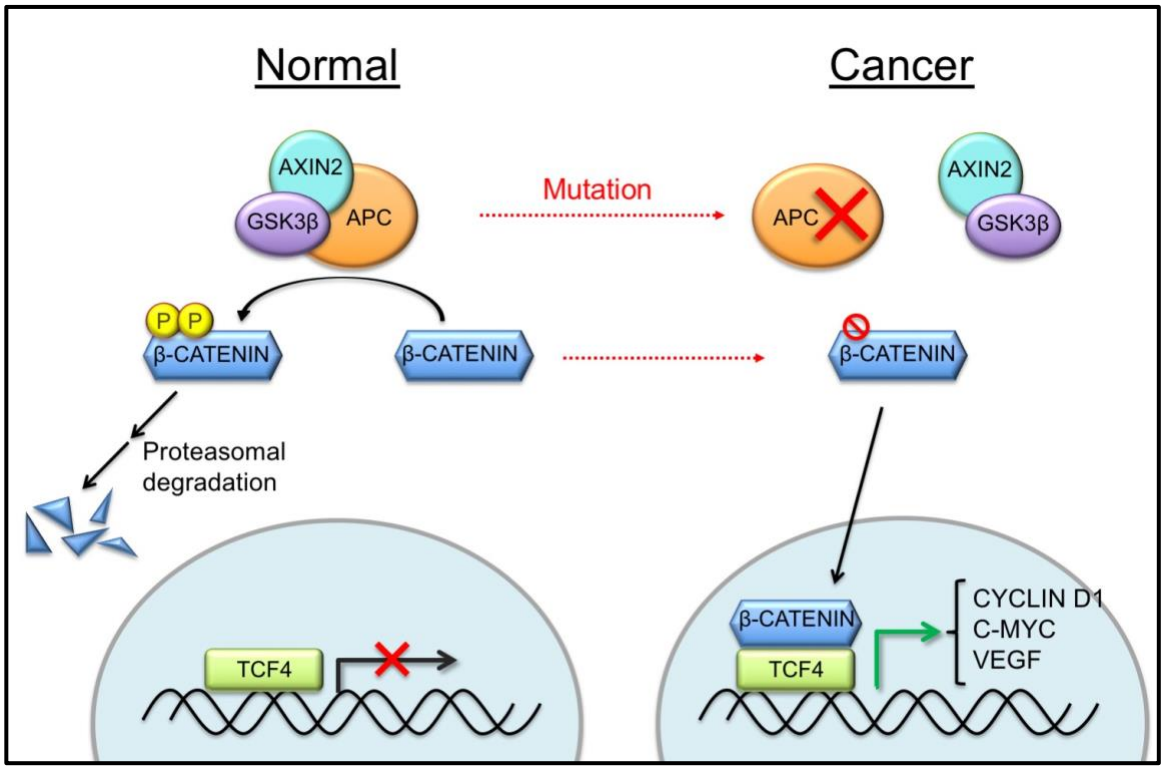


Figure 3. Mechanism of APC/β-CATENIN pathway in normal and cancer cells.
In a normal cell, β-CATENIN is phosphorylated by a protein complex containing adenomatous polyposis coli (APC), axin-like protein 2 (AXIN 2), and glycogen synthase kinase 3 beta (GSK3β). Phosphorylated β -CATENIN is subsequently degraded by the proteasome. Loss-of-function mutation of APC or gain-of-function mutation of CTNNB1 can be found in a cancer cell. These mutations allow β-CATENIN to evade proteasomal degradation, enter the nucleus, and activate target genes together with T cell factor 4 (TCF4). Some examples of β-CATENIN target gene that are oncogenic are CYCLIN D1, C-MYC, and vascular endothelial growth factor (VEGF).

Although *APC* mutations can initiate colon polyp formation, they do not promote carcinogenesis alone. Accumulation of additional mutations is required for further progression (Markowitz and Bertagnolli, 2009; Mármol et al., 2017). The MAPK pathway is another frequently mutated pathway in colon cancer that is associated with metastasis. Near half of colon cancer patients carry a mutation in the *KRAS* gene, predominantly at amino acid 12 and 13, and a smaller fraction of patients alternatively carry mutant *BRAF*, predominantly V600E (Kandoth et al., 2013; Markowitz and Bertagnolli, 2009). Most established human colon cancer cell lines have mutant *KRAS* or *BRAF* as well (Ahmed et al., 2013). These mutations in *KRAS* and *BRAF* constitutively activate their GTPase and kinase activity, leading to activation of the downstream

MAPK/ERK signaling. In addition, mutations in *PIK3CA* gene of the PI3K/AKT signaling pathway, *SMAD4* in TGFβ pathway, and *TP53* are all likely to happen during later stages of cancer progression, and further aid tissue invasion and metastasis (Kandoth et al., 2013; Kuipers et al., 2015; Markowitz and Bertagnolli, 2009).

Non-mutational alterations of protein expression contribute to colon cancer as well. For instance, Cyclooxygenase 2 (COX2), an enzyme that governs prostaglandin synthesis, is up-regulated in about two-thirds of human colon tumors. Inhibition of this enzyme by nonsteroidal anti-inflammatory drugs (NSAIDs) helps control early-stage tumor formation (reviewed in Clevers, 2006). Colon tumors also express increased level of EGFR, which activates MAPK and PI3K/AKT pathways, and can be targeted in colon cancer chemotherapy (Kuipers et al., 2015). Colon cancer cells also expresses unique stem cell markers, such as leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), CD133, and CD44. Higher expression of these proteins indicates a higher stem-cell population in a tumor, which is positively related to chemo-resistance and invasion (Medema, 2017; Munro et al., 2018).

1.3.3. PPARβ/δ and the APC/β-CATENIN pathway

The first study to relate PPARβ/δ with cancer was published by He et al. in 1999. This study observed that expression of functional APC or dominant-negative TCF4 in *APC*-mutant colon cancer cell lines decreased the expression of PPARβ/δ. The promoter-binding activity of PPARβ/δ onto a putative PPRE sequence was also reduced by APC or dominant dominant-negative TCF4, and was enhanced by overexpression of β-CATENIN. Together with the observation that PPARβ/δ inhibits NSAID-induced apoptosis and that NSAIDs reduce PPARβ/δ functionality, this study concluded that PPARβ/δ is a target gene of the oncogenic β-CATENIN pathway, which is up-regulated in colon cancer to promote cancer cell survival (He et al., 1999).

In contrast to these results, several studies did not support the theory that PPARβ/δ is a β-CATENIN target gene. Disruption of *APC* up-regulated β-CATENIN but resulted in decreased PPARβ/δ protein and mRNA expression (Reed et al., 2004). Another study found that there was no difference between protein levels of PPARβ/δ in normal colon tissue from $Apc^{+/+}$ and $Apc^{+/min}$ mice, and colon polyps from $Apc^{+/min}$ mice (Foreman et al., 2009). PPARβ/δ expression was also not different between human colon-cancer cell lines with wild-type or mutant *APC*/*CTNNB1* genes, although these mutations coincide with CYCLIN D1, a verified target of β-CATENIN (Foreman et al., 2009). Surprisingly, PPARβ/δ expression was significantly lower in colon tumors compared to controls collected from human patients or $Apc^{+/min\text{-}FCCC}$ mice. CYCLIN D1 was up-regulated in both cases, suggesting further activation of β-CATENIN in the majority of human and mouse tumor samples (Foreman et al., 2011). *PPARD* was also not identified as a target gene of β-CATENIN and TCF4 by ChIP-seq screenings (Bottomly et al., 2010; Yochum et al., 2007). In addition, repression of PPARβ/δ expression or activity was not found with NSAIDs; ligand activation of PPARβ/δ facilitated apoptosis and repressed proliferation with or without NSAIDs in both *APC*/*CTNNB1* wild-type and mutant colon cancer cell lines (Foreman et al., 2009, 2011).

Although these studies did not support the hypothesis that the expression of PPARβ/δ is regulated by β-CATENIN, whether its activity is altered by β-CATENIN, as part of the report by He et al. (1999) suggested, is currently unknown. The idea that β-CATENIN promotes colon carcinogenesis through activation of PPARβ/δ is still believed by many researchers to this day.

A few studies have proposed other types of relationships between PPARβ/δ and the β-CATENIN pathway. It was reported that ligand activation of PPARβ/δ changes the DNA-binding pattern of β-CATENIN on its target gene *VEGFA* and promotes its expression (Hwang et al., 2012). However, a former study did not observe a change in VEGF expression related to

ligand activation of PPARβ/δ in colon and liver cancer cell lines (Hollingshead et al., 2007b). Recently, activation of PPARβ/δ was observed to increase four non-canonical β-CATENIN target genes: *BMP4*, *JAG1*, *JAG2* and *EDN3*, in mouse. Ligand activation of PPARβ/δ also increased nuclear staining of β-CATENIN, while high fat diet induced protein-level interaction between PPARβ/δ and β-CATENIN (Beyaz et al., 2016). Similarly, a later study observed that overexpression of PPARβ/δ increased the level of β-CATENIN and its target gene mRNA in *APC*-mutant mouse colon, while the opposite effect was observed with knockout of *PPARD* (Liu et al., 2019). These studies suggested that, in contrast with the finding of He et al. (1999), PPARβ/δ may facilitate β-CATENIN to potentiate colon cancer tumorigenicity and stemness.

1.3.4. PPARβ/δ expression in human and mouse colon tumors

Along with the controversial role of PPARβ/δ in colon cancer, studies have reported up- or down-regulation of PPARβ/δ in colon tumors in both human patients and mouse models (reviewed in Peters et al., 2008, 2011a). The majority of these studies analyzed PPARβ/δ expression on mRNA level. The two earliest studies on this subject used northern blot technique, and observed that expression of PPARβ/δ mRNA was higher in tumors obtained from colon cancer patients compared to control samples (He et al., 1999; Gupta et al., 2000). In these studies, relatively small sample sizes were used (n = 4 and 6 respectively), but quantification of expression was not performed. Similar findings of higher mRNA expression of PPARβ/δ in human colon cancer or cancerous mucosa samples compared to normal controls were described by three later studies using quantitative PCR (qPCR) analysis, and the sample numbers were 20, 12, and 22, respectively (Delage et al., 2007; Wang et al., 2012; Zuo et al., 2017). In contrast to the above studies, qPCR results from several reports suggested that PPARβ/δ is down-regulated in colon tumors. One study observed lower PPARβ/δ mRNA level in the normal mucosa

adjacent to tumors from nine colon cancer patients compared to mucosa from non-cancer patients, and in polyps from *Apc*$^{+/min}$ mice compared to normal mucosa from wild-type littermates (Chen et al., 2004). Another study reported a down-regulation of PPARβ/δ mRNA in colon tumors compared to normal controls from 10 familial adenomatous polyposis (FAP) patients, as well as from an *Apc*$^{+/min}$ mouse model ($p = 0.06$ for human samples) (Modica et al., 2010). Similarly, one study also observed that the mRNA expression of PPARβ/δ were lower in colon tumors compared to matched controls from 19 patients and 9-12 *APC*$^{+/min\text{-}FCCC}$ mice (Foreman et al., 2011). The colon cancer study of The Cancer Genome Atlas, containing RNA-seq data of more than 270 tumor and 41 normal samples, showed a significant decrease of PPARβ/δ mRNA in colon tumors to about 2/3 of its level in normal tissue (The Cancer Genome Atlas Network, 2012) (data visualized using online portals described by Chandrashekar et al., 2017; Tang et al., 2017). Additionally, some studies found no difference of PPARβ/δ mRNA expression between cancer samples and normal controls — this includes a study of matched tumor and normal samples from 17 colorectal cancer patients using qPCR analysis (Feilchenfeldt et al., 2004), and qPCR data from a study of 86 rectal cancer patients (which is often undistinguished from colon cancer) (Yang et al., 2006). Moreover, there is a large variance of PPARβ/δ expression between individual patients (Feilchenfeldt et al., 2004; Wang et al., 2012).

Protein analysis of PPARβ/δ may not generate agreeable results to mRNA analysis, since protein expression is subject to additional translational and degradational regulation. A number of studies used solely immunohistochemistry (IHC) to examine PPARβ/δ protein expression in colon cancer. However, IHC analysis for PPARβ/δ expression has been suggested to be inaccurate due to errors caused by non-specific bindings (reviewed in Peters and Gonzalez, 2009; Peters et al., 2011b). As a semi-quantitative method, proper controls should always be used in IHC assay. A valid IHC result should be accompanied by three controls: a positive control,

which is a sample known to express the target protein at a high level; a negative control, where the target protein is not or minimally expressed; an isotype-specificity negative control, which is the experimental sample stained with pre-immune serum or isotype-specific immunoglobulin instead of the primary antibody, to demonstrate minimal non-specific staining and background signal. Additionally, western blot analysis should be performed using the tissue of interest to verify the target protein by size. Unfortunately, these essential technical controls were rarely completed in existing IHC data for PPARβ/δ. (Hewitt et al., 2014) One study of 32 non-familial colorectal cancer patients that observed higher PPARβ/δ immunostaining in colorectal carcinoma compared to adenoma, without comparing to normal tissue, and that no significant relevance between PPARβ/δ expression and tumor size, differentiation, or metastasis was found (Takayama et al., 2006). A recent study found increased PPARβ/δ immunostaining in colon tumors from 152 patients compared to normal control. However, a control experiment for antibody specificity, or an alternative analysis using western blot, was not shown (Zuo et al., 2017). A study of rectal patients also found a higher frequency of strong PPARβ/δ protein expression in primary tumors compared to normal control. Both IHC and western blot was used and generated similar results in this study, however, whether PPARβ/δ was targeted was not verified using a positive or negative control (Yang et al., 2011). In mouse experiments, two studies suggested that PPARβ/δ expression is increased in tumors from *Apc*$^{+/min}$ mice, or in normal tissues in *Apc*$^{+/min}$ mice compared to wild-type mice (Knutsen et al., 2005; Ouyang et al., 2006). These studies either used solely IHC, or statistically incomplete when alternative methods such as qPCR or western blot were used. In fact, a later study analyzed the same samples as Ouyang et al. (2006) using western blot and a positive control, but found no difference of PPARβ/δ expression between *Apc*$^{+/min}$ and control mice, in contrast to the original IHC data described by Ouyang et al. This demonstrated the inaccuracy of quantifying PPARβ/δ using IHC.

In contrast, western blot analysis, confirmed with a PPARβ/δ-overexpression positive control sample, revealed that PPARβ/δ protein is significantly down-regulated in colon tumors compared to normal controls in 19 colon cancer patients, as well as 9-12 *Apc*$^{+/min\text{-}FCCC}$ mice (Foreman et al., 2011). Since the colon is one of the highest PPARβ/δ-expressing organs in the human body, high PPARβ/δ expression may be necessary for maintaining normal colon function (Escher et al., 2001; Girroir et al., 2008; Modica et al., 2010; Uhlén et al., 2015).

Overall, inconsistencies exist on both protein and mRNA expression pattern of PPARβ/δ in colon cancer from human and mouse models. Large variances between individual patients have been commonly observed. Whether changes of PPARβ/δ expression in colon cancer have tumorigenic functions, or as a result of altered metabolism or inflammation in the cancer environment still remain to be investigated.

1.3.5. Role of PPARβ/δ in in vivo tumorigenesis

Genetic alteration and ligand activation of PPARβ/δ have been studied in *in vivo* colon tumorigenesis, using transgenic mouse models (*Apc*$^{+/min}$ or *Apc*$^{+/min\text{-}FCCC}$), chemical carcinogens such as azoxymethane (AOM), or xenograft of human colon cancer cell lines. While some studies suggested that PPARβ/δ colon tumorigenesis in mice, others generated opposite results. It was first described by Park et al. (2001) that knockout of PPARβ/δ suppressed ectopic xenograft tumor growth of HCT116 cells. By contrast, a later study showed that PPARβ/δ knockdown in KM12C cells increased ectopic xenograft tumor size, as well as the expression of Ki67, a cell proliferation-related protein, in these tumors (Yang et al., 2013). In mouse tumor models, Barak et al. (2002) first showed that knockout of PPARβ/δ had a tendency of decreasing polyp number and size in the *Apc*$^{+/min}$ mouse intestine, although these results were not statistically insignificant (Barak et al., 2002). In line with this study, another group observed that knockout of PPARβ/δ

did not change polyp numbers, but increased polyp size, in *Apc*$^{+/min}$ mice. Ligand activation of PPARβ/δ using GW501516 also increased the number and size of polyps in the *Apc*$^{+/min}$ mouse colon (Gupta et al., 2004; Wang et al., 2004a, 2006). Focusing on the COX2 pathway, the same group found that PPARβ/δ expression was higher in AOM-induced mouse colon tumors compared to normal controls, as a result of COX2 activation (Gupta et al., 2000). AOM induced colon tumorigenesis was found to be reduced in mice with targeted deletion of PPARβ/δ in the intestinal epithelium (Zuo et al., 2009, 2014). On the other hand, several studies have suggested that PPARβ/δ prevents tumorigenesis in genetic or chemical mouse models. Previously published studies from our lab found that *Ppard* $^{-/-}$ mice developed more colon polyps compared to *Ppard* $^{+/-}$ and *Ppard*$^{+/+}$ mice in both *Apc*$^{+/min}$ and AOM-induced tumor models (Harman et al., 2004; Reed et al., 2004), and administration of a PPARβ/δ ligand further reduced the number of polyps in *Ppard*$^{+/+}$*Apc*$^{+/min}$ mice (Marin et al., 2006).

