MACROPHAGE PPAR-GAMMA INHIBITS GPR132 TO MEDIATE THE ANTI-TUMOR EFFECTS OF ROSIGLITAZONE

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DEDICATION

This dissertation is dedicated to my parents, Yee Nok and Wah Jing, for their endless support.

I would like to thank my mentor and the members of my Graduate Committee.

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Tumor-associated macrophage (TAM) significantly contributes to tumorigenesis. Human cancer is enhanced by PPARgamma loss-of-function mutations, and inhibited by the thiazolidinedione (TZD) class of synthetic PPARgamma agonists and type 2 diabetes drugs such as rosiglitazone. However, it remains enigmatic whether and how macrophage contributes to PPARgamma tumor-suppressive functions. Here we uncover that macrophage PPARgamma deletion in mice exacerbates mammary tumor development by increasing the number and pro-inflammatory property of TAMs, which in turn stimulate cancer cell proliferation. Macrophage PPARgamma loss also impairs the anti-tumor effects of

rosiglitazone. Mechanistically, we identify Gpr132 as a novel direct PPARgamma target in macrophage whose expression is enhanced by PPARgamma loss but repressed by PPARgamma activation. Functionally, macrophage Gpr132 is pro-inflammatory and pro-tumor. Genetic Gpr132 deletion not only retards inflammation and cancer growth but also abrogates the anti-tumor effects of PPARgamma and rosiglitazone. Pharmacological Gpr132 inhibition significantly impedes mammary tumor malignancy. These findings identify macrophage PPARgamma and Gpr132 as critical TAM modulators, new cancer therapeutic targets, and essential mediators of TZD anti-cancer effects.

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LIST OF ABBREVIATIONS

9RA: 9-cisretinoic acid

ApoA1: Apolipoprotein A1

BC: Breast cancer

BLI: Bioluminescence imaging

BM Mf: Bone marrow-derived macrophage

ChIP: Chromatin-immunoprecipitation

CRP: C-reactive protein

DCs: Dendritic cells

DKO mice: Double knockout mice

DMBA: 7,12-dimethylbenz[a]anthracene

G2A: G2 accumulation

GPR132: G protein-coupled receptor 132

Het: Heterozygous

hPBMN: Human peripheral blood mononuclear cells

IL-6: Interleukin-6

KO: Knockout

LPS: Lipopolysaccharide

LyzCre: Lysozyme-Cre

Mf-g-KO: macrophage PPARgamma knockout

micro-PET: micro-positron emission tomography

MMP-9: Metalloproteinase-9

MMTV: Mouse mammary tumor virus

NHE1: Na(+)/H(+) exchanger 1

PH3: Phospho histone H3

PPARgamma: Peroxisome Proliferator-Activated Receptor gamma

PPRE: Peroxisome proliferator response element

Rosi: Rosiglitazone

rtTA: reverse tetracycline-controlled transactivator

RXR: Retinoid X receptor

Sp Mf: Spleen-derived macrophage

TAM: Tumor-associated macrophage

TZD: Thiazolidinedione

UCP-1: Uncoupling protein 1

VSMCs: Vascular smooth muscle cells

WT: Wild type

INTRODUCTION

About two decades ago, breast cancer accounted for more deaths of American women than any other cancer (Menck 1997) and most of the breast tumors arise from the duct epithelium (Harris 1992). However, significant advancement in biomedical research in the past decades leads to the development of more effective treatments and improves the outcome of breast cancer patients. According to the statistics from the American Cancer Society, breast cancer is the most common cancer among American women, except for skin cancer. Breast cancer is also the second leading cause of cancer death in women, exceeded by lung cancer. Currently, breast cancer patients receive single or a combination of treatments including surgical resection, radiation, chemotherapy, and targeted therapy. However, associated with significant toxicity, chemotherapy may benefit only 20%-25% of patients. Another challenge physicians encounter is drug resistance in patients. Thus, physicians and researchers are motivated to investigate the mechanisms involved in cancer biology and to find novel therapeutic approaches for breast cancer.

How immune cells in the tumor microenvironment modulate cancer malignancy is a fundamental and fascinating question with tremendous therapeutic significance for cancer intervention. Emerging evidence supports a functional association between inflammation and cancer. Chronic inflammation is implicated in >15% of cancers (Coussens 2002) and shown to promote tumorigenesis (Shacter 2002; Kuper 2000; Blaser 1995; Scholl 1994). As a key player in inflammation and cancer progression, tumor-associated macrophage (TAM) strongly correlates with poor cancer prognosis (Noy 2014; Ruffell 2012; Qian 2010; Bingle 2002). For example, overexpression of macrophage colony-stimulating factor 1 (CSF1 or M-

CSF) leads to accelerated tumor progression in mice and human (Lin 2001; Scholl 1994). Moreover, macrophages also modulate therapeutic responses (Ruffell 2015). Although numerous clinical studies and experimental mouse models support that macrophages generally play a pro-cancer role, anti-tumor property has also been reported for certain subtypes of macrophages, suggesting that macrophage regulation of cancer malignancy is pleotropic and context-dependent (Krzeszinski 2015; Ruffell 2015; Noy 2014; Ruffell 2012; Qian 2010).

Understanding the molecular mechanisms of cancer cells as well as various types of stromal cells composing the tumor micro-environment is crucial for the development of effective treatments of breast cancer. Basic research and clinical data suggested that TAMs played an important role in breast cancer progression by producing excessive amounts of pro-angiogenic factors, as well as growth factors. Thus, targeting the macrophages may inhibit tumor growth. This idea is supported by various studies that examine the effects of macrophage depletion on cancer growth (Germano 2013). Recently, using murine macrophage cell line RAW264.7 and murine 4T1 breast tumor model, Li and colleagues demonstrated that Huaier extract inhibited macrophage infiltration and angiogenesis (Li 2016). Trametes robiniophila Murr (Huaier) was a sandy beige mushroom found on the truck of trees and had been widely used in traditional Chinese medicine. The anti-tumor properties of Huaier extract were highlighted in previous studies (Zheng 2014; Sun 2013; Wang 2012). The new findings by Li et al. revealed a novel mechanism of the anti-tumor effect of Huaier extract which inhibited angiogenesis by targeting TAMs, and supported Huaier as a

promising drug for clinical treatment of breast cancer (Li 2016).

My project investigated the role of stromal PPARgamma, particularly macrophage PPARgamma, in breast cancer development and progression. Previous studies largely focused on the direct effects of PPARgamma on cancer cells. For instance, PPARgamma activation by ligands was shown to promote terminal differentiation, reduce proliferation and trigger lipid accumulation in human breast cancer cells and liposarcoma cells (Mueller 1998; Tontonoz 1997). Similar inhibitory effects on cell growth were observed in a variety of cancer cells, including colon, lung, ovary, breast, thyroid and prostate cancer cells, treated with PPARgamma ligands (Han 2007). However, whether and how PPARgamma in macrophages modulates cancer progression is unknown. Moreover, previous studies heavily relied on the usage of PPARgamma ligands, which may exert PPARgamma-independent and/or physiologically irrelevant effects; whereas *in vivo* genetic dissection of the specific PPARgamma functions in each cell type in the cancer milieu is lacking.

The Wan lab previously reported that female mice with PPARgamma deletion in the hematopoietic and endothelial cells developed inflammation in their lactating mammary gland. This led to the production of inflammatory milk, which triggered systemic inflammation in the nursing neonates manifested as a transient fur loss (Wan 2007). These intriguing observations suggest that PPARgamma plays an anti-inflammatory role in macrophage and mammary gland, which may influence breast cancer. I hypothesize that PPARgamma in macrophages impedes breast cancer development by inhibiting inflammation. Using a series of genetic and pharmacological, gain- and loss-of-function, *in*

vitro and *in vivo* approaches, here I uncover macrophage PPARgamma as an important suppressor of breast cancer progression and a key mediator of the anti-tumor effects of rosiglitazone that functions by repressing a novel target Gpr132 in macrophages.