One of the mechanisms by which PPARβ/δ regulates colon cancer is through inhibition of inflammation, which is a known factor that promotes cancer. In contrast to one study in which knockout of PPARβ/δ in mice prevented dextran sulfate sodium (DSS)-induced colitis and proinflammatory signaling (Wang et al., 2014), a previous study observed the opposite effect (Hollingshead et al., 2007a). Utilizing an intestinal epithelial-targeted PPARβ/δ knockout strain, another report found that PPARβ/δ was protective against tumor injury and mediated the anti-inflammatory effect of fish oil induced by co-treatment of AOM and DSS (Monk et al., 2012). Despite this controversy, numerous reports from non-colon cancer models strongly suggested the protective effect of PPARβ/δ against inflammation and carcinogenesis, in addition to the knowledge that PPARβ/δ down-regulates the NFκB proinflammatory signaling pathway (reviewed in Peters et al., 2011a, 2015b). Besides inflammation, more connections between PPARβ/δ and colon tumorigenesis are waiting to be explored.

1.3.6. PPARβ/δ and cancer cell invasion and metastasis

A limited number of studies have examined the relationship between PPARβ/δ and colon cancer cell invasion *in vitro* and in vivo. Results from one study suggested that PPARβ/δ inhibits colon cancer malignancy, that, knockdown of PPARβ/δ increased colony size, malignant phenotypes, and integrin linked kinase (ILK) expression in KM12C, KM12SM, and KM12L4a colon cancer cell lines, although no change of migration or invasion was observed (Yang et al., 2010). On the other hand, two recent reports published by one group suggested that PPARβ/δ potentiates cancer-cell invasion by activating EMT. In the first study, knockdown or knockout of PPARβ/δ inhibited, while ligand activation of PPARβ/δ promoted, metastasis of colon cancer cell lines, as well as melanoma, pancreatic cancer, and lung cancer cell lines (Zuo et al., 2017). However, except for a pancreatic cancer model where an orthotopic xenograft model was used, only tail vein injection was used to study the other types of cancer, including colon cancer. This method has limitation, since it allows the cancer cells to enter the circulatory system without the need for tissue invasion from a primary tumor location (Rashid et al., 2013). The same study also found that knockout of PPARβ/δ profoundly altered the expression of EMT markers E-CADHERIN and VIMENTIN, and further reduced migration and invasion in the HCT116 cell line (Zuo et al., 2017). The second study found that targeted overexpression and/or ligand activation of PPARβ/δ increased tumorigenesis and potentiated local tumor invasion in an *Apc*-mutant mouse model. This study further suggested that PPARβ/δ increases activity of BMP7/β-CATENIN and AKT pathways, as potential mechanisms through which PPARβ/δ promotes tumor invasion (Liu et al., 2019). These studies together suggest that PPARβ/δ regulates colon cancer cell EMT, invasion, and metastasis.

The studies described above conflict with a number of other knowledge of PPARβ/δ. First, our previous studies examined the expression of AKT, phospho-AKT, and VEGF (which is also a β-CATENIN target), in both *Ppard*$^{+/+}$ and *Ppard*$^{-/-}$ mice and the HCT116 human colon cancer cell line, with or without ligand activation of PPARβ/δ. No change in any of these proteins was observed with different PPARβ/δ expression or activity. Secondly, PPARβ/δ has been shown to promote terminal differentiation in colon epithelial cells (Marin et al., 2006) and many other types of cells (reviewed in 1.2.2 and reviewed by Burdick et al., 2006; Peters et al., 2011b), which is opposite from the stem cell-like features generally observed with metastasis and EMT. PPARβ/δ has also been shown to inhibit expression of BMP2 and BMP4 to promote differentiation (Simonini et al., 2010), which is contradictory to the upregulation of BMP7 found by Liu et al. In addition, PPARβ/δ has been shown to reduce MMP activity, inhibit anchorage-independent growth, down-regulate stem cell marker expression, decrease migration and invasion rate, and reduce stem cell marker expression in other types of cancer cell lines (Yao et al., 2015a, 2017). Antagonizing PPARβ/δ has also been shown to promote EMT, invasion, and migration in melanoma cell line (Lim et al., 2018). Furthermore, a number of studies have shown that PPARβ/δ is protective against genetic or chemically induced tumorigenesis, as was discussed in chapter 1.4.2.

Contradictory results have also been published for the relationship between PPARβ/δ expression and stage, metastatic status, and survival in colon cancer patients. The aforementioned study by done by Yang et al. (2011) observed that, although PPARβ/δ expression is stronger in primary rectal tumors compared to normal mucosa, its expression became lower in advanced stage tumors, and patients with relatively higher PPARβ/δ expression were more likely to survive (Yang et al., 2011). Another study, observed increased malignant morphology in samples with higher scores of PPARβ/δ immunostaining, although there was no

difference related to metastasis or tumor stage (Takayama et al., 2006). In contrast, a recent study found that mRNA expression of PPARβ/δ was higher in colon cancer with metastasis than without, while PPARβ/δ was associated with a poor prognosis in colon cancer patients (n=33), as well as breast cancer and liposarcoma patients (Zuo et al., 2017). Finally, RNA-seq data of 276 patients from The Cancer Genome Atlas database did not identify PPARβ/δ as a prognostic marker of colon cancer (The Cancer Genome Atlas Network, 2012; Uhlen et al., 2017).

Taken together, the published studies added value to the understanding of PPARβ/δ in colon cancer EMT, invasion, and metastasis; however, our knowledge is still limited in this topic, and it remains to be elucidated in the future.

1.3.7. Selective repression of PPARβ/δ in cancer

Studies have shown promising chemopreventive or chemotherapeutic values of selective repressive PPARβ/δ ligands. Due to the relatively short history of these ligands, their role in colon cancer has not been examined. One study found that selective repression of PPARβ/δ using DG172 and ST247 suppresses breast cancer cell invasion, potentially by interfering with the TGFβ pathway and reducing ANGPTL4 expression (Adhikary et al., 2013). Studies from another group showed that DG172 inhibited migration and proliferation of breast cancer cells, and also reduced dexamethasone-induced survival of chronic lymphocytic leukemia cells (Li et al., 2017; Sun et al., 2018; Wang et al., 2016). It is important to use controls when studying selective repression of PPARβ/δ, since PPARβ/δ-independent effects have been reported previously (Lieber et al., 2015).

1.4. Objective and hypothesis

This overall aim of these studies is to elucidate the functional roles of PPARβ/δ in colon cancer, including its relationship with the APC/β-CATENIN pathway, colon cancer cell growth, and malignancy-related features. Early studies led to the theory that PPARβ/δ, as a target of the APC/β-CATENIN pathway, function to promote colon cancer. While other evidence suggested that PPARβ/δ expression is not likely regulated by β-CATENIN, and is inhibitory against colon carcinogenesis, the remaining possibility that functionality of PPARβ/δ may be regulated by β-CATENIN has not been examined. This led to the main objective of the study in Chapter 2. Since activation of PPARβ/δ involves nuclear translocation and regulation of target genes, we hypothesize that cellular retention and/or target gene regulation of PPARβ/δ are different between colon cancer cell lines with inactive or active APC/β-CATENIN pathway, and that ligand activation and/or selective repression of PPARβ/δ affect growth human colon cancer cell lines with or without mutant *APC*/*CTNNB1* genes. Previous reports have also focused less on and generated conflicting results for the role of PPARβ/δ in malignancy and metastasis of colon cancer. Selective repression of PPARβ/δ has been recently shown to reduce cancer cell invasion. Considering the inconsistent and insufficient knowledge on this topic, the study in Chapter 3 will further explore the role of PPARβ/δ in malignancy-related features of colon cancer cells. In particular, whether ligand activation or selective repression of PPARβ/δ inhibits anchorage-independent growth, migration, MMP activity, and EMT of colon cancer cell lines, will be examined.

Colon cancer is one of the most lethal types of cancer as there has not been many effective treatments up to date. PPARβ/δ, on the other side, has demonstrated therapeutic value, yet is concerned for potential risk of carcinogenesis. Results from this study will help to further solve the relationship between PPARβ/δ and key oncogenic pathways of colon cancer, evaluate

safety of PPARβ/δ ligands, and provide insights for targeting PPARβ/δ in colon cancer chemotherapy.

Chapter 2. Functional relationship between PPARβ/δ and the APC/β-CATENIN pathway and the role of PPARβ/δ in colon cancer cell growth

2.1. Abstract

The role of PPARβ/δ in colon cancer remains controversial. Early evidence suggested that PPARβ/δ is a target of the APC/β-CATENIN pathway, and is up-regulated by its activation in colon cancer. Up-regulation of PPARβ/δ was also thought to facilitate colon tumorigenesis and colon cancer cell growth. In contrast, results from other studies did not support the existence of regulation of PPARβ/δ expression by APC/β-CATENIN, while decreased or unchanged expression of PPARβ/δ in colon cancer was observed. Further studies showed that expression or ligand activation of PPARβ/δ inhibited colon tumorigenesis and colon cancer cell survival. It remains a question, however, whether PPARβ/δ is regulated on the functionality level rather than expression level by the APC/β-CATENIN pathway, and whether PPARβ/δ facilitates colon cancer cell line proliferation. To investigate these questions, this study examined the influence of β-CATENIN activation, caused by pre-existing *APC*/*CTNNB1* mutations or transient overexpression, on the cytosolic/nuclear retention of PPARβ/δ, as well as the rate of ligand-induced target gene activation in human colon cancer cell lines. Additionally, growth of colon cancer cells with wild-type or mutant *APC*/*CTNNB1* under the influence of ligand activation or selective repression of PPARβ/δ was also monitored. We observed that, while expression of a β-CATENIN target gene was increased in the presence of *APC*/*CTNNB1* mutations, cytosolic and nuclear retention of PPARβ/δ were not different between the groups. Change of nuclear retention of PPARβ/δ by ligand activation was not detected in cell lines with either genotype. Interestingly, ligand-induced activation of PPARβ/δ target genes occurred in *APC*/*CTNNB1* mutant cell lines compared to an *APC*/*CTNNB1* wild-type cell line. However, when repeating the

test in the β-CATENIN overexpression model, elevation of the β-CATENIN target was seen, but a difference in ligand responsiveness of PPARβ/δ was not observed. On the other hand, ligand activation and selective repression of PPARβ/δ both inhibited growth of *APC*/*CTNNB1* mutant cell lines, but not the *APC*/*CTNNB1* wild-type cell line. Collectively, these results suggest that the functionality of PPARβ/δ, including subcellular localization and gene regulation, is not directly regulated by β-CATENIN. Although differences exist for PPARβ/δ activity between cells with wild-type or mutant *APC*/*CTNNB1*, the direct cause of this is not β-CATENIN, and the cause requires future investigation. In fact, cell lines that were more sensitive to ligand-induced PPARβ/δ activation were also prone to growth inhibition caused by PPARβ/δ ligands, suggesting that PPARβ/δ is a good target to treat colon cancer with *APC*/*CTNNB1* mutations.

2.2. Introduction

Colon cancer is the second leading cause of death in the United States among all types of cancer (Siegel et al., 2019). Mutations in the adenomatous polyposis coli (*APC*) and β-CATENIN (*CTNNB1*) genes exist in, and are the initiating event in, more than 80 percent of colon cancer occurrences (Kandoth et al., 2013; and reviewed in Markowitz and Bertagnolli, 2009). In a normal epithelial cell, β-CATENIN is constantly phosphorylated by a protein complex containing APC, Axin-like protein 2 (AXIN 2), and glycogen synthase kinase 3β (GSK3β), which targets β-CATENIN for proteasomal degradation and leaves the downstream pathway inactivated. However, mutations in colon cancer cells produce either inactive APC or constitutively active β-CATENIN with abolished phosphorylation sites. This further causes β-CATENIN to accumulate in the cytosol, enter the nucleus, and regulate target gene expression together with T cell factor 4 (TCF4). β-CATENIN and TCF4 up-regulate a number of target genes that are involved in cancer cell growth and development, such as CYCLIN D1, C-MYC,

and vascular endothelial growth factor (VEGF) (reviewed in MacDonald et al., 2009). Additionally, peroxisome proliferator-activated receptor β/δ (PPARβ/δ, gene name *PPARD*) has been proposed as a β-CATENIN target gene, although this mechanism was not supported by subsequent studies.

PPARβ/δ, an important nuclear receptor in the regulation of fatty acid and glucose metabolism, has a controversial role in colon cancer (reviwed in Peters et al., 2008, 2012, 2015). PPARβ/δ has been proposed to be an APC/β-CATENIN target gene, based on an observation that conditionally induced overexpression of wild-type APC or dominant-negative TCF4 reduced PPARβ/δ mRNA expression in the HT29 colon cancer cell line, which has mutations in both copies of *APC* genes (He et al., 1999). However, a later study found that the protein level of PPARβ/δ was not different between colon cancer cell lines with wild-type or mutant *APC/CTNNB1* genes, although β-CATENIN target CYCLIN D1 expression was higher in the *APC/CTNNB1* mutant cells, including the HT29 cell line (Foreman et al., 2009). The original study, as well as several later studies, reported increased expression of PPARβ/δ in colon tumor samples compared to normal controls, supporting the theory that PPARβ/δ is up-regulated by β-CATENIN (Gupta et al., 2000; He et al., 1999). However, more studies reported decreased or unchanged PPARβ/δ expression in human colon tumors compared to controls, although a general higher expression of CYCLIN D1 in these colon tumors indicated the abundance of active β-CATENIN (Foreman et al., 2011; Modica et al., 2010; Yang et al., 2006). The unclear relationship between PPARβ/δ and the APC/β-CATENIN pathway, and the obscure expression pattern of PPARβ/δ in colon tumors, have led to its controversial role in colon cancer. Evidence can be found on both sides, e.g., that PPARβ/δ either promotes or inhibits colon cancer cell proliferation, survival, *in vivo* tumorigenesis, and patient prognosis (reviewed in Peters et al., 2011a, 2011b, 2012).

He et al. (1999) also observed that expression of APC or dominant negative TCF4 reduces binding affinity of PPARβ/δ to a potential PPRE sequence, suggesting a functional relationship between PPARβ/δ and the β-CATENIN pathway. This has not been examined by other studies. Therefore, there remains a gap of knowledge whether PPARβ/δ functionality instead of expression is regulated by the APC/β-CATENIN pathway. To investigate this relationship, we hypothesized that PPARβ/δ functionally, such as its nuclear/cytosol retention and target gene expression following ligand activation, is modulated by functional β-CATENIN and thus also by *APC*/*CTNNB1* mutations. Along with this hypothesis, whether PPARβ/δ regulates colon cancer cell growth as a potential functional target of β-CATENIN was also examined.

2.3. Materials and methods

Cell culture and spheroid culture

RKO, DLD1, HCT116, HT29 and LS-174T cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). RKO and LS174T cells were cultured in Eagle's Minimum Essential Medium (EMEM) (Sigma), DLD1 cells were cultured in RPMI 1640 medium (Gibco), and HCT116 and HT29 cell lines were cultured in McCoy's 5A medium (Sigma). All cell culture media were supplemented with 5% fetal bovine serum (FBS) (Atlanta) and 1% Penicillin-streptomycin (Gibco), unless otherwise specified. When ligand activation was used to examine PPARβ/δ activity, Cells were treated with 1 μM GW0742 or vehicle control (0.01% DMSO) for 0-24 hours.

Plasmids and transient transfection

The pCMV3-CTNNB1 plasmid was purchased from Sino Biological. pCMV3-CTNNB1 -S45 delete mutant (S45Δ) plasmid was generated from the pCMV3-CTNNB1 plasmid using QuikChange II Site-directed mutagenesis kit (Agilent), forward primer (5'-CACTACCACAG CTCCTTTTCTGAGTGGTAAAG-3') and reverse primer (5'CTTTACCACTCAGAAAAGG AGCTGTGGTAGTG-3'). Empty vector control was kindly provided by Dr. Gary Perdew. Transient plasmid transfections were done using the Lipofectamine 3000 transfection kit (Thermo Fisher Scientific), following the manufacture's protocol. Typically, 2 x 10^5 RKO cells plated 1 day before transfection were transfected with 1 μg plasmid (or equivalent amount of water in mock control), 2 μl p3000 reagent and 2.5 μl lipofectamine 3000 reagent.

Protein collection

Total soluble protein lysates were collected by lysing cells in RIPA buffer (50 mM Tris-Cl, pH 8.6, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, and 1% NP-40) containing fresh protease inhibitors (Roche), and centrifugation at 15000 xg for 15 minutes to remove insoluble parts. For cytosolic and nuclear protein fractionation, crude cytosolic proteins were collected by lysing trypsinized cells with buffer A (10 mM HEPES, pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM MgCl2, 1mM DTT, and protease inhibitors) plus 0.5 % NP-40, centrifugation at 200 x g for 20 mins, and collecting the supernatant. The remaining nuclear pellets were washed twice with Buffer A, and lysed with buffer C (10 mM HEPES, pH 7.9, 0.5 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, and protease inhibitors) to obtain nuclear proteins. Both cytosolic and nuclear samples were centrifuged finally at 15000 x g for 30 minutes to remove insoluble parts. Protein concentration were determined by bicinchoninic acid (BCA) assay (Thermo Scientific).

Western blot analysis

Western blot was performed as previously described (Hollingshead et al., 2007b). Generally, 15 to 30 μg of each protein sample was resolved using SDS-PAGE (10%), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was then blocked with either 3% bovine serum albumin (BSA) (Sigma) (for PPARβ/δ, CYCLIN D1, and β-CATENIN) or 5% milk (for LDH and LAMIN). Blocked membranes were then incubated with 1 μg/ml primary antibodies for PPARβ/δ (Abcam #21209), β-CATENIN (Abcam #32572), CYCLIN D1 (Cell Signaling Technology #2922), LAMIN (Santa Cruz #376248), or LDH (Rockland #200-1173) at 4 ℃ overnight, followed by 1:10000-diluted biotin-conjugated secondary antibodies (Jackson ImmunoResearch) at room temperature for 1 hour, and Streptavidin-I^{125} (approximately 3 μl /10 ml, kindly provided by Dr. Gary Perdew) at room temperature for 10 minutes. Three 10-minute washes were done between each of these steps. Finally, the membranes were exposed to a storage phosphor screen, from which the proteins bands were detected and quantified using the Cyclone phosphorimager system (PerkinElmer).