The following chapters provide critical reviews on PPARgamma, the application of its ligands, and Gpr132 which is a novel target of PPARgamma I have identified, explaining the rational for my investigations. The first chapter explores the functions of PPARgamma and its implication in cancers, especially breast cancer. The second chapter delineates the effects of the thiazolidinediones (TZDs) which are synthetic ligands of PPARgamma, and discusses the therapeutic potential of TZDs in the treatment of cancers. The third chapter highlights the effects of PPARgamma on inflammation and atherosclerosis. The fourth chapter examines the role of Gpr132 in cancers, as well as inflammation, and evaluates the significance of targeting Gpr132 in cancers, as well as inflammatory diseases, such as atherosclerosis.

LITERATURE REVIEW

Chapter 1

A REVIEW OF PPAR-GAMMA AND ITS ROLE IN BREAST CANCER

A critical review of PPARgamma and its role in breast cancer development and progression is included in this chapter. Peroxisome proliferator-activated receptor gamma (PPARgamma) , also known as the glitazone receptor, is a nuclear receptor that acts as a transcription factor modulating a myriad of physiological processes (Lefterova 2014; Ahmadian 2013), including glucose and fat metabolism (Barak 1999; Kubota 1999; Rosen 1999), inflammation (Glass 2006; Ogawa 2005), and tumorigenesis (Fenner 2005; Tontonoz 1997). PPARgamma loss-of-function mutations have been associated with human cancer development (Aldred 2003; Sarraf 1999).

In addition, PPARgamma is the molecular target of the approved anti-diabetic drugs known as the thiazolidinediones (TZDs) (Lehmann 1995; Forman 1995). These synthetic PPARgamma agonists such as rosiglitazone and pioglitazone have been implicated to inhibit tumor malignancy (Apostoli 2015; Frohlich 2015; Monami 2014; Bosetti 2013; Skelhorne-Gross 2012; Uray 2012; Drzewoski 2011; Feng 2011; Kumar 2009; Fenner 2005).

Due to its important functions in many biological processes, as well as the widespread clinical use of TZDs, PPARgamma has been the subject of intense study and a lot of effort has been put into elucidating the detailed mechanism of PPARgamma signaling, and the effects of PPARgamma on tumor development and progression.

Chapter 1A. PPAR-GAMMA' MECHANISMS OF ACTION

PPARgamma functions as a heterodimer with RXRs (Kliewer 1992) and this dimerization is essential for the binding to DNA by PPARgamma. The C-terminal region is responsible for dimerization with RXR and contains the major transcriptional activation domain (AF2 domain). The N-terminal region has equally important functions. Domain-swap experiments provided evidence that the adipogenic function of PPARgamma depends on its N-terminal region. PPARdelta has no adipogenic action in fibroblasts. However, fusion of the N-terminal region of PPARgamma on to the appropriate region of PPARdelta enabled PPARdelta to acquire adipogenic functions (Brun 1996). Subsequent studies revealed that this region of PPARgamma-2 binds PGC-2, a small protein with adipogenic action (Castillo 1999). Modification such as phosphorylation by MAP kinase of PPARgamma-2 significantly decreases the biological actions of the receptor (Adams 1997; Hu 1996). Further investigations demonstrated that phosphorylation of the N terminus reduced ligand-binding affinity, negatively regulating the transcription of PPARgamma (Shao 1998).

Chapter 1B. LIGANDS, COACTIVATORS, AND COREPRESSORS

PPARgamma functions as a transcription factor in a ligand-dependent manner and cofactors play an important role in its activity. Some of the coactivators that interact with PPARgamma include CBP/p300, the SRC family, and TRAP220 (Powell 2007). A few examples of corepressors include NCoR, RIP140, and SMRT (Debevec 2007). Previous work showed that these coactivators were highly regulated at the transcriptional and post-

transcriptional levels. For instance, PGC-1 was induced in brown fat cells by cold exposure, and it increased the transcriptional activity of PPARgamma on the uncoupling protein (UCP-1) promoter, regulating mitochondrial gene expression and thermogenesis (Puigserver 1998). PGC-1 and other coactivators are also regulated by protein-kinase cascades. p38 MAP kinase stabilized and activated PGC-1 by phosphorylation at three sites (Knutti 2001; Puigserver 2001). Modified PGC-1 was then recruited to the PPARgamma-binding site on the UCP-1 promoter. In contrast, phosphorylation of PGC-1 by AKT-2 decreased its activity in liver (Li 2007).

Chapter 1C. PPAR-GAMMA AND CANCER

The function of PPARgamma to mediate adipocyte differentiation, which is coupled with a complete cessation of cell proliferation, stimulates researchers' interest in this receptor's effect on tumor growth (Tontonoz 2008). Due to the role of PPARgamma in adipose cell formation, PPARgamma ligands are used in the treatment of liposarcoma, which is a malignant tumor arises in fat cells in deep soft tissue (Hatton 2008; Tontonoz 1997). Early preclinical studies on human liposarcoma cells demonstrated the therapeutic potential of PPARgamma ligands in the treatment of liposarcoma. Tontonoz and colleagues showed that PPARgamma was expressed at high levels in human liposarcoma and treatment with PPARgamma ligands induced primary human liposarcoma cells to undergo terminal differentiation, which was characterized by accumulation of intracellular lipid, induction of adipocyte-specific genes, and withdrawal from the cell cycle. Based on their work, Tontonoz

et al. suggested that induction of terminal differentiation by PPARgamma ligands might be a promising therapeutic approach to treating liposarcoma (Tontonoz 1997).

To assess the therapeutic potential of PPARgamma ligands in the treatment of cancer, a phase II clinical trial was conducted by Demetri and colleagues. They examined the effects of troglitazone, a synthetic ligand of PPARgamma, on tumor differentiation in patients with advanced or metastatic liposarcoma (Demetri 1999). Tumors from patients receiving troglitazone exhibited extensive lipid accumulation and enhanced transcription of genes involved in adipocyte differentiation. Furthermore, a significant reduction in the expression of Ki-67 in these tumor cells indicated that troglitazone suppressed tumor cell proliferation *in vivo* (Demetri 1999). In their study, Demetri et al. also addressed the safety and tolerability of troglitazone in patients. They reported that no adverse events were observed in patients receiving the treatment. All patients tolerated the daily dosing of troglitazone with no side effect problems (Demetri 1999). This small pilot trial of six subjects showed a differentiating effect of short-term troglitazone therapy in advanced stage liposarcomas and provided the rationale for further clinical evaluation of troglitazone, as well as other PPARgamma ligands, in the treatment of liposarcomas (Hatton 2008; Demetri 1999). Furthermore, these findings in liposarcoma patients might have important relevance for broader groups of patients with neoplastic diseases based on the expression of PPARgamma. For instance, preclinical studies demonstrated that some breast, as well as colon, carcinomas expressed PPARgamma. PPARgamma activation by ligands induced differentiation and repressed tumor cell proliferation *in vitro* in both breast cancer and colon cancer cell lines (Demetri 1999; Mueller

1998; Sarraf 1998).

A second phase II trial was conducted to evaluate the effects of rosiglitazone, another ligand of PPARgamma. In this study, nine patients with advanced, unresectable liposarcomas were treated with rosiglitazone twice a day (Debrock 2003). However, analysis of tumors did not show any differentiating effect. Tumor cell proliferation measured by Ki67 immunostaining did not decrease significantly with the treatment. Based on these observations, Debrock and colleagues concluded that rosiglitazone was not effective as an anti-tumoral drug in the treatment of liposarcomas (Debrock 2003). It is interesting that the two clinical studies yield opposite results. The discrepant findings may relate to differences in the patient cohorts, tumors, and study agents (Hatton 2008).

Better understanding of PPARgamma effects on tumors may expand the use of PPARgamma ligands to treating other types of cancer. Several studies supported the use of PPARgamma ligands in the treatment of breast cancer. In breast cancer cells, PPARgamma ligands had been shown to inhibit proliferation and induce apoptosis both *in vitro* and *in vivo*. In addition, PPARgamma ligands suppressed tumor angiogenesis and invasion (Fenner 2005). Despite the profound effects PPARgamma ligands had on cancer cells, the underlying mechanisms remained to be elucidated. Several mechanisms had been proposed. For instance, Kumar et al. identified Na(+)/H(+) exchanger 1 (NHE1) as a downstream target of PPARgamma signaling by ligands. They demonstrated that a peroxisome proliferator response element (PPRE) existed in the promoter region of NHE1 and treatment with PPARgamma ligands in breast cancer cells led to a downregulation of NHE1 transcription as