Real time PCR (qPCR)

Total RNA samples were collected from cells using Ribozol (VWR)/chloroform extraction following manufacture's protocol. The samples were then precipitated using 50% isopropanol and cleaned with 70% ethanol. Quantification of RNA was done using the NanoDrop instrument (Thermo Scientific). cDNA were synthesized from RNA (50 ng/μl) using M-MLV reverse transcriptase (Promega). qPCR reactions were prepared to contain 0.2 μL (10 ng RNA-equivalent of) cDNA, 0.5 μM forward and reverse primers, and 50% volume of SYBR

Green master mix (Quanta) in a total of 20 μL each. qPCR was performed using the MyiQ2 Real Time PCR System (Bio-Rad Laboratories), as previously described (Foreman et al., 2009). The following primer pairs were used for qPCR: *ADRP*: (5'-CTGCTCTTCGCCTTTC GCT-3', 5'-ACCACCCGAGTCACCACACT-3') *ANGPTL4*: (5'-TCACAGCCTGCAGACA CAACTCAA-3', 5'-CCAAACTGGCTTTGCAGATGCTGA-3') and *GAPDH*: (5'-TGCACCACCAACTGCT TAGC-3', 5'-GGCATGGACTGTGGTCATGAG-3'). *GAPDH* was used as a housekeeping control.

Real time proliferation assay

Cell proliferation was monitored using the xCELLigence system (ACEA Biosciences, Inc., San Diego, CA), following the manufacture's manual. On day 0, background readings of E-plate-16 chambers were measured with 50 μl media per well. RKO (1000 cells), DLD1 (4000 cells), HCT116 (4000 cells) or HT29 (4000 cells) were then seeded with 100 μl media per well, allowed to attach at room temperature for 30 minutes, and placed into the xCELLigence machine located inside a cell culture incubator. The cells numbers were determined by pre-experiment titration to be the highest numbers showing sustained growth for the experiment duration. On day 1, 50 μl of media containing 4 x concentrated of desired treatment or vehicle control (4 x 0.01 % DMSO) was added into each well already containing 150 μl media, to achieve 1 x final concentration. Growth of cells were continued to me monitored for four days.

Statistical analysis

Statistical analysis was performed using student's t-test (for western blot results) or two-way ANOVA with Tukey's test (for qPCR and proliferation assay results). Significance was

considered when $p \leq 0.05$. Statistical analysis was performed using the GraphPad Prism software (version 5.0).

2.4. Results

2.4.1. Mutation in the APC or CTNNB1 genes does not alter PPARβ/δ activity in colon cancer cell lines

To understand whether *APC/CTNNB1* genotype causes differences in the functionality of PPARβ/δ, cytosol and nuclear retention of PPARβ/δ in a cell line with wild-type *APC/CTNNB1* was compared against cell lines with mutant *APC/CTNNB1*. Mutation status of the *APC* and *CTNNB1* genes of these cell lines are summarized in Table 3. Note that the same cell lines were examined in a previous study, in which no difference in total PPARβ/δ protein level was found (Foreman et al., 2009). As a positive control, β-CATENIN and its target protein CYCLIN D1 were also analyzed. The results showed that β-CATENIN is expressed in the cytosol and nucleus in all cell lines with mutant *APC* (DLD1, HT29) (Fig. 3) or mutant *CTNNB1* (HCT116, LS174T) (Fig. 4), but was not detected in the RKO cell line, which possesses wild-type *APC* and *CTNNB1*. CYCLIN D1 is also two to three times higher in DLD1, HCT116, and LS174T cells compared to RKO, which is expected as a result of functional β-CATENIN. The level of CYCLIN D1 in HT29 compared to RKO, however, was not significantly higher in these results, although has been shown to be higher at whole cell level in a previous study (Foreman et al., 2009). PPARβ/δ, on the other hand, was mainly observed in the cytosol samples in all cell lines tested, and its retention in cytosol samples in the HT29, DLD1, and LS174T cell lines was not different from RKO, although a moderately higher expression in HCT116 cells (1.4 times) was observed. Nuclear retention of PPARβ/δ, on the other hand, was much lower in all of the cell

lines (less than 0.5 times of cytosolic intensity), and was likely affected by cytosol contamination, since similar levels of LDH were detected in the nuclear samples. Overall, there was no trend of change in nuclear or cytosol retention of PPARβ/δ in these cell lines. These results confirm that β-CATENIN is functionally activated in the colon cancer cell lines with mutant *APC* or *CTNNB1* genes, but also suggest that subcellular localization of PPARβ/δ is not likely affected by the activation of β-CATENIN.

Cell Line	***APC* status**	***CTNNB1* status**
RKO	Wild-type	Wild-type
DLD1	Mutant (different mutations on both copy)	Wild-type
HT29	Mutant (different mutations on both copy)	Wild-type
HCT116	Wild-type	Mutant (heterozygous S45del)
LS174T	Wild-type	Mutant (homozygous S45F)

Table 3. Status of *APC* and *CTNNB1* genotype in cell lines used in this study.

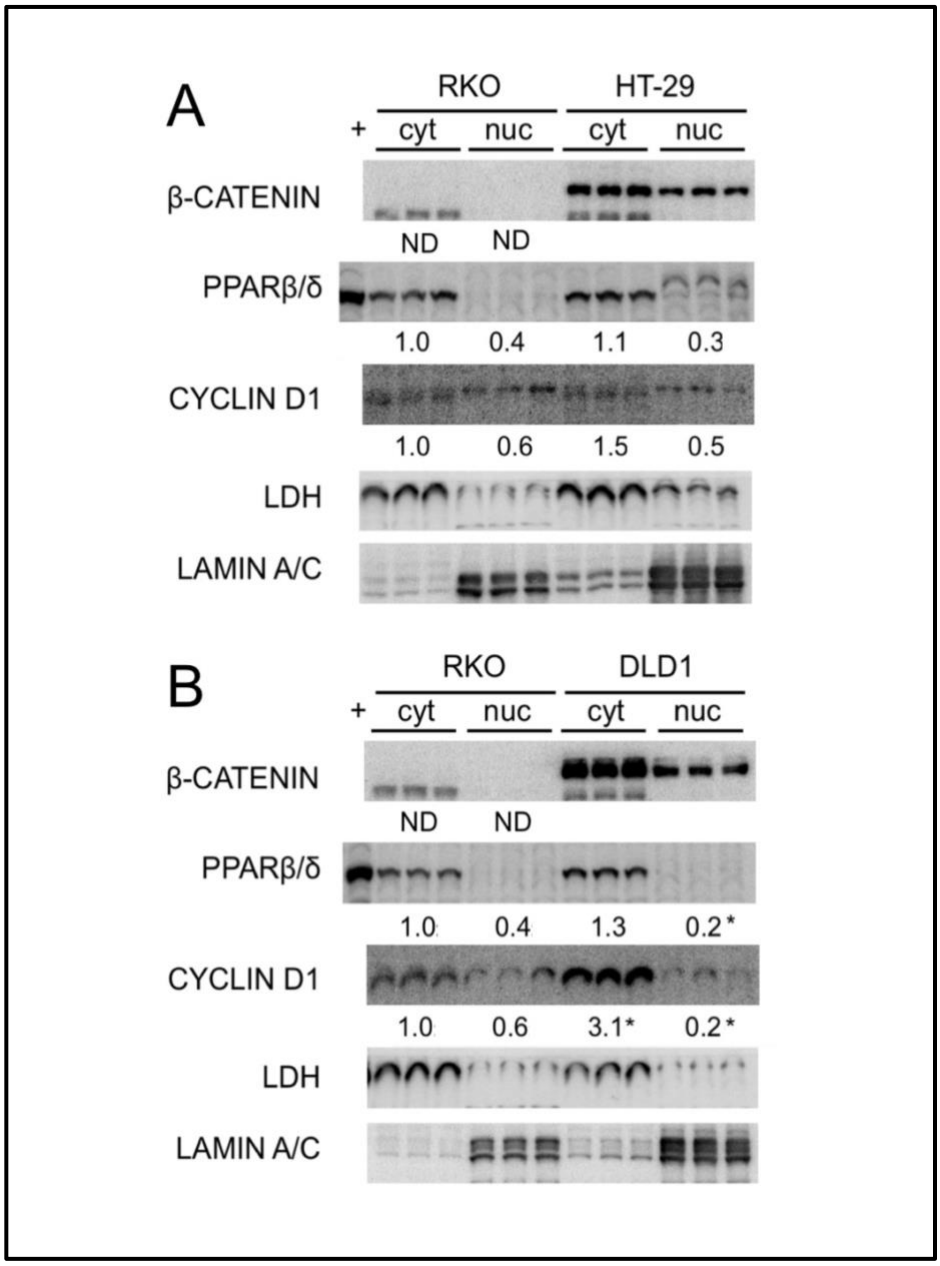


Figure 4. Mutant *APC* increases CYCLIN D1 expression but does not affect nuclear retention of PPARβ/δ in human colon cancer cell lines.
The expressions of β-CATENIN, CYCLIN D1, and PPARβ/δ in the cytosolic and nuclear fractions were compared between RKO (wild-type APC and CTNNB1) and DLD1 (mutant APC) (A), or between RKO and HT29 (mutant APC) (B) human colon cancer cell lines, using western blot. Expression of these proteins were normalized using LDH if in the cytosol, or LAMIN if in the nuclear. Numbers below each blot indicate mean normalized to RKO cytosol. The pooled overall SEM is 0.12. The average amount of protein was assumed to be the same between cytosol and nuclear samples since same amount of protein were loaded for each sample based on BCA assay. +, positive control for PPARβ/δ: lysate from COS1 cells transiently transfected with a PPARβ/δ expression vector. N=9 per group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to RKO (using student's t-test).

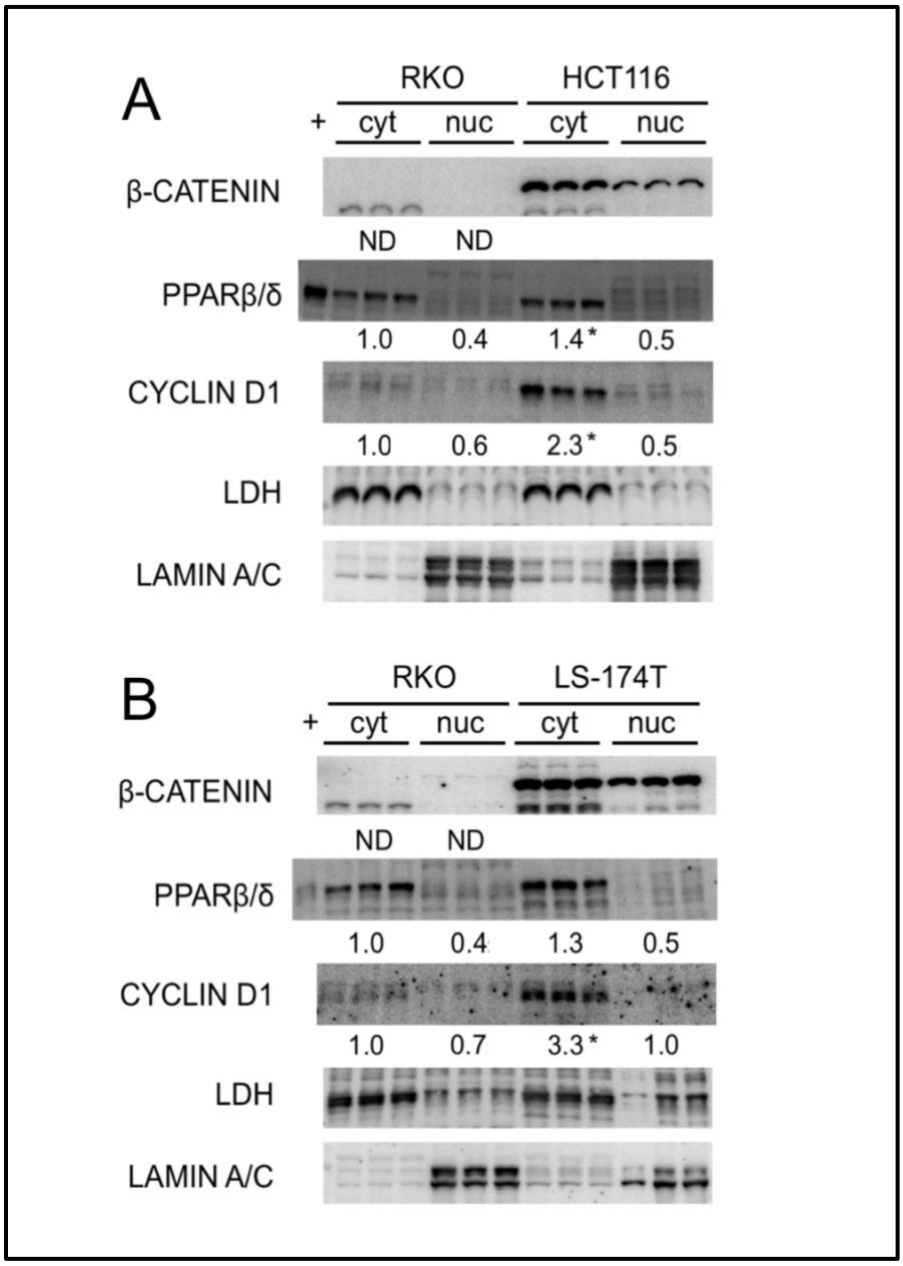


Figure 5. Mutant *CTNNB1* increases CYCLIN D1 expression but does not affect nuclear retention of PPARβ/δ in human colon cancer cell lines.
The expressions of β-CATENIN, CYCLIN D1, and PPARβ/δ in the cytosolic and nuclear fractions were compared between RKO (wild-type *APC* and *CTNNB1*) and HCT116 (mutant *CTNNB1*) (A), or between RKO and LS174T (mutant *CTNNB1*) (B) human colon cancer cell lines, using western blot. Expression of these proteins were normalized using LDH if in the cytosol, or LAMIN if in the nuclear. Numbers below each blot indicate mean, normalized to RKO cytosol. The pooled overall SEM is 0.14. The average amount of protein was assumed to be the same between cytosol and nuclear samples. +, positive control for PPARβ/δ: lysate from COS1 cells transiently transfected with a PPARβ/δ expression vector. N=9 per group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to RKO (using student's t-test).

2.4.2. Ligand activation of PPARβ/δ does not change its nuclear retention in colon cancer cell lines with either wild-type or mutant APC or CTNNB1

To further examine the relationship between *APC*/*CTNNB1* gene mutations and PPARβ/δ localization, cytosol and nuclear proteins were collected over time after the addition of 1 μM GW0742 or vehicle control, and the level of PPARβ/δ was examined. Since nuclear translocation of nuclear receptors generally occurs within 1 to 2 hours, retention of PPARβ/δ at 0 to 2 hours after adding ligand was analyzed. However, none of the cell lines showed a change in nuclear or cytosolic retention of PPARβ/δ after ligand activation (Fig. 6). Preliminary results from samples collected up to 24 hours after addition of ligand also did not reveal an increase in nuclear retention (data not shown). Thus, these results did not generate evidence that the mutant *APC*/*CTNNB1* affects PPARβ/δ activity in colon cancer cells. However, it remains possible that western blot is not a sensitive enough method to detect ligand-induced nuclear accumulation of PPARβ/δ.

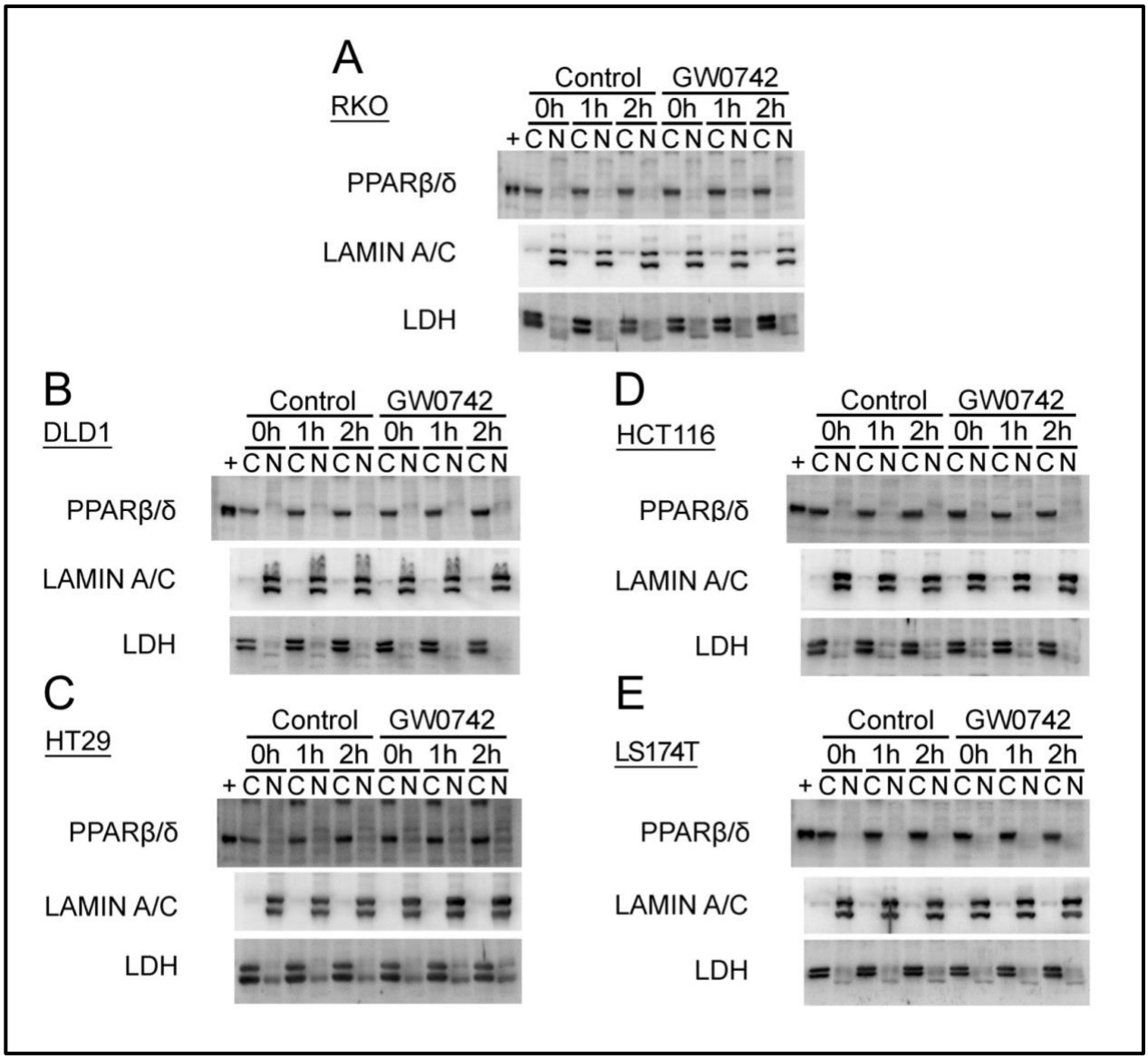


Figure 6. Change in nuclear retention of PPARβ/δ is not observed in colon cancer cell lines with either wild-type or mutant *APC*/*CTNNB1* after ligand activation.
RKO (A), DLD1 (B), HT29 (C), HCT116 (D), and LS174T (E) cells were treated with vehicle control (0.01% DMSO) or 1 μM GW0742 for 0, 1 or 2 hours. Cytosolic and nuclear fractions were collected immediately after treatment, and levels of PPARβ/δ protein in these samples were examined using western blot. Three repeats were performed for each cell line, and one set of representative results is shown. No significant change of nuclear/cytosol level of PPARβ/δ was found using student's t-test, therefore quantification is not shown.