well as protein expression. Overexpression of NHE1 attenuated the inhibitory effect of PPARgamma on tumor colony-forming ability while gene silencing of NHE1 by siRNA enhanced the sensitivity of cancer cells to growth-inhibitory stimuli. Kumar et al. also analyzed breast cancer biopsies from patients with type II diabetes treated with rosiglitazone. The results showed that rosiglitazone significantly repressed NHE1 in the tumor tissue (Kumar 2009). The work by Kumar and colleagues provided evidence for tumor-selective downregulation of NHE1 by PPARgamma activation *in vitro* as well as in breast cancer patients. Furthermore, a few studies supported that the overexpression of NHE1 was associated with carcinogenesis (Amith 2013; Loo 2012). Another potential target of PPARgamma signaling was Bid. Bonofiglio et al. indicated that treating human breast cancer cells with PPARgamma ligands resulted in an upregulation of Bid expression and increased the association between Bid/p53 in cytosol as well as mitochondria. Knocking down Bid attenuated apoptosis induced by rosiglitazone and 9-cisretinoic acid (9RA). These data highlighted the role of p53/Bid complex at the mitochondria in promoting breast cancer cell apoptosis upon low doses of PPARgamma and RXR ligands, suggesting that Bid may be a potential target in the novel therapeutical strategies for breast cancer (Bonofiglio 2011).

Numerous studies had demonstrated that PPARgamma ligands generally suppressed tumor growth. However, treatment with PPARgamma ligands in APC-min mice, a mouse strain harboring a mutation in the *APC* gene that is prone to colon polyps, led to increased polyps (Tontonoz 2008; Lefebvre 1998; Saez 1998). This observation suggested that specific mutations may make organisms resistant to the anti-cancer effects of PPARgamma ligands;

these mutations could possibly exacerbate tumor frequency or progression (Tontonoz 2008).

Several genetic studies in mice reveal consistent results demonstrating that loss of one allele of the PPARgamma gene predisposes mice to cancer (Tontonoz 2008; Kato 2006; McAlpine 2006; Nicol 2004; Girnun 2002). These genetic studies suggest that PPARgamma may be a tumor suppressor gene. For instance, in an earlier investigation, researchers treated mice harboring global heterozygosity with a carcinogen that induced tumors only in the colon. They observed that PPARgamma deficiency led to increased frequency of colon tumors, as well as larger tumors (Girnun 2002). Similar effect is also reported in studies with human tumors. The PPARgamma gene is sequenced in various types of human tumors to determine if there are any alterations in the gene (Tontonoz 2008). The results of one of the studies demonstrated that the PPARgamma gene had been mutated in a small but significant number of colon tumors. These mutations causing a substantial loss of activity suggested that these mutations were functional in the context of human cancer (Sarraf 1999). Recently, Skelhorne-Gross and colleagues treated adipocyte-specific PPARgamma knockout mice and wild-type controls with 7,12-dimethylbenz[a]anthracene (DMBA) to initiate breast tumorigenesis. They reported a higher mammary tumor incidence and decreased tumor latency in PPARgamma knockout mice. Mammary tumor volumes were reduced by 50% with rosiglitazone treatment. Further analysis by Skelhorne-Gross et al. suggested that *in vivo* PPARgamma in mammary stromal adipocytes attenuated breast tumorigenesis through BRCA1 upregulation and reduced leptin secretion (Skelhorne-Gross 2012). This study strongly supported a protective effect of PPARgamma activation in adipocytes and potential

use of PPARgamma ligands in the treatment of breast cancer.