2.4.3. APC/CTNNB1 mutant cell lines are more responsive to ligand activation of PPARβ/δ compared to RKO cells

We next examined ligand-induced PPARβ/δ target gene activation over time in the same cell lines. As shown before, increased expression of functional PPARβ/δ should result in increased sensitivity to ligand activation (Foreman et al., 2011). Cell lines with wild-type or mutant *APC/CTNNB1* were treated with 1 μM GW0742 or vehicle control for 0, 2, 4, 8, 12, and 24 hours, and the levels of PPARβ/δ target gene mRNA were analyzed using qPCR. Adipose differentiation-related protein (ADRP) and angiopoietin-like 4 (ANGPTL4) are two well-studied direct target genes of PPARβ/δ. Since a previous study showed that ANGPTL4 is not inducible with GW0742 in RKO and ADRP is not inducible with GW0742 in DLD1 (Foreman et al., 2009), we chose to analyze PPARβ/δ activity using ANGPTL4 in the DLD1 line, and ADRP in the other cell lines. We observed that ADRP mRNA in HCT116, HT29, and LS174T cell lines, and ANGPTL4 mRNA in the DLD1 cell line, increased within 2 to 4 hours after the addition of ligand, whereas the same treatment took more than 12 hours to increase ADRP mRNA in the RKO cell line (Fig. 7). These results support the hypothesis that PPARβ/δ functionality is increased in colon cancer cell lines with active β-CATENIN. However, whether β-CATENIN activation is the direct cause of increased PPARβ/δ activity needs further investigation.

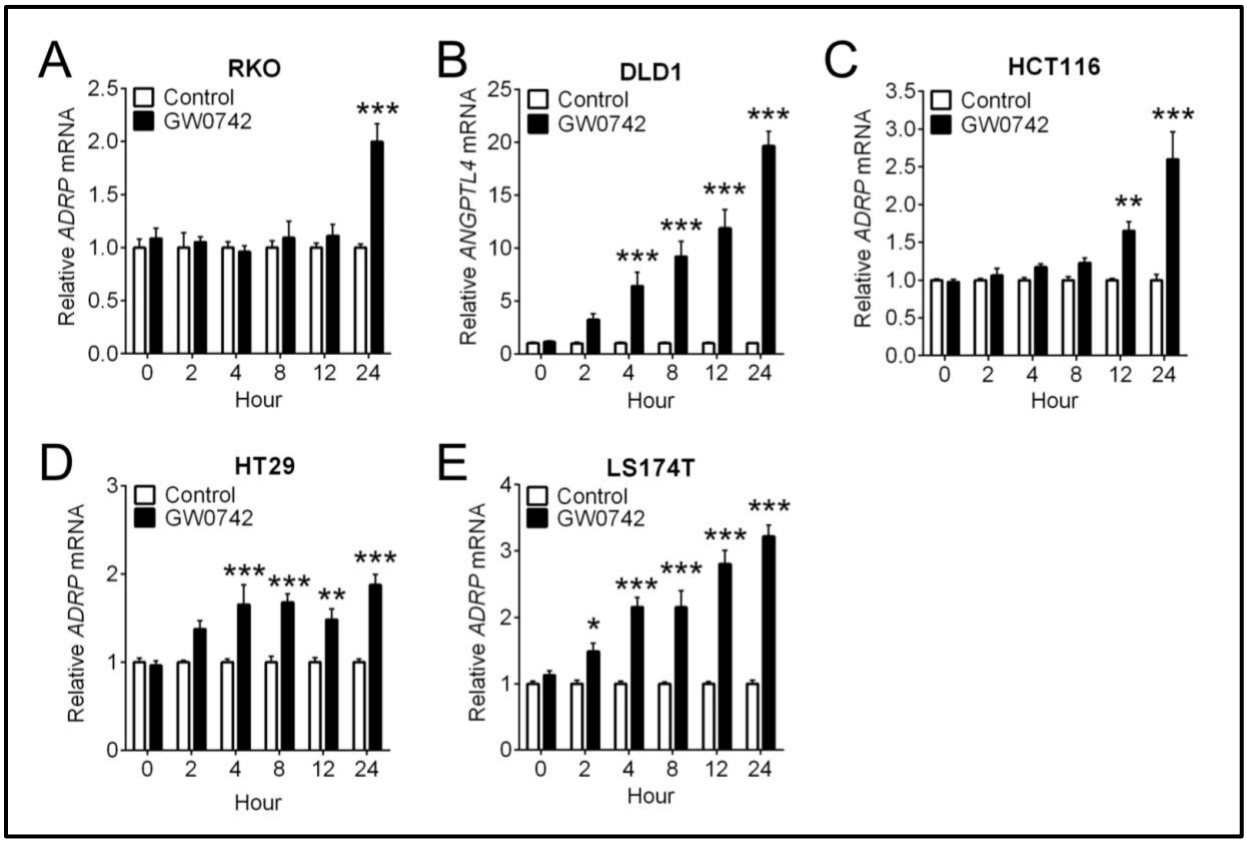


Figure 7. Target gene activation of PPARβ/δ in response to ligand activation takes a longer time in an *APC*/*CTNNB1* wild-type cell line compared to *APC*/*CTNNB1* mutant cell lines.
RKO (A), DLD1 (B), HCT116 (C), HT29 (D), and LS174T (E) cells were treated with vehicle control (0.01% DMSO) or 1 μM GW0742 for up to 24 hours. mRNA expression of PPARβ/δ target genes ADRP or ANGPTL4 was analyzed using qPCR and normalized to GAPDH. Fold induction compared to the control group of each time point is shown. N=9 per group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the vehicle control-treated groups (using two-way ANOVA with Tukey's test).

2.4.4. Activation of β-CATENIN in an APC/CTNNB1 wild-type colon cancer cell line did not affect PPARβ/δ activity

To understand whether a direct regulation exists between β-CATENIN and PPARβ/δ functionality, a β-CATENIN expression vector was obtained, and from which a constitutively active mutant β-CATENIN expression vector was generated via site-directed mutagenesis. The use of constitutively active β-CATENIN avoids its degradation by the wild-type APC protein complex. The specific mutation used to maintain β-CATENIN was serine 45 deletion (S45del or

S45Δ). S45 is one of several critical phosphorylation sites that regulates β-CATENIN degradation (Liu et al., 2002). Mutations at this position were also found in two of the *APC* wild-type cell lines used in this study (HCT116 and LS174T), suggesting that this mutation alone may be sufficient for constitutive activation of β-CATENIN. The RKO cell line, which carries wild-type *APC* and *CTNNB1*, was transiently overexpressed with β-CATENIN$^{S45\Delta}$, and was compared against wild-type β-CATENIN-, empty vector-, or reagent-only- transfected control. The effect of β-CATENIN activity on ligand-induced target gene expression of PPARβ/δ, as well as CYCLIN D1 expression as a positive control, were examined. Twenty-four hours after the transfection, increase of β-CATENIN expression, as well as its target gene product CYCLIN D1, were seen (Fig. 8 A). Therefore, at this time point, 1 μM GW0742 or vehicle control was added to the transfected cells, and the mRNA expression of *ADRP* was analyzed for the next 24 hours. Results showed that, in both control and β-CATENIN-activated RKO cells, an increase of *ADRP* mRNA occurred at 12 to 24 hours after ligand activation, and the levels of increase were similar between these cells (Fig. 8 B). These observations indicate that β-CATENIN does not directly alter PPARβ/δ functionality in colon cancer cells.

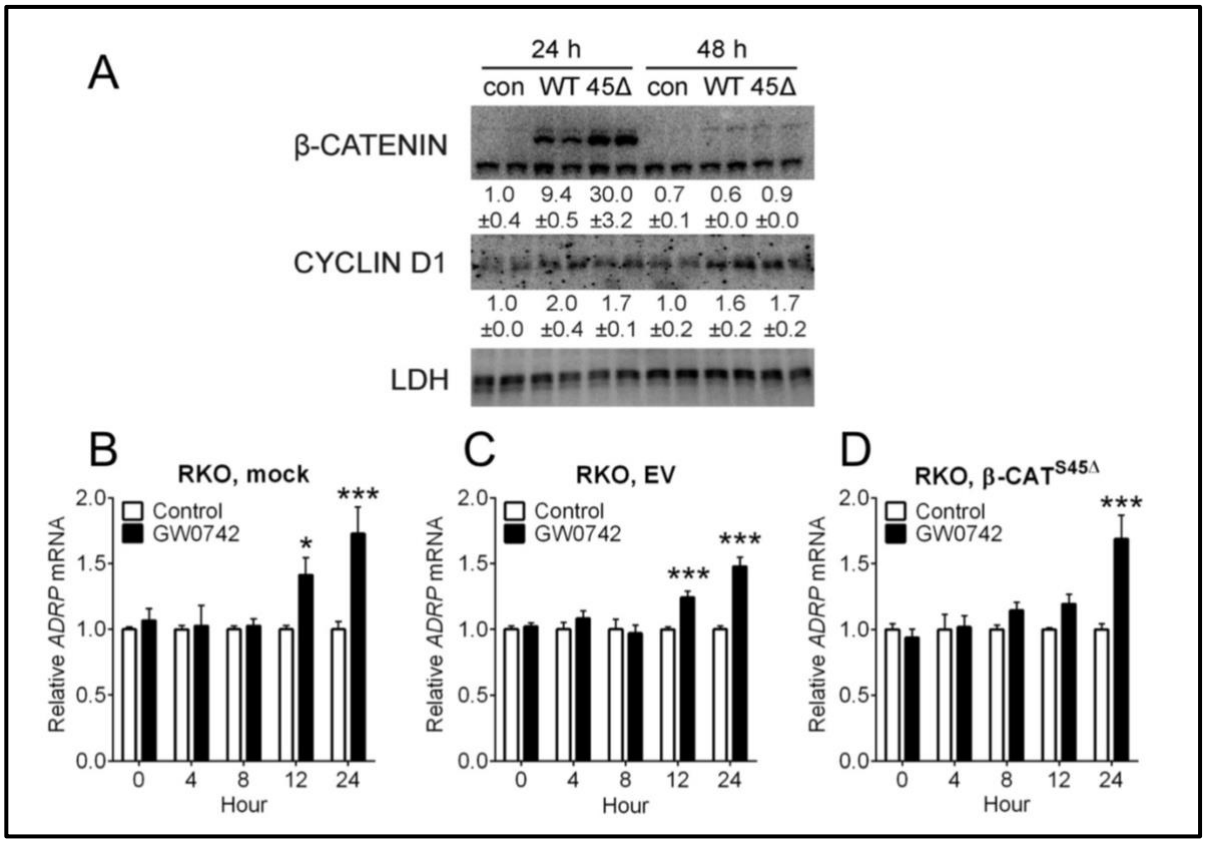


Figure 8. β-CATENIN activation does not alter PPARβ/δ activity in the RKO cell line.
A. RKO cells were transiently transfected with no DNA (mock), empty vector (EV), wild-type β-CATENIN (WT), or an active mutant of β-CATENIN (45Δ). Either 24 or 48 hours after the transfection, expression of β-CATENIN and its target gene product CYCLIN D1 was analyzed using western blot. Values represent mean, and the pooled overall SEM is 0.12. B-D. Twenty-four hours after RKO cells were transfected with mock (B), EV (C), or β-CATENIN-S45Δ (β-CAT$^{S45\Delta}$) (D), vehicle control or 1 μM GW0742 was added to cell culture media for an additional 0 to 24 hours. The relative expressions of *ADRP* mRNA, analyzed with qPCR using *GAPDH* housekeeping control, are displayed as values normalized to the control-treated groups. N=2 for panel A and N=9 for panel B-D. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the control-treated groups (using two-way ANOVA and Tukey's test).

2.4.5. Ligand activation and selective repression of PPARβ/δ inhibit colon cancer cell line growth

To understand whether PPARβ/δ potentiates colon cancer cell growth, growth of colon cancer cell lines, with or without ligand activation or selective repression of PPARβ/δ, was examined using the xCELLigence real-time cell analysis system. The difference between ligand activation and selective repression of PPARβ/δ lies in that, ligand activation induces both activation and repression of its target genes, while selective repression only induces repression of

its target genes (Khozoie et al., 2012; Naruhn et al., 2011). GW0742, a subtype-specific synthetic agonist for PPARβ/δ, was used for its ligand activation. DG172 and PTS264, two synthetic ligands known to induce target gene repression but not activation by PPARβ/δ, were used to selectively repress PPARβ/δ. The treatments were only present in the cell culture since day 1. For ligand activation of PPARβ/δ, growth of RKO and HCT116 cells was not affected by GW0742 at 5 μM concentration (Fig. 9 A and C). However, growth of DLD1 and HT29 cells was inhibited by GW0742 (Fig. 9 B and D). On the other hand, selective repression of PPARβ/δ with 0.5 μM PTS264 inhibits growth of DLD1, HCT116, and HT29 cells (Fig. 10 D-F). DG172 at 5 μM also inhibited growth of DLD1 cells (Fig. 10 B). Growth of RKO was again unaffected by either of these ligands (Fig. 10 A and C). In addition, PPARβ/δ knockout RKO and DLD1 cell lines were tested as a control, and were not sensitive to either compound, suggesting that their growth-inhibitory effects are PPARβ/δ-dependent (data not shown). Interestingly, these data suggest that growth inhibition by ligand activation or selective repression of PPARβ/δ is effective only in the *APC/CTNNB1* mutant cell lines that have more efficient ligand-induced PPARβ/δ target activation (Fig. 7). Together, these results suggest that although PPARβ/δ activity is higher in the *APC/CTNNB1* mutant cell lines compared to the *APC/CTNNB1* wild-type cell line, activation of PPARβ/δ in fact slows down colon cancer cell growth.

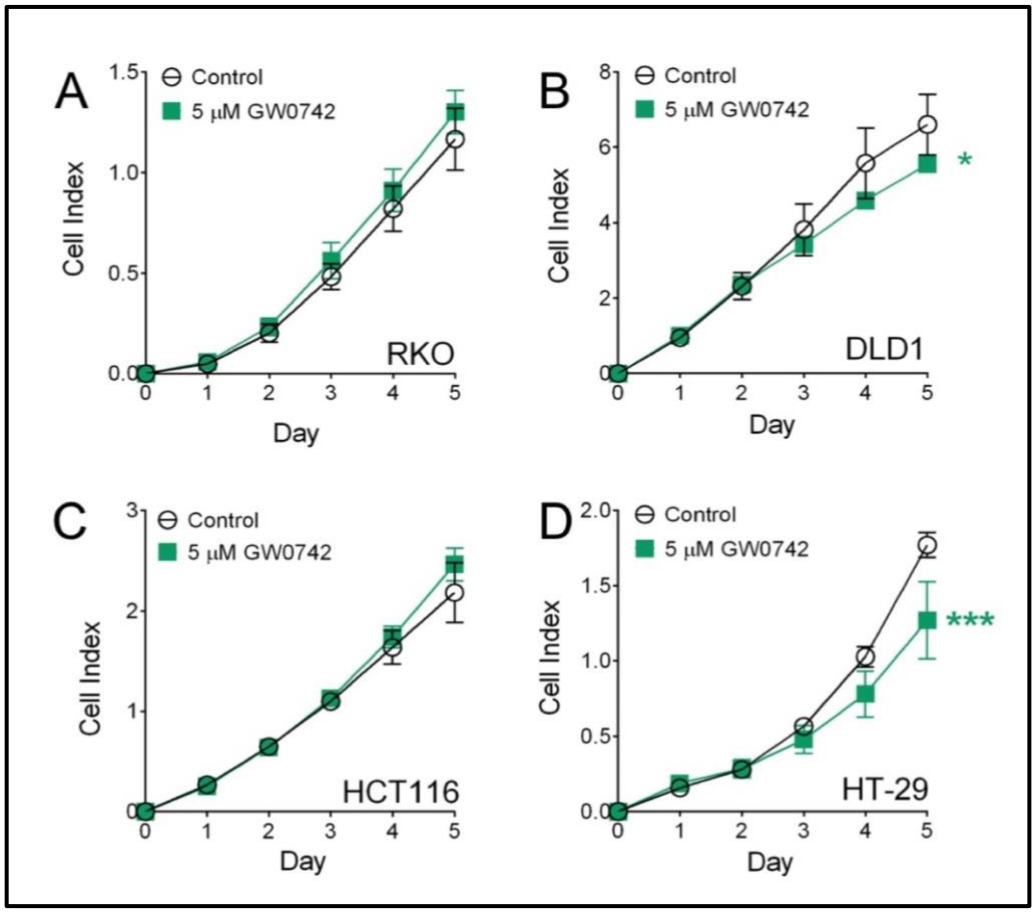


Figure 9. Activation of PPARβ/δ with an agonist inhibits growth of colon cancer cell lines.
Growth of RKO (A), DLD1 (B), HCT116 (C), and HT29 (D) cells with the indicated treatments was monitored using the xCELLigence system. Cells were plated on day 0 and the indicated treatments were started from day 1. Error bar represents SEM. N=3 per group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the vehicle control-treated groups (using two-way ANOVA and Tukey's test).

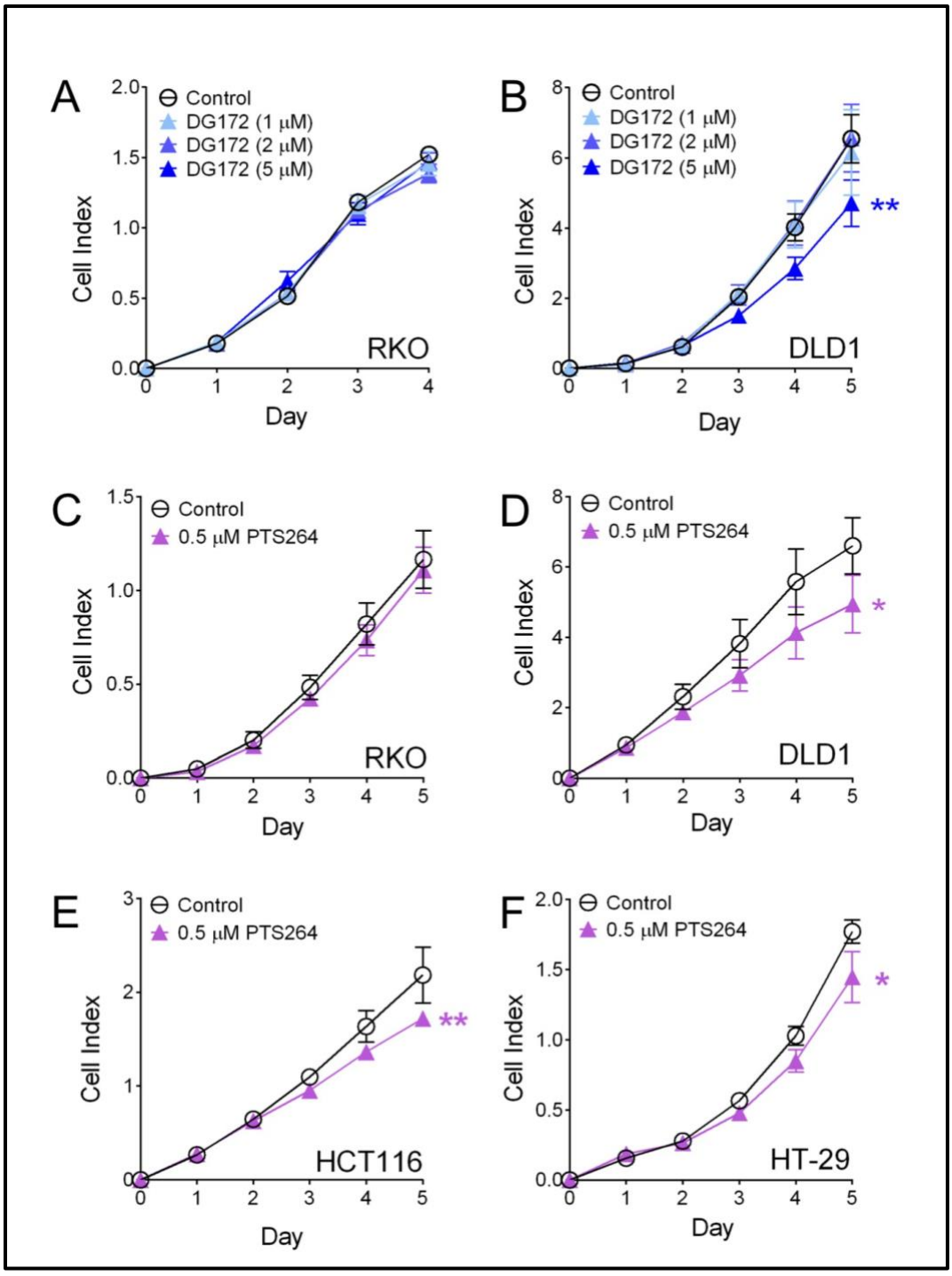


Figure 10. Selective repression of PPARβ/δ inhibits growth of colon cancer cell lines.
Effect of two selective repressive PPARβ/δ ligands, DG172 (A-B) and PTS264 (C-F), on growth of colon cancer cell lines was examined using the xCELLigence system. Cells were plated on day 0 and the indicated treatments began on day 1. Error bar represents SEM. N=3 per group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the control-treated groups (using two-way ANOVA and Tukey's test).

2.5. Discussion

Consistent with previous studies showing that protein expression of PPARβ/δ was not affected by activation of the β-CATENIN pathway in human colon cancer cells and tumor samples (Foreman et al., 2009), results from the current study showed that functionality of PPARβ/δ is also not directly regulated by the APC/β-CATENIN pathway. The first evidence is that, although β-CATENIN and its target protein were present at higher level in cell lines with mutant *APC/CTNNB1* compared to a cell line with wild-type *APC/CTNNB1*, cytosol retention and nuclear retention of PPARβ/δ were not different between these two types of cell lines, with or without an exogenous ligand. Results from the western blot analysis suggest that PPARβ/δ is mainly retained in the cytosol, while ligand activation does not further change nuclear retention of PPARβ/δ in any of these cell lines. The lack of nuclear translocation following ligand activation was an unexpected result, since it had been shown previously that a ligand induces nuclear translocation of nuclear receptors including other isoforms of PPAR, and this change can usually be detected within an hour (Nishi et al., 2004; Ochiai et al., 2004; Tan et al., 2010; Trebble et al., 2013). The majority of these studies visualized nuclear receptors using immunostaining or fluorescent-tagged receptor protein. Compared to these techniques, it remains possible that western blot may not be sensitive enough to detect changes in nuclear retention. Therefore, alternative approaches, such as designing a fluorescently-tagged and functional PPARβ/δ fusion protein, should be taken in the future, to examine more precisely whether ligand-induced nuclear translocation of PPARβ/δ takes place in colon cancer cell lines, and whether differences exist for this process between cells with normal or mutant APC/β-CATENIN pathway. Additionally, our finding that PPARβ/δ is mainly detected in the cytosolic fraction of colon cancer cells differs from a previously published western-blot result, in which PPARβ/δ

was detected at higher levels in the nucleus fraction compared to the cytosolic fraction in several mouse tissues, including colon and small intestine (Girroir et al., 2008). While this difference may be due to the biological differences between *in vitro* cell culture and *in vivo* mouse tissue, it is still worth exploring whether PPARβ/δ localization is different between normal and cancerous colon epithelial cells.

The second piece of evidence suggesting that PPARβ/δ is not functionally regulated by β-CATENIN is that expression of active β-CATENIN in the RKO cell line, which has wild-type APC and β-CATENIN, did not change the efficacy of ligand-induced target gene expression of PPARβ/δ. On the other hand, CYCLIN D1 expression was up-regulated by the overexpression of β-CATENIN$^{S45\Delta}$, proving the functionality of exogenous β-CATENIN$^{S45\Delta}$ protein. Interestingly, however, faster target gene activation of PPARβ/δ following ligand activation was observed in the *APC/CTNNB1* mutant cell lines compared to RKO. These two results together suggest that there may be an indirect relationship between activation of the APC/β-CATENIN pathway and PPARβ/δ activity. One related example is that increased efficacy of ligand-induced target gene activation occurs when PPARβ/δ is overexpressed (Borland et al., 2011, 2017; Foreman et al., 2011; Yao et al., 2014, 2015a, 2017). This can be explained as follows: When a higher concentration of PPARβ/δ is present in a cell, more receptor-ligand complexes can be formed in the same amount of time, leading to a higher level of target gene activation. However, data from previous studies and the current study show that there is no difference in total PPARβ/δ expression between the cell lines used in this study. Therefore, the difference in ligand response can be explained by: 1) a higher portion of PPARβ/δ proteins in the *APC/CTNNB1* mutant cells being in an ready-to-be-activated form (e.g., locational proximity with RXRα, co-activators, or target gene); 2) presence of an inhibitory mechanism on PPARβ/δ activity in the *APC/CTNNB1* wild-type colon cancer cell line; or 3) a lower portion of PPARβ/δ proteins are ligand-occupied

in these cells, therefore providing a greater potential to be activated by an exogenous ligand. Additionally, since mRNA expression is determined by both rate of transcription and rate of mRNA degradation, mRNA stability may also have contributed to the differences between these cell lines. In addition to the genotype of *APC* and *CTNNB1* genes, other genetic traits that are different among these cell lines, including mutations to the *KRAS, BRAF, PIK3CA, TP53, CDKN2A,* and *SMAD4* genes, should also be taken into consideration (see Appendix). Therefore, the underlying mechanism behind this observation remains to be developed in the future.

Since described as a target gene of β-CATENIN, PPARβ/δ has been believed by many to function as an oncoprotein in colon cancer. Contradictory roles of PPARβ/δ in cancer cell proliferation and apoptosis have been found (reviewed in Peters and Gonzalez, 2009; Peters et al., 2011a). In the current study, inhibition of growth by ligand activation of PPARβ/δ was observed in two *APC*-mutant cell lines, DLD1 and HT29, while no effect on growth was observed for the HCT116 cell line, which carries heterozygous *CTNNB1* mutation, or the RKO cell line, which has wild-type *APC* and *CTNNB1*. Interestingly, the cell lines whose growth was inhibited by PPARβ/δ ligands also exhibited faster ligand-induced target gene activation of PPARβ/δ compared to those that were not affected. These results suggest that not only does PPARβ/δ not potentiate colon cancer growth, but ligand activation of PPARβ/δ may have chemotherapeutic potential in colon cancer, particularly in the cases with mutant *APC/CTNNB1* genes. It should be mentioned that a previous study (Hollingshead et al., 2007b) showed that 1 μM GW0742 inhibited proliferation of HCT116 cell line within three days, which was not observed in the current study. This may be a result of a technical difference, i.e., that cell numbers were counted using a particle counter in the previous study, but measured using a real-time cell analysis instrument in the current study.

Another finding of this study is that selective repression of PPARβ/δ inhibits colon cancer cell growth. This is the first time that the effect of selective repression of PPARβ/δ on growth of colon cancer cell lines has been reported. Selective repressive ligands reinforce target gene repression of PPARβ/δ by facilitating its interaction with co-repressors, thereby causing different gene regulation compared to a traditional agonist (Lieber et al., 2012; Naruhn et al., 2011). However, similar to PPARβ/δ agonist GW0742, selective repressive ligands DG172 and PTS264 also inhibited growth in DLD1 and HT29 cell lines, but not in HCT116 and RKO cell lines. While the underlying mechanism of agonist and selective repressive ligand can be different, this result indicates again that *APC/CTNNB1* mutant colon cancer cells are more sensitive to selective repression of PPARβ/δ. Compared to a limited number of studies that examined the effect of selective repressive PPARβ/δ ligands in other models, our results are consistent with another study in which inhibition of growth by DG172 was observed in a breast cancer cell line (Wang et al., 2016). We also observed from a preliminary xenograft study that DG172 delayed the incidence of DLD1 and RKO xenograft tumor formation (data not shown). Our data suggest that selective repressive PPARβ/δ ligands may have promising chemotherapeutic effects on colon cancer. However, their effect on growth of normal colon epithelial cells and *in vivo* tumor growth should be carefully examined in the future.

In contrast to our observations, a previous study reported that knockout of PPARβ/δ in the HCT116 cell line reduced its proliferation, suggesting that PPARβ/δ facilitates cancer cell growth (Park et al., 2001). One should keep in mind that activation of PPARβ/δ with a ligand does not create the same effect as increasing its expression, or the opposite effect of knocking down/out this receptor. In fact, studies have revealed that expression of some PPARβ/δ target genes are only changed by genetic manipulation or ligand activation of the receptor, but not by both; and a number of PPARβ/δ target genes are regulated in the opposite way by PPARβ/δ, with

or without an exogenous ligand (Adhikary et al., 2011; Khozoie et al., 2012). This is possibly due to the existence of endogenous agonists or selective repressive ligands. Therefore, results from over-expression, knockdown/knockout, or ligand activation of PPARβ/δ should be distinguished from each other.

In contrast to the studies that suggested that expression or functionality of PPARβ/δ is up-regulated by the APC/β-CATENIN pathway in colon cancer cells to facilitate their growth, results from the current study failed to prove PPARβ/δ as a direct functional target of the APC/β-CATENIN pathway. Although this study observed a higher ligand inducibility of PPARβ/δ in cell lines with mutant *APC/CTNNB1*, modulation of this receptor with an agonist or selective repressive ligand actually inhibited growth of these cells. Results from this study are in line with the others who observed that PPARβ/δ inhibits colon tumorigenesis, inhibits colon cancer cell growth, and reduces colon cancer cell survivability. The reason for higher PPARβ/δ inducibility in *APC/CTNNB1* mutant cell lines, and the mechanism of growth inhibition by PPARβ/δ, remain to be investigated.

Chapter 3. Investigating of the role of PPARβ/δ in colon carcinogenesis, epithelial-to-mesenchymal transition, and invasion

3.1. Abstract

There are limited and inconsistent reports of the role of PPARβ/δ in colon cancer growth, invasion, epithelial-to-mesenchymal transition (EMT), and metastasis. Recent publications have suggested that selective repression of PPARβ/δ inhibits cancer cell migration and invasion. In this study, the hypothesis that selective repression or ligand activation of PPARβ/δ inhibits anchorage-independent growth, migration, invasion, and EMT of human colon cancer cell lines was examined. Anchorage-independent colony formation and growth, migration, EMT marker expression, and MMP activity of colon cancer cell lines were evaluated in the absence or presence of the PPARβ/δ agonist GW0742 or selective repressive ligand DG172. We observed that selective repression of PPARβ/δ inhibits anchorage-independent clonogenicity and spheroid growth of colon cancer cell lines by inducing apoptosis. Selective repression of PPARβ/δ also reduced EMT marker expression. No change in these phenotypes was observed with ligand activation of PPARβ/δ. On the other hand, ligand activation of PPARβ/δ inhibited colon cancer cell migration at a relatively lower concentration, while selective repression of PPARβ/δ did not. Concordant with this observation, reduced TNFα/TGFβ-induced MMP activity in cell culture media with ligand activation of PPARβ/δ was observed. Combined, these results suggest that, while selective repression of PPARβ/δ can efficiently reduce anchorage-independent survival and growth of colon cancer cell lines, ligand activation of PPARβ/δ has more potential in controlling tumor cell malignancy.

3.2. Introduction

This study explores the role of PPARβ/δ in invasion-related features using colon cancer cell lines, including anchorage-independent growth, migration, invasion, and epithelial-to-mesenchymal transition (EMT). Sustained growth, suspension of apoptosis, tissue invasion, and metastasis are important hallmarks of cancer (Hanahan and Weinberg, 2000, 2011). Metastasis is often the direct cause of death by a solid tumor, and the means through which cancer becomes hard to eliminate or even control. Several features of cancer cells are related to metastasis potential. First, anchorage-independent growth—the ability of cells to survive and grow without attaching to an extracellular matrix—has been shown to positively correlate with metastasis potential (Mori et al., 2009). Second, migration and invasion, referring to the ability of cells to move freely or through the extracellular matrix, are also related to metastasis. During invasion, cancer cells secrete matrix metalloproteinases (MMPs) into the environment to help digest the extracellular matrix and move through. Another important feature in cancer malignancy in adenocarcinomas is EMT, during which a cell alters expression of junction proteins by switching from E-CADHERIN (E-CAD) to N-CADHERIN (N-CAD) to reduce cell adherence, and at the same time alters structural proteins such as vimentin (VIM) and fibronectin (FN) to gain migration and invasion capacity (reviewed in Brabletz et al., 2018; Lamouille et al., 2014).

The role of PPARβ/δ in cancer migration, invasion, and metastasis potential of colon cancer cells and other types of cancer cells has not been elucidated to this day. Two studies published from one group found that PPARβ/δ promotes colon cancer metastasis in mouse models. In the first study, a PPARβ/δ knockdown HCT116 colon cancer cell line, as well as PPARβ/δ knockdown melanoma, lung, pancreatic, and mammary gland cancer cell lines, were generated. These cells were observed to produce fewer lung and/or liver metastasis sites

compared to control cells after being injected into immunocompromised mice, while ligand activation of PPARβ/δ resulted in more metastasis (Zuo et al., 2017). It should be noted that this study used solely tail-vein injection to study metastasis, which omitted the process of invasion from a primary location. The same study also found a negative relationship between PPARβ/δ expression and metastasis-free survival in 66 colorectal patients, 486 breast cancer patients, and 240 liposarcoma patients. The second study observed that targeted overexpression of PPARβ/δ increased local invasion of intestinal tumors formed in an *APC* mutant mouse model, and shortened their overall survival compared to control mice (Liu et al., 2019).