PPARgamma ligands had been used as a monotherapy in a few advanced forms of human cancer, such as breast and prostate. However, no beneficial effect was observed in these trials. Based on the results, investigators claimed that troglitazone and rosiglitazone had little apparent clinical value among patients with metastatic breast cancer and prostate cancer respectively (Tontonoz 2008; Smith 2004; Burstein 2003). Nonetheless, these clinical trials did not examine the effects of PPARgamma ligands in combination with other anti-cancer drugs. Most drugs that are used in the treatment of cancers are used in combination with other drugs. The addition of PPARgamma ligands, which have relatively low toxicity profile, may improve the outcome of cancer patients receiving combination therapy. To determine whether PPARgamma ligands exert a synergistic effect with other anti-cancer drugs, Girnun and colleagues treated cancer cells with PPARgamma ligands and a number of classical chemotherapeutic agents, and examined cancer cell growth. Girnun et al. demonstrated that the combination of PPARgamma ligands and the platins, including carboplatin and cisplatin, resulted in a remarkable synergy (Girnun 2007). It is important to note that the synergistic interactions between rosiglitazone and carboplatin were observed *in vivo*, in transplantable tumors in nude mice, as well as in chemically induced colon carcinogenesis in mice (Tontonoz 2008; Girnun 2007). Microarray analyses of gene expression in tumor cells were used to determine the mechanisms of interactions between these drugs. The results showed that PPARgamma activation by ligands suppressed the metallothionein family, which had been implicated in resistance to the platin-based drugs (Tontonoz 2008; Girnun 2007).

Recently, a phase I trial of exemestane in combination with metformin and rosiglitazone was carried out in nondiabetic obese postmenopausal women with hormone receptor-positive metastatic breast cancer. The trial indicated that oral daily administration of exemestane and metformin with and without rosiglitazone was well tolerated. Exemestane pharmacokinetics were not altered by metformin and rosiglitazone (Esteva 2013). Further investigations are needed to determine if metformin and/ or thiazolidinediones have a beneficial effect on patients with metastatic breast cancer.

Overall, the genetic data revealing PPARgamma as a tumor suppressor gene, and the low toxicity profile of PPARgamma ligands support further investigations into the possibility of combining these drugs with other cancer therapies. Moreover, since millions of people are taking TZD drugs for diabetes, it will be interesting to examine how these drugs affect the incidence of various tumors (Tontonoz 2008).

Chapter 2

A REVIEW OF THIAZOLIDINEDIONES (TZDS)

Thiazolidinediones (TZDs) are a class of medications used to treat type II diabetes, which is characterized by insulin resistance. These drugs function by increasing insulin sensitivity. However, the use of certain TZDs had been restricted because of concerns over side effects and adverse events (Soccio 2014). Although the TZDs were shown to be insulin-sensitizing drugs in the early 1980s , their mechanism of action was not clearly understood until the mid-1990s (Soccio 2014; Fujita 1983). Research in subsequent years confirmed that the TZDs were ligands for PPARgamma (Lehmann 1995).

Chapter 2A. PPAR-GAMMA AS THE BIOLOGICAL RECEPTOR FOR THE TZD ANTI-DIABETIC DRUGS

Initially, the TZDs were discovered due to their ability to lower glucose levels in rodents. They were later confirmed to improve insulin sensitivity in human (Nolan 1994). Rosiglitazone and pioglitazone, two members of the TZD drugs, are being used for the treatment of type II diabetes. Harris and Kletzien were the first to make a connection between TZDs and PPARgamma. They demonstrated that pioglitazone increased the transcriptional activity of the ARF6 DNA-binding complex on the aP2 promoter (Harris 1994). ARF6 was subsequently identified as the PPARgamma/RXRalpha heterodimer (Tontonoz 1994). The work by Lehmann et al. provided the key evidence, supporting that TZDs were ligands for PPARgamma (Lehmann 1995).

The TZDs function by activating PPARgamma (Lehmann 1995; Forman 1995). When activated, the receptor binds to DNA in complex with the retinoid X receptor (RXR), and regulates the transcription of target genes. The activated PPARgamma/RXR heterodimer binds to peroxisome proliferator hormone response elements upstream of target genes and a number of coactivators, such as nuclear receptor coactivator 1 and CREB binding protein, is recruited to the site to facilitate the transcription of target genes.

Being a master regulator of glucose and fat metabolism, activated PPARgamma has been shown to decrease insulin resistance, and increase adipocyte differentiation. When the storage of fatty acids in adipocytes increases, the amount of fatty acids present in circulation consequently decreases. Moreover, PPARgamma modulates the level of particular adipokines. For instance, PPARgamma activation increases adiponectin level while decreasing the level of leptin.

The TZDs exert their biological effects on insulin sensitivity through binding to PPARgamma. Willson and colleagues showed that the clinical potencies of different TZDs were closely correlated with potency of receptor activation (Willson 2001; Willson 1996). Non-TZD agonists for PPARgamma were also shown to improve insulin sensitivity (Henke 1998). Mutations in PPARgamma in both rodents and humans were associated with insulin resistance (Agostini 2006; He 2003; Barroso 1999).

Chapter 2B. EFFECTS OF TZDS IN HUMANS

With a high expression of PPARgamma, adipose tissue is the primary site of TZD action in diabetic patients (Semple 2006). Studies with the euglycemic-hyperinsulinemic clamp in humans demonstrated that treatment with troglitazone improved whole-body insulin sensitivity by increasing glucose disposal and reducing hepatic glucose output (Inzucchi 1998; Nolan 1994). Troglitazone also lowered FFA levels in diabetic patients (Maggs 1998).

Some investigators proposed that the TZDs improved insulin resistance by regulating expression of various adipokines. Insulin-resistant patients have reduced levels of plasma adiponectin and increased levels of inflammatory mediators including TNFalpha and resistin. Treatment of diabetic patients with TZDs enhanced the expression of adiponectin while repressing the expression of resistin and TNFalpha (Sharma 2007; Trujillo 2006; Trujillo 2005; Wellen 2005).

In a recent review, Drzewoski and colleagues examined available data to determine if there was an association between cancer incidence and antidiabetic drug therapy. The data, although inconclusive, suggested that some hypoglycemic medications increased cancer risk. In contrast, metformin and rosiglitazone might suppress cancer progression, especially in breast cancer (Drzewoski 2011). Using human cancer cell lines, Feng et al. also demonstrated that metformin and rosiglitazone inhibited cancer cell growth and induced apoptosis. The combination of metformin or rosiglitazone with gemcitabine or doxorubicin led to an additional decrease in live cancer cells, as well as increase in apoptosis (Feng 2011). Another review article by Frohlich and Wahl summarized the results of several studies evaluating the

effect of combined therapies with TZDs and other agents. For instance, rosiglitazone sensitized hepatocellular carcinoma cell lines to 5-fluorouracil antitumor activity by upregulating PTEN (Frohlich 2015; Cao 2009). The combination of gefitinib and rosiglitazone increased growth inhibition of lung cancer cells (Lee 2006). Moreover, herceptin sensitized breast cancer cells to the inhibitory effects of troglitazone (Yang 2003).

The mechanism of action of the TZDs in breast cancer cells is not fully elucidated, but various research studies suggested the involvement of interactions with other nuclear hormone receptors, transcriptional co-activators and repressors. It is of paramount importance to study the PPARgamma-independent effects exerted by TZDs since a thorough understanding about the TZDs' functions is needed to ensure beneficial outcome in patients.

Overall, a better understanding of the mechanism of PPARgamma signaling, as well as the effects of TZDs, is crucial for PPARgamma ligands to be useful in the treatment of breast cancer. The goal of my project is to elucidate the role of PPARgamma in breast cancer and shed light on the therapeutic potential of PPARgamma agonist drugs.

Chapter 3

THE ROLE OF PPAR-GAMMA IN INFLAMMATION AND ATHEROSCLEROSIS

Chapter 3A. THE REGULATION OF MYELOID GENE EXPRESSION BY PPAR-GAMMA

The earliest studies focused on the effects of PPARgamma in adipose tissue; however, subsequent work had indicated that PPARgamma is also a crucial regulator of gene expression in myeloid cells. This receptor is induced during monocyte differentiation into macrophages and is highly expressed in activated macrophages, such as the foam cells of atherosclerotic lesions (Marx 1998; Ricote 1998; Tontonoz 1998). An induction of PPARgamma is also observed during the differentiation of monocytes into dendritic cells (DCs) (Szatmari 2004; Gosset 2001).

A number of studies suggested that oxidized lipids and lipoproteins, such as oxidized LDL, 9-HODE and 13-HODE, activated PPARgamma signaling in myeloid cells (Han 2000; Huang 1999; Nagy 1998). Moreover, Huang and colleagues showed that the action of 12/15-lipoxygenase on fatty acid substrates provided by lipoproteins may produce PPARgamma ligands in macrophages (Huang 1999).