In contrast, other studies did not support the above findings. One study of 141 primary rectal cancer patients observed that patients who express higher levels of PPARβ/δ were less likely to develop metastasis, and had a significantly higher rate of survival, compared to those who expressed lower levels of PPARβ/δ (Yang et al., 2011). Another study of 32 colorectal cancer patients observed that, although higher PPARβ/δ expression coincided with malignant phenotypes, there was no correlation between PPARβ/δ expression and cancer stage or metastasis (Takayama et al., 2006). Data from The Human Protein Atlas database also suggest no correlation between PPARβ/δ expression and colorectal cancer prognosis (Uhlen et al., 2017).

Despite the works described above, little research has been done *in vitro* to study the relationship between PPARβ/δ and colon cancer migration and invasion. Ligand activation or selective repression of PPARβ/δ have also been demonstrated to reduce migration, invasion, MMP activity, or stem cell marker expression in breast cancer, testicular cancer, and neuroblastoma cell lines (Adhikary et al., 2013; Yao et al., 2015a, 2017). In contrast, a number of studies done using keratinocytes suggested that PPARβ/δ promotes wound healing or migration (Di-Poï et al., 2003; Ham et al., 2010; Michalik et al., 2001). It is uncertain whether the same roles for PPARβ/δ can be observed in colon cancer cell lines.

We evaluated in preliminary studies the role of PPARβ/δ in the malignant potential of human colon cancer cell lines. In the current study, we hypothesized that ligand activation or selective repression of PPARβ/δ would inhibit the malignancy-related properties of colon cancer cells, including anchorage-independent colony formation, spheroid growth, migration, MMP activity, and EMT marker expression.

3.3. Materials and methods

Cell culture and spheroid culture

RKO, DLD1, HCT116, and HT29 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cell lines were maintained in Eagle's Minimum Essential Medium (EMEM) (Sigma) (RKO), RPMI 1640 medium (Gibco) (DLD1), or McCoy's 5A medium (Sigma) (HCT116 and HT29), supplemented with 5% fetal bovine serum (FBS) (Atlanta) and 1% Penicillin-streptomycin (Gibco). Spheroid were generated using 96- (for observation) or 384- (for protein/RNA collection) well Ultra-Low Attachment Spheroid Microplates (Corning).

Protein collection and western blot

Total soluble protein was collected by lysing cells with RIPA buffer (50 mM Tris-Cl, pH 8.6, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, and 1% NP-40) containing fresh protease inhibitors (Roche), and centrifugation at 15000 x g for 15 minutes to remove the insoluble parts. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo Scientific). Western blot were done as previously described (Hollingshead et al., 2007b). Briefly, 15 to 30 μg of each protein sample was resolved using SDS-PAGE (10%), and

transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were then blocked with 5% milk at room temperature for 1 hour, incubated with primary antibodies for PARP (Cell Signaling #9542), E-CADHERIN (Cell Signaling #5296), VIMENTIN (Abcam #8978), or LDH (Rockland #200-1173) at 4 ℃ overnight, followed by biotin-conjugated secondary antibodies (Jackson ImmunoResearch) at room temperature for 1 hour, and finally Streptavidin-I^{125} (kindly provided by Dr. Gary Perdew) at room temperature for 10 minutes. Three 10-minute washes were done between each of the incubation steps. Finally, the membranes were exposed to a storage phosphor screen, from which the proteins bands were detected and quantified using the Cyclone phosphorimager system (PerkinElmer).

Soft agar growth assay

This assay was performed as described previously (Yao et al., 2015a). DLD1 cells were pre-treated with desired concentration of DG172 for 24 hours. On the day of experiment, $1x10^4$ cells, suspended in 2 ml of 0.35% agarose were plated into each well of 6-well plates, pre-coated with 1 ml of 0.5% agar. Both top and bottom agar were prepared to contain 1x concentration of cell culture media and the desired treatment. After solidification of the top agar, 2 ml extra media with consistent treatment was added on top of each well to keep the agar moisturized. The plates were further cultured for two weeks, while the top media were refreshed twice per week. After two weeks, colony number was counted and averaged from six random fields per well (three wells per group), and colony size was quantified from photomicrographs using the ImageJ software (version 1.8).

Wound healing migration assay

RKO and DLD1 cells were plated into 6-well plates at 0.5 x 10^6 cells/well and pre-treated with indicated dose of PPARβ/δ ligands for 24 hours. On the day of experiment, the cells should reach 80-90% confluency. A cell-free zone was created through the middle of each of well using a 1 ml pipet tip, and cells that detached were rinsed away using PBS. The cells were cultured for additional 24 hours under the same condition. Pictures (6 per well) were taken under the microscope both at the beginning and the end of the 24-hours period. Wound area was measured from pictures using the wound healing assay tool of ImageJ, and migration area was calculated as the difference of wound size between 0 and 24 hours.

Gelatin zymography

Gelatin zymography was performed as previously described (Yao et al., 2015a). Briefly, cells were cultured with indicated concentrations of PPARβ/δ ligands. 24 hours later, TNFα and TGFβ (10 ng/ml each, R&D systems) were added and growth was continued for another 24 hours. Next, cells were conditioned for an additional 24 hours with serum-free media containing the same treatments. Conditioned media were collected, centrifuged to get rid of cell debris, and diluted using serum-free media to the same concentration based on cell count. Conditioned media were then resolved through SDS-PAGE (8%) containing 1 mg/ml porcine gelatin (sigma) at 4 ℃. The gels were then renatured in renaturing buffer (50 mM Tris-HCl, pH 7.5, 2.5 % Triton X-100) for 3 x 20 minutes, and incubated in developing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 100 mM $CaCl_2$, 0.02 % NaN_3) for 36 hours. Proteolytic bands were further detected by Coomassie blue staining. Then, the gels were imaged using a gel documentation system (Bio-Rad) and quantified using ImageJ (version 1.8).

Statistical analysis

Statistical analysis was performed using either one-way ANOVA or two-way ANOVA followed with Tukey's test as indicated in the figure legends. Significance was considered when $p \leq 0.05$. Statistical analysis was performed using the GraphPad Prism software (version 5.0).

3.4. Results

3.4.1. Selective repression of PPARβ/δ inhibits anchorage-independent colony formation of DLD1 cell line

The effect of selective repression of PPARβ/δ on anchorage-independent colony formation of DLD1 cells was assessed using soft agar colony formation assay. DG172, a selective repressive ligand for PPARβ/δ was used. The result shows that DG172 at 1 μM concentration decreased colony size, and at 2.5 μM concentration reduced the number of colonies significantly (Fig 11. A-C). Although all visible colonies were counted, the majority of colonies in the 1 μM and 2.5 μM groups were more irregularly shaped and darker compared to the control group, possibly indicating cell death. This result suggests that selective repression of PPARβ/δ using DG172 inhibits anchorage-independent survival and growth of DLD1 colon cancer cells, and therefore may have potential for reducing malignancy.

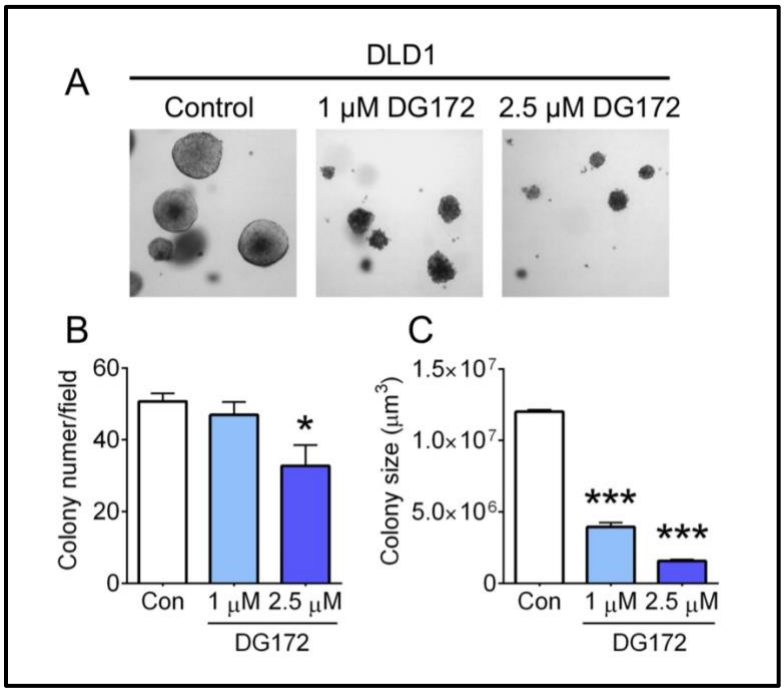


Figure 11. Preliminary study showing that selective repression of PPARβ/δ inhibits anchorage-independent growth of DLD1 cells.
DLD1 cells, pretreated with the indicated concentration of DG172 for 24 hours, were suspended in soft agar and cultured with the same treatments for an additional two weeks. Representative photomicrographs (A), average number of colonies per field under a 40x microscope (B), and average size of colonies (C) are displayed. N = 6. Error bar represents SEM. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to control-treated group (using one-way ANOVA and Tukey's test).

3.4.2. Selective repression of PPARβ/δ inhibits spheroid growth through induction of apoptosis

We then studied selective repression and ligand activation of PPARβ/δ in three-dimensional (3D) spheroid culture. In the case of DLD1, spheroids generated using the ultra-low attachment plates exhibited similar morphology to colonies formed in soft agar. Moreover, consistent with the soft agar assay, we observed that 1 μM DG172 decreased, while 2.5 μM DG172 completely suppressed, the growth of DLD1 spheroids (Fig. 12 A and B). A preliminary test using PPARβ/δ knockout DLD1 cell spheroids showed less response to DG172, suggesting that this effect was mediated through PPARβ/δ (data not shown). Note that in a 2D cell culture proliferation assay, DG172 affected DLD1 cell growth only at a relatively higher concentration

(5 μM) (Chapter 1, Fig. 10 B). This indicates that selective repression of PPARβ/δ inhibits anchorage-independent growth in a 3D tumor-like environment more effectively than anchorage-dependent proliferation in a simple monolayer culture. DG172 also inhibited HCT116 spheroid growth; however, a relatively higher concentration (i.e., 5 μM) was required to fully repress its growth (Fig. 13). On the other hand, spheroid size could not be quantified in the RKO cell line, since these cells form loosely attached, grape-shaped colonies instead of spheroids. However, RKO cells were able to grow anchorage independently with 2.5-5 μM DG172, suggesting that anchorage-independent growth of RKO is not as sensitive as DLD1 and HCT116 cell spheroids (data not shown). In contrast, ligand activation of PPARβ/δ with a high-affinity agonist GW0742 did not affect spheroid growth at 1-10 μM concentration (data not shown).

Furthermore, we observed that 2.5 μM DG172 increased poly (ADP-ribose) polymerase (PARP) cleavage in DLD1 spheroids within 48 hours (Fig. 12 C). This suggests that selective repression of PPARβ/δ inhibits anchorage-independent growth of colon cancer cells at least partially by promoting apoptosis.

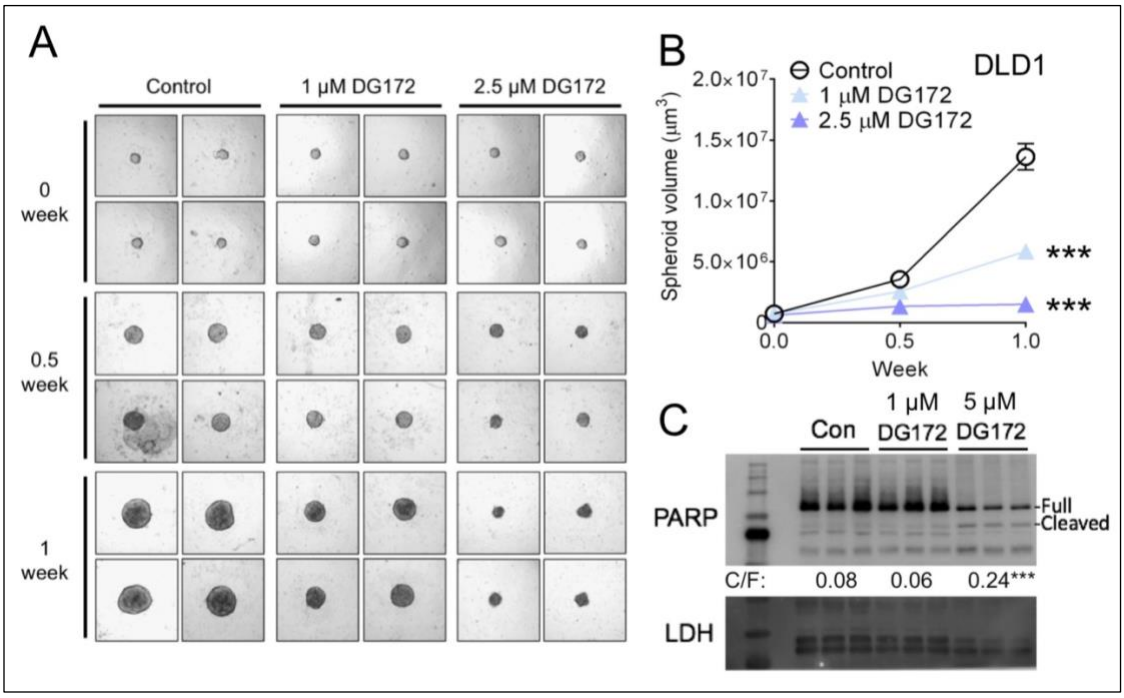


Figure 12. Selective repression of PPARβ/δ inhibits DLD1 spheroid growth by inducing apoptosis. DLD1 spheroids were generated by plating 100 cells/well in Corning ultra-low attachment plates. The indicated treatments were applied to growth media 24 hours after spheroid plating (at the 0-week time point). Growth of spheroids under these conditions was monitored for one week. A. Representative photomicrographs of the spheroids. B. Size of spheroids quantified from the photomicrographs using ImageJ software (N=12). Error bar represents SEM. Statistical analysis: $p \leq 0.05$ between groups analyzed using two-way ANOVA and Tukey's test. C. Preliminary western blot analysis of PARP in DLD1 spheroids treated as indicated for 48 hours (N=3). Quantification indicates the average of cleaved/full sized PARP ratio. The pooled SEM is 0.01. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the control-treated groups, using two-way ANOVA (spheroid size) or one-way ANOVA (western blot) followed by Tukey's test.

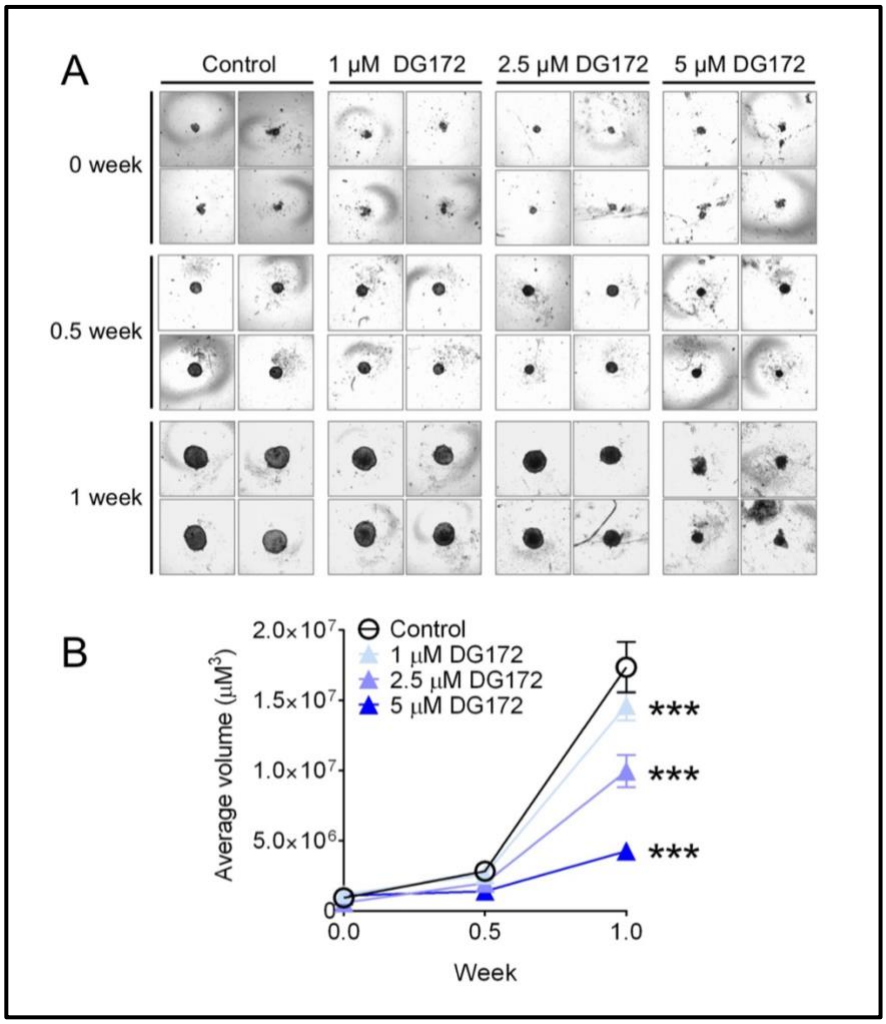


Figure 13. Selective repression of PPARβ/δ inhibits HCT116 spheroid growth.
HCT116 spheroids were generated by plating 100 cells/well in Corning ultra-low attachment plates, and grew under the indicated conditions for one week. A. Representative photomicrographs of the spheroids. B. Size of spheroids quantified from the photomicrographs using ImageJ software. N=12 per group. Error bar represents SEM. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001, compared to the control-treated group (using two-way ANOVA and Tukey's test).

3.4.3. Ligand activation of PPARβ/δ inhibits colon cancer cell line migration

We next examined the effect of GW0742 and DG172 on the migration ability of RKO and DLD1 cell lines using wound healing assay. Results described here are preliminary. For both cell lines, DG172 inhibited migration, but only at relatively higher concentrations (Fig. 14). Since more dead cells were observed with 10 μM DG172, the reduction of migration seen at this concentration could be possibly affected by cell death. On the other hand, GW0742 inhibited

migration of both cell lines by about 20% at 1 μM concentration, and this concentration was found to have no effect on cell growth (Fig. 15). Preliminary results of transwell migration and invasion assays (in which the transwell inserts were coated with Matrigel) generated similar results: Ligand activation of PPARβ/δ reduced migration and invasion of colon cancer cell lines, while selective repression of PPARβ/δ did not affect these properties, at relatively lower concentrations (data not shown).

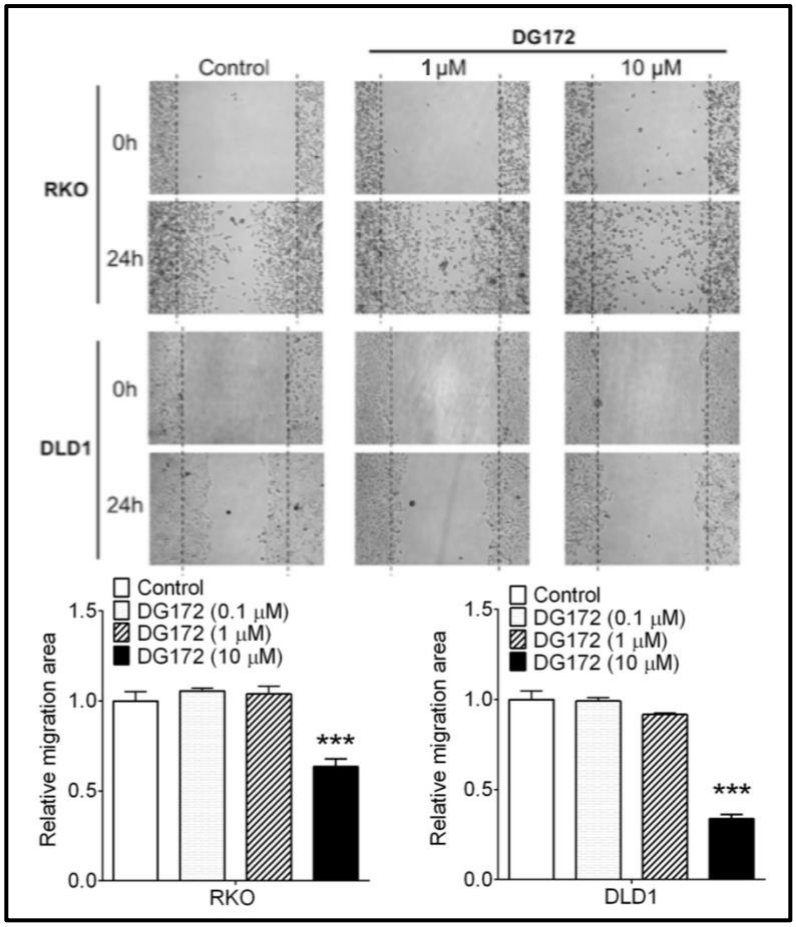


Figure 14. Preliminary results suggesting that selective repression of PPARβ/δ inhibits migration of colon cancer cell lines.
Wound healing assay was performed with RKO and DLD1 cell lines, pre-treated for 24 hours with indicated concentrations of DG172. Relative migration area was calculated as the average difference of wound size between 0h and 24h, using six random pictures per well, and three wells per group. Wound sizes were quantified from these photos using wound healing assay tool of the ImageJ software. Final values were normalized to the control group. N=3 per group. Error bar represents SEM.
*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the control-treated groups (using one-way ANOVA and Tukey's test).

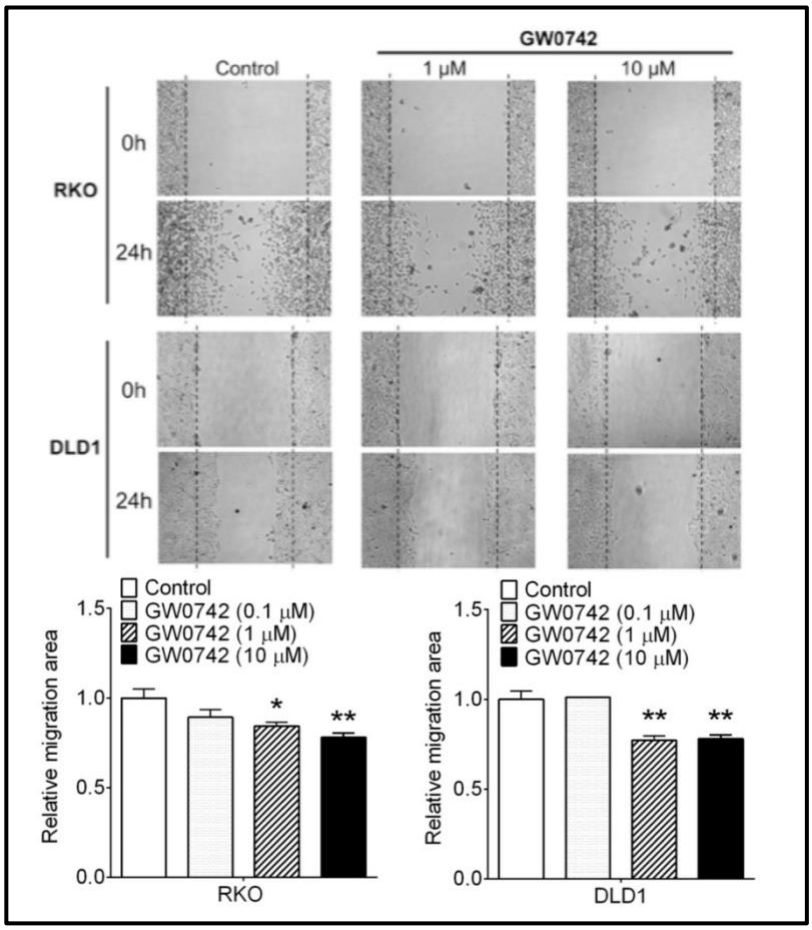


Figure 15. Preliminary results suggesting that ligand activation of PPARβ/δ inhibits migration of colon cancer cell lines.
Wound healing assay was performed with RKO and DLD1 cell lines, pre-treated for 24 hours with indicated concentrations of GW0742. Relative migration area was calculated as the average difference of wound size between 0h and 24h, using six random pictures per well, and three wells per group. Wound sizes were quantified from these photos using wound healing assay tool of the ImageJ software. Final values were normalized to the vehicle control group. N=3 per group. Error bar represents SEM. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the control groups (using one-way ANOVA and Tukey's test).

3.4.4. Selective repression of PPARβ/δ reduces EMT marker expression in colon cancer cell lines

E-CADHERIN and VIMENTIN expression were then used to analyze the effect of ligand activation and selective repression of PPARβ/δ on colon cancer EMT. E-CADHERIN, a tight

junction protein, is normally expressed in epithelial cells and is down-regulated during EMT. VIMENTIN, an intermediate filament protein, is usually expressed in mesenchymal cells and up-regulated during EMT. A comparison of their expression in colon cancer cell lines reveals that the level of EMT ranks from highest to lowest in RKO, HCT116, HT29, and DLD1 cells, respectively (Fig. 16 A).

The expression of E-CADHERIN and VIMENTIN in RKO and HCT116 cells was then examined following treatment with either GW0742 or DG172. From preliminary results, we observed down-regulation of VIMENTIN in RKO cells and up-regulation of E-CADHERIN in HCT116 cells, one day after treating cells with 1 to 5 μM DG172. Additionally, expression of E-CAD in RKO was not detectable with or without a PPARβ/δ ligand, while VIMENTIN expression in HCT116 was not changed by this treatment (data not shown). In contrast, no change in E-CADHERIN and VIMENTIN expression in RKO or HCT116 was observed with GW0742 (data not shown). These results indicate that selective repression of PPARβ/δ inhibits EMT in colon cancer cell lines, while ligand activation of PPARβ/δ does not affect EMT.

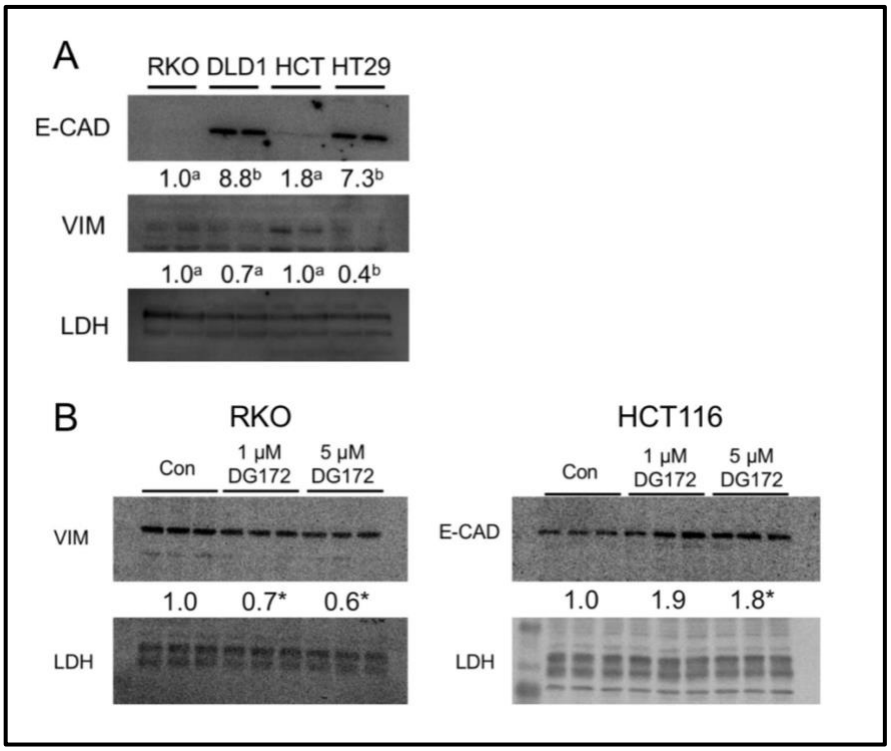


Figure 16. Preliminary results suggesting that selective repression of PPARβ/δ inhibits EMT marker expression in RKO and HCT116 cells.
Expression of E-CADHERIN (E-CAD) and VIMENTIN (VIM) from whole cell lysates collected from non-treated colon cancer cell lines (A), or RKO (B) and HCT116 (C) cells cultured with indicated treatments of DG172 for 24 hours were analyzed using western blot. N=3 per group. Quantification represents mean, and the pooled SEM is 0.12. Statistical analysis: significantly different between groups ($p \leq 0.05$ using) (panel A), or: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared to the control groups (using one-way ANOVA and Tukey's test) (Panel B).

3.4.5. Ligand activation of PPARβ/δ reduces TNFα/TGFβ-induced MMP activity in HCT116 cell line

To understand if activating PPARβ/δ affects MMP activity, conditioned media from a monolayer cell culture, pretreated with GW0742 or DG172, was assessed using gelatin zymography. Since all of the cell lines tested do not express high level of MMPs, TNFα and TGFβ, which are known inducers of invasion and MMP activity, were used at the same time to induce MMP expression. RKO, DLD1, and HCT116 cell lines were tested, but only HCT116 cells expressed a detectable amount of MMP9 after induced with TNFα and TGFβ; therefore, only the results from this cell line are shown here. We observed that GW0742, at 5 μM

concentration, reduced TNFα/TGFβ-induced MMP9 activity in the conditioned media, while DG172 did not alter TNFα/TGFβ-induced MMP9 activity at up to 2.5 μM. These results suggest that ligand activation of PPARβ/δ inhibits TNFα- and TGFβ-induced MMP9 expression or secretion, therefore potentially inhibiting the invasion capacity of this cell line.

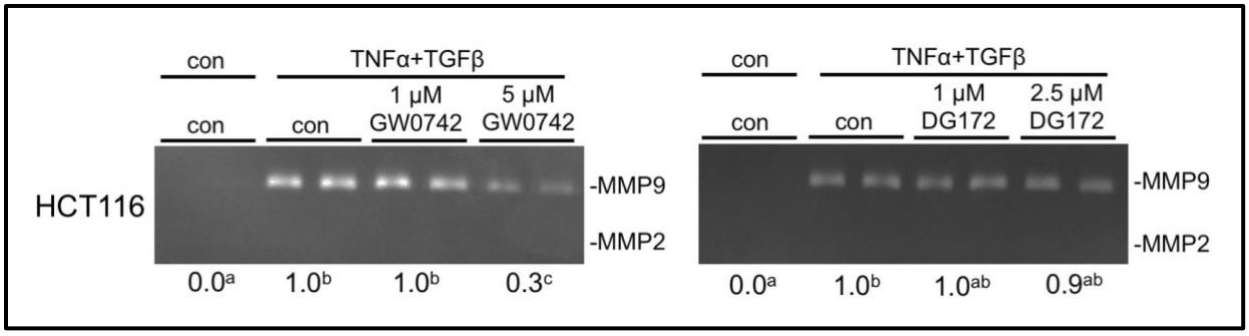


Figure 17. Ligand activation, but not selective repression of PPARβ/δ reduces MMP activity in HCT116 cell culture.
HCT116 cells, pre-treated with indicated concentrations of GW0742 or DG172 for 24 hours followed by additional TNFα and TGFβ (10 ng/ml each) for 24 hours, were conditioned for 24 hours. Conditioned media, normalized using cell count, were then analyzed using gelatin zymography. Pictures of stained gels were taken using a gel imager, and the intensity of zymolytic bands was quantified using ImageJ. N=2 per group (preliminary). Pooled SEM was 0.1. Statistical analysis: $p \leq 0.05$ between groups (one-way ANOVA and Tukey's test).

3.5. Discussion

Studies of the relationship between PPARβ/δ and colon cancer malignancy in cell culture, mouse models, and patients have led to contradictory findings. While some suggest that higher expression or ligand activation of PPARβ/δ facilitates colon cancer invasion and metastasis, others found that PPARβ/δ expression is not correlated or is negatively associated with metastasis. Evidence also suggests that selective repression of PPARβ/δ inhibits cancer cell invasion. The current study was conducted based on the hypothesis that selective repression or

ligand activation of PPARβ/δ inhibits the malignancy potential of colon cancer cell lines, which include anchorage-independent growth, migration, MMP activity, and EMT.

The first important finding of this study is that selective repression of PPARβ/δ with DG172 inhibits anchorage-independent colony formation and growth in spheroid-forming colon cancer cell lines—DLD1 and HCT116—through induction of apoptosis. Moreover, observed from DLD1 cells as an example, the efficacy of growth inhibition by selective repression of PPARβ/δ in spheroid models was more notable than in monolayer culture. On the other side, ligand activation of PPARβ/δ with GW0742 did not affect spheroid growth, although the same ligand was observed to inhibit growth of monolayer DLD1 cells (Chapter 2, fig. 9). These observations suggest that ligand activation and selective repression of PPARβ/δ inhibit monolayer growth through different mechanisms, and that the mechanism related with selective repression of PPARβ/δ may have an important role in anchorage-independent growth. Note that GW0742 have only been tested in the spheroid growth assay but not in the soft agar assay; thus, we are still uncertain whether ligand activation of PPARβ/δ affects anchorage-independent colony formation. Nevertheless, spheroids generated using ultra-low attachment plates and colonies formed in soft agar appeared to have similar morphology, indicating that these models share similar biological features and are both driven by anchorage-independent growth.

While no other published studies have examined the relationship between PPARβ/δ and anchorage-independent growth in colon cancer models; results from this study are congruent with findings from two other studies, that overexpression of PPARβ/δ inhibited anchorage-independent growth and invasive capacity in prostate or testicular cancer cell lines (Martín-Martín et al., 2018; Yao et al., 2015a). Although one of these studies also found that ligand activation of PPARβ/δ inhibits anchorage-independent growth in a testicular cancer cell line (Yao et al., 2015a). In contrast, it has also been reported that relatively higher expression or

ligand activation of PPARβ/δ facilitates anchorage-independent growth in lung cancer, breast cancer, and melanoma models (Pedchenko et al., 2008; Wang et al., 2016; Zuo et al., 2017). Despite the morphological observations, the mechanism through which PPARβ/δ regulates anchorage-independent growth was not explicated by these studies. It remains possible that PPARβ/δ might affect different types of cancer in distinct ways. Therefore, the molecular mechanism through the role of PPARβ/δ in anchorage-independent growth of colon cancer models has not yet been examined by other studies, and is worth investigating in the future.

3D spheroid models have become increasingly important in studies of cancer mechanisms and chemotherapy screening. The dynamic structure and molecular profile of 3D models are more similar to tumors, compared to monolayer (2D) cell cultures (Luca et al., 2013; Riedl et al., 2017). Cancer cell spheroids contain proliferative cells on the periphery and apoptotic cells at the core; and, in general, they are less proliferative and more apoptotic compared to monolayer cultures (Luca et al., 2013; Mohanty et al., 2013; Riedl et al., 2017). Differences in molecular pathways between spheroid and monolayer cultures may provide clues for understanding how selective repression of PPARβ/δ exhibits stronger inhibition of anchorage-independent growth compared to monolayer growth in colon cancer cells. Consistent findings suggest that activities of protein kinase B (AKT) and mitogen-activated protein kinases (MAPK)/ extracellular-signal-regulated kinase (ERK) pathways are down-regulated in colon cancer spheroids compared to monolayer cultures (Luca et al., 2013; Riedl et al., 2017; Tabusa et al., 2013). In addition, various inhibitors of the AKT and ERK pathways inhibited DLD1 spheroid growth more efficiently than monolayer growth, similar to the effect of DG172 in our results (Riedl et al., 2017). Interestingly, it coincides with knowledge from studies of our and other labs, that PPARβ/δ has complicated relationships with the AKT and ERK signaling pathways in various models (Burdick et al., 2007; Jeong et al., 2014; Liu et al., 2013; Pollock et

al., 2011; Yao et al., 2015b; Zhu et al., 2014a, 2014b). These data together suggest a possible link between PPARβ/δ and AKT/ERK pathways in regulating anchorage-independent growth of colon cancer cells, which will be investigated in our future studies.

It is worth noting that growth of a non-spheroid-forming cell line (RKO) did not appear to be inhibited by selective repression of PPARβ/δ. Revealed by results from Chapter 2, the growth of this cell line was also unaffected by PPARβ/δ ligands in 2D proliferation assay. This coincide with the observation that ligand activation of PPARβ/δ took longer time to elevate target gene expression in RKO, which has wild-type *APC/CTNNB1*, compare to the other cell lines that have mutant *APC/CTNNB1*. These results together suggest a possibility that the *APC*/*CTNNB1* genotype may play a role in growth-regulation by ligand activation or selective repression of PPARβ/δ. On the other hand, the non-spheroid morphology of RKO colonies may provide another explanation to its lack of response to PPARβ/δ ligands. RKO cells form irregularly shaped, grape-like colonies in spheroid plates, coinciding with their low expression of tight junction proteins (e.g., E-CADHERIN); therefore, these colonies have different molecular and structural features from tightly packed spheroids. Whether spheroid feature is required for selective repression of PPARβ/δ to inhibit anchorage-independent growth of a colon cancer cell line remained to be understood.

Our results also produce evidence that selective repression of PPARβ/δ inhibits EMT, based on the observation that DG172 reduced expression of VIMENTIN in RKO, and increased E-CADHERIN expression in HCT116. However, these results are still preliminary, because VIMENTIN and E-CADHERIN are only two of many downstream markers of EMT, and these results have not been repeated in other colon cancer cell lines. In future experiments, the expression of other EMT markers, including N-CADHERIN, FIBRONECTIN, ZO1, CLAUDIN, and OCCLUDIN, will be analyzed with or without exogenous PPARβ/δ ligands. Cell lines with

relatively lower EMT level will also be used. To further understand the mechanism of PPARβ/δ in regulating EMT, the expression and activity (e.g., nuclear localization) of EMT-inducing transcription factors, including SNAI1/2, TWIST, and Zinc finger E-box binding homeobox 1/2 (ZEB1/2), should be analyzed. Additionally, transforming growth factor beta (TGFβ) is known as one of the major inducers of EMT (Lamouille et al., 2014; Singh et al., 2018). It has been suggested that the anti-invasion property of selective repressive PPARβ/δ ligands in breast cancer cell lines is potentially due to their repression on TGFβ signaling (Adhikary et al., 2013). Relationships between PPARβ/δ and the TGFβ pathway have been pointed out by a number of other studies, although in these studies ligand activation or knockout of PPARβ/δ was used (Ham et al., 2010; Kaddatz et al., 2010; Stockert et al., 2011; Tan et al., 2005). Based on this evidence, the possibility that selective repression of PPARβ/δ inhibits EMT through TGFβ pathway in colon cancer cells worth evaluating in the future.

To our surprise, selective repression of PPARβ/δ did not inhibit colon cancer cell migration or TNFα/TGFβ-induced MMP activity, at the relatively lower concentration that reduced EMT. This is contradictory to a study, which found inhibition of migration and invasion, and reversal effect on the TGFβ pathway, by the same ligand in breast cancer cell lines (Adhikary et al., 2013). On the other hand, ligand activation of PPARβ/δ reduced migration and MMP activity, while the same treatment did not alter EMT marker expression or spheroid growth. This coincides with our previous observation that overexpression and ligand activation of PPARβ/δ inhibits migration and MMP expression in a testicular cancer cell line (Yao et al., 2015a). These data indicate that, although correlations exist between migration, invasion and EMT, migration and MMP activity of colon cancer cells are possibly driven by mechanisms independent from EMT. Besides the aforementioned relationship between PPARβ/δ and the TGFβ pathway, the PPARβ/δ-TNFα/NF-κB axis might also be involved in the anti-migration/

invasion property of PPARβ/δ. PPARβ/δ is known to antagonize NF-κB, a major downstream effector of TNFα, through sequestering its subunit p65 (reviewed in Peters et al., 2011a).

It should be emphasized that most of the present studies, especially the analysis of migration, MMP activity, and EMT marker expression, are preliminary due to low sample numbers used and a lack of biological repeats. Therefore, results and conclusions obtained from current repeats may be subject to changes until sufficient repeats are achieved. Alternative approaches and control models for PPARβ/δ specificity, such as loss-of-function or gain-of-function cell lines will also be needed to determine the role of PPARβ/δ in regulating colon cancer cell malignancy.

Overall, results from this study suggest that selective repression or ligand activation of PPARβ/δ can reduce tumorigenesis and malignancy-related phenotypes, including anchorage-independent growth, migration, EMT, and MMP activity, in human colon cancer cell lines. Our results also provide evidence that synthetic PPARβ/δ ligands have chemotherapeutic potentials in controlling colon cancer, and are unlikely to have a carcinogenic side effect for their other therapeutic applications.

Chapter 4. Discussion and future directions

For the past two decades, researchers have been trying to understand the role of PPARβ/δ in colon and many other types of cancer, yet conflicting results have emerged. Not only is the functional relevance of PPARβ/δ to the APC/β-CATENIN pathway—the major contributor to colon cancer—unclear, it is also not known whether activation of this receptor potentiates tumor cell initiation, survival, proliferation, and invasion. It is vitally important to understand the relationship between PPARβ/δ and cancer, since there is great pharmaceutical potential for targeting this receptor, for example, in treating metabolic diseases.

The present studies focused on two aims: to examine the functional relationship between PPARβ/δ and the APC/β-CATENIN pathway, and to investigate the effect of activating this receptor PPARβ/δ with different types of ligands on anchorage-dependent and -independent growth, and on the malignancy potential of colon cancer cells. Experiments were conducted mainly in colon cancer cell lines that have wild-type or mutant *APC/CTNNB1* genotypes. PPARβ/δ was activated using two types of high-affinity, subtype-specific ligands: a classic agonist, GW0742, which causes both up- and down-regulation of PPARβ/δ target genes; and selective repressive ligands DG172 and PTS264, which only induce repression of PPARβ/δ target genes. Synthetic PPARβ/δ agonists have been studied for nearly two decades, and controversial results have been generated for their effect on cancer. Selective repressive ligands have been developed relatively recently, and exhibited promising results for cancer inhibition.

Results from our studies suggest that, first, while PPARβ/δ is more easily activated in cells with mutant APC/β-CATENIN pathway, the activity of PPARβ/δ is not directly affected by activation of β-CATENIN. These results did not support the findings of a previous study, where

PPARβ/δ was found to be functionally regulated by the APC/β-CATENIN pathway (He et al., 1999). Second, ligand activation and selective repression of PPARβ/δ inhibited growth only in the *APC*/*CTNNB1*-mutant cell lines with higher inducibility of PPARβ/δ, suggesting that activation of PPARβ/δ inhibits, rather than facilitates *APC*/*CTNNB1*-mutant colon cancer cell growth, and that carcinogenic side effects should not be a concern for the therapeutic use of PPARβ/δ ligand. Our observations are in line with other studies demonstrating that PPARβ/δ inhibits proliferation, reduces clonogenicity, or promotes apoptosis in colon cancer cells, as well as reduces colon tumor formation and tumor growth in mouse models (Foreman et al., 2011; Harman et al., 2004; Hollingshead et al., 2007b; Marin et al., 2006; Reed et al., 2004; Yang et al., 2008, 2013). By contrast, our findings challenge the results that PPARβ/δ inhibits apoptosis and promotes growth of colon cancer cells *in vitro* or *in vivo* (Barak et al., 2002; Gupta et al., 2004; Park et al., 2001; Wang et al., 2006, 2012; Zuo et al., 2009, 2014).

We further found that selective repression of PPARβ/δ inhibited anchorage-independent growth by enhancing apoptosis in spheroid-forming colon cancer cell lines, and inhibited EMT marker expression in monolayer cultures. Ligand activation of PPARβ/δ, on the other hand, inhibited migration and invasion-related MMP9 activity. The distinct phenotypic effects caused by ligand activation and selective repression of PPARβ/δ may be mediated by different modes of target gene regulation, partially different set of target genes, and potential competition with endogenous PPARβ/δ ligands. Although ligand activation and selective repression of PPARβ/δ have shown distinct effect on anchorage-independent growth, migration, MMP activity and EMT, we have not observed significant potentiation of these tumorigenesis- and malignancy-related features by either ligand of PPARβ/δ. Taken together, our results in part support the other studies in which inverse relationships between PPARβ/δ and colon cancer malignancy were demonstrated (Yang et al., 2010, 2011), while questioning the findings that PPARβ/δ promotes

colon cancer malignant phenotype, invasion, or metastasis (Liu et al., 2019; Takayama et al., 2006; Zuo et al., 2017).

Results from the current studies brought up new questions as well. First of all, the cause of increased efficacy of ligand activation of PPARβ/δ in cell lines with mutant *APC/CTNNB1* is unknown. In order to understand the specific cause of differential PPARβ/δ functionality among these cell lines, it may be helpful to further compare the response of these cell lines to a PPARβ/δ antagonist or a selective repressive ligand over time, or to study the interaction of PPARβ/δ with co-activators, co-repressors, or physical proximity to the regulatory region of its target genes using immunoprecipitation assays. If a lower baseline activity of PPARβ/δ in the *APC/CTNNB1* mutant cell lines caused their higher sensitively to ligand activation, these cells would exhibit weaker response to a selective repressive ligand or an antagonist, compare to *APC/CTNNB1* wild-type cell lines. Alternatively, if PPARβ/δ is indeed more active in the *APC/CTNNB1* mutant cell lines, a higher level of association between PPARβ/δ and co-activators or target genes should be observed.

There were differences in the responses observed in cancer cells following modulation of PPARβ/δ activity in *APC*/*CTNNB1* mutant cells as compared to their wild-type counterpart (RKO cells). However, the mechanism by which this is mediated is unclear. One possibility is that this is mediated by changes in lipid oxidation/energy metabolism. A number of recent studies suggested that cancer cells, including colon cancer cells, have increased level of fatty acid oxidation compared to normal cells (reviewed in Corbet and Feron, 2017; Ma et al., 2018). Since PPARβ/δ is known to regulate lipid metabolism, and cancer cells require high level of ATP generated by lipid catabolism (Carracedo et al., 2013), it remains possible that this difference is related to this interaction.

The mechanism through which selective repression of PPARβ/δ promotes apoptosis in colon cancer cell spheroids is also unclear. Since selective repression of PPARβ/δ inhibits growth in spheroid models more significantly than in monolayer cultures, the underlying mechanism may be differentially regulated in these two models as well. As discussed in Chapter 3.4, ERK and AKT pathways are some of the major known differences between spheroid and monolayer models. Since PPARβ/δ has been previously shown to interact with the ERK and AKT pathways, it might be feasible to examine whether PPARβ/δ promotes apoptosis by interacting with these pathways.

Furthermore, alteration of downstream EMT marker expression by selective repression of PPARβ/δ was seen, but the upstream mechanism has not been investigated. It is currently known that EMT-inducing pathways, including TGFβ and TNFα pathways, can promote EMT by stimulating the SNAIL1/2, ZEB1/2, and/or TWIST transcription factors. Thus, the influence of selective repression of PPARβ/δ on the expression and activity of each of these factors should be examined in order to understand the full picture. Moreover, reduced migration rate and TGFβ/TNFα-induced MMP9 activity in cell culture media by ligand activation of PPARβ/δ were observed. Whether reduced MMP9 activity was a result of down-regulated TGFβ/TNFα signaling, decreased MMP9 mRNA/protein expression, or secretion, can be analyzed as the first step to understand its mechanism. It is also important to examine the effect of ligand activation and selective repression of PPARβ/δ on the invasion of colon cancer cell lines in both spheroid and monolayer cultures.

Limitations exist in the current study as well. First, although preliminary data has indicated specificity for PPARβ/δ, this has not been definitely proven in all of the experiments. This is especially important for the selective repressive ligands, since PPARβ/δ-independent effects of DG172 have been previously reported (Lieber et al., 2015). In future studies,

PPARβ/δ-overexpression and knockout models, as well as PPARβ/δ antagonists, should be applied as controls in places where PPARβ/δ specificity is uncertain. The use of PPARβ/δ-overexpression and knockout models will also add to the understanding of how the endogenous functions of PPARβ/δ are different from synthetic ligand-mediated effects in terms of cancer cell growth and malignancy. Another limitation of the present studies is that the experiments were done only *in vitro*. Although convenient for studying cancer mechanisms, these models lack an environmental complexity such as the tumor stroma and the immune system, and have different availability of nutrients, compared to *in vivo* models. Hence, the current findings of PPARβ/δ in colon cancer cell growth, invasion, and metastasis should be repeated in *in vivo* experiments. Performing orthotopic xenograft is one of our future goals. Using this model, the role of PPARβ/δ in *in situ* growth, invasion, metastasis, as well as other processes of colon cancer development can be examined. Patient-derived orthotopic xenograft would be an even more advanced model to compare the functional roles PPARβ/δ in colon cancer between a broad spectrum of genetic background.

One of the major questions about PPARβ/δ in colon cancer that has not been elucidated to this day is whether its expression is elevated, unchanged, or down-regulated in colon tumors compared to normal controls. To understand this from a cell culture perspective, we plan to study whether differences exist for PPARβ/δ expression, localization, and regulatory activity between normal colon epithelial cell lines and colon cancer cell lines.

In contrast to the original theory that β-CATENIN regulates PPARβ/δ, a new mechanism emerged recently suggesting that PPARβ/δ may actually facilitate β-CATENIN activity. This mechanism further relates PPARβ/δ to colon tumorigenicity, cancer stemness, and invasion. One study found that activation of PPARβ/δ in mice increased the population of Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5)-positive intestinal stem cells, potentiated their

organoid formation capability, and increased the expression of several β-CATENIN target genes that are involved in stem cell functions (Beyaz et al., 2016). The study therefore suggested that PPARβ/δ mediates high fat induced colon carcinogenesis by promoting β-CATENIN activity. However, the direct effect of ligand activation of PPARβ/δ on β-CATENIN activity was not examined in this study. Another study suggested that modulation of PPARβ/δ expression in mice correlated with change of β-CATENIN expression and β-CATENIN target gene expression, and further demonstrated a positive role between PPARβ/δ and colon cancer invasion and metastasis (Liu et al., 2019). Results from these studies are contradictory with results from this study and the evidence that PPARβ/δ promotes terminal differentiation. Since cancer cell stemness is also highly associated with malignancy and was not examined in this study, we aim to explore the role of PPARβ/δ in colon cancer stem cell properties in future studies. In particular, the effect of genetic manipulation or activation of PPARβ/δ on the expression of colon cancer stem cell markers, such as LGR5 (LGR5), Octamer-binding transcription factor 4 (OCT4), Homeobox protein NANOG (NANOG), SRY-box 2 (SOX2), should be analyzed (Munro et al., 2018). Among these proteins, it has been shown previously that PPARβ/δ regulates the expression of OCT4 and SOX2 (Yao et al., 2015a, 2017). Whether modulation of PPARβ/δ affects the $CD133^+$ / $CD44^+$ stem cell population within a cell line or tumor sample, or the growth and malignant potential of sorted $CD133^+$/ $CD44^+$ cancer stem cells can also be investigated for this purpose.

Overall, results from our current studies contribute to the understanding of PPARβ/δ in its relationship with the colonic oncogenic β-CATENIN pathway, and its role in colon cancer growth and malignancy. The results suggest that activation of PPARβ/δ with synthetic agonist or selective repressive ligands can inhibit, rather than promote, colon cancer. These results further support the idea that the use of PPARβ/δ ligands in treating metabolic diseases is likely to have

no risk of carcinogenesis. On the other hand, synthetic PPARβ/δ ligands or selective repressive ligands may have chemotherapeutic value in treating colon cancer.

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Appendix

Cell line	Gender	MSI	CIN	APC/β-CATENIN pathway		MAPK pathway		PI3K/Akt pathway	Cell cycle regulation	TGFβ pathway	
				APC	*CTNNB1*	*KRAS*	*BRAF*	*PIK3CA*	*TP53*	*CDKN2A*	*SMAD4*
RKO	N/A	MSI	-	Wild-type	Wild-type	Wild-type	V600E	H1047R	Wild-type	Wild-type	Wild-type
DLD1	M	MSI	-	Mutant	Wild-type	G13D	Wild-type	D545K, D549N	S241F	Wild-type	Wild-type
HCT116	M	MSI	-	Wild-type	S45del	G13D	Wild-type	H1047R	Wild-type	p.R24fs*20	Wild-type
LS174T	F	MSI	-	Wild-type	S45F	G12D	WT	H1047R	Wild-type	Wild-type	Wild-type
HT29	F	MSS	+	E853*, T1556 fs*3	Wild-type	Wild-type	V600E	P449T	R273H	Wild-type	Q311*

Genetic traits of the human colon cancer cell lines used in this study.
Abbreviations: MSI, microsatellite instability/instable; MSS, microsatellite stable; CIN, Chromosome instability. N/A, information is not available.

Reference:
Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknæs, M., Hektoen, M., Lind, G.E., and Lothe, R.A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2, e71.