Although there is evidence showing PPARgamma ligands promote the expression of genes including CD36 and aP2, which are markers of macrophage differentiation, several studies using PPARgamma-null cells indicated that PPARgamma was not essential for macrophage or DC differentiation per se (Szatmari 2004; Chawla 2001; Moore 2001;

Tontonoz 1998). Nonetheless, PPARgamma regulates the expression of several key genes characteristic of mature macrophages. Established fat target genes are also responsive to PPARgamma ligands in macrophages and DCs (Castrillo 2004). Similar to adipose cells, most of the genes positively regulated by PPARgamma in myeloid cells have functions linked to lipid metabolism (Tontonoz 2008). For instance, the class B scavenger receptor CD36 functions as both a transporter of fatty acids and a receptor for oxidized lipid epitopes found on apoptotic cells and modified lipoproteins. Short-term treatment of macrophages with PPARgamma ligands promotes lipid uptake (Nagy 1998; Tontonoz 1998).

These findings support the important role of PPARgamma in macrophage differentiation and macrophage lipid metabolism. Subsequent studies confirmed that the scavenger receptor CD36 was a target gene for PPARgamma (Chawla 2001). Retroviral expression of PPARgamma in macrophages facilitated induction of CD36 in response to PPARgamma ligands (Chawla 2001). Chawla et al. reported their observation, which was consistent with previous findings, that TZDs induced CD36 expression in wild-type but not in PPARgamma-deficient macrophages, and again emphasized the induction of CD36 was PPARgamma dependent (Chawla 2001).

PPARgamma not only controls lipid metabolism, it also has important secondary consequences for myeloid cell immune functions (Tontonoz 2008). Global analysis of gene expression in developing human DCs illustrated that the class of transcripts changed in early stages of DC maturation was comprised of genes involved in lipid metabolism, and genes involved in immune responses were altered at later stages of maturation (Szatmari 2007).

Chapter 3B. THE ROLE OF PPAR-GAMMA AS A MODULATOR OF INFLAMMATORY SIGNALING

Several strands of evidence suggest that PPARgamma ligands exert anti-inflammatory effects in monocytes and macrophages. Treating monocytes and macrophages with PPARgamma agonists reduces secretion of inflammatory cytokines, including tumor necrosis factor alpha, interleukin-1 beta, interleukin-6 and MMP-9, and inhibits macrophage activation (Jiang 1998; Ricote 1998; Spiegelman 1998). Treating activated macrophages with PPARgamma ligands, similarly, promotes a resting phenotype and represses inducible nitric oxide synthase, gelatinase B and scavenger receptor A (Ricote 1998). Altogether, these observations support that PPARgamma might represent a target for anti-inflammatory therapy. However, there are reports indicating that the anti-inflammatory effects of PPARgamma ligands are PPARgamma independent (Chawla 2001). In their study, Chawla et al. stimulated wild-type and PPARgamma-deficient macrophages with lipopolysaccharide (LPS) and examined the inhibition of cytokine secretion in response to PPARgamma ligands. Stimulation with LPS resulted in an equivalent increase in the secretion of pro-inflammatory cytokines, including TNF alpha and IL-6, in both wild-type and PPARgamma-deficient macrophages. PPARgamma ligands inhibited the secretion of TNF alpha and IL-6 in wild-type macrophages. Intriguingly, this suppression by PPARgamma ligands was equally effective in PPARgamma-deficient macrophages. In conclusion, Chawla et al. indicate that PPARgamma expression is not crucial for PPARgamma ligands to exert anti-inflammatory effects in macrophages and therefore the receptor may not be an appropriate target for anti-

inflammatory drugs (Chawla 2001). Moreover, some studies used the naturally occurring prostanoid 15d-PGJ2 as the PPARgamma ligand. Subsequent experiments indicated that 15d-PGJ2 had effects independent of PPARgamma, such as covalently modifying components of the NF-κB signaling pathway (Castrillo 2001; Straus 2000).

Over the years, researchers investigate the mechanism by which PPARgamma inhibits inflammation, and propose a cross talk between PPARgamma and transcription factors, including NF-κB, on the promoters of inflammatory genes (Glass 2006; Ogawa 2005). Since PPREs have not generally been found in the proximal promoters of genes suppressed by PPARgamma ligands, researchers believe an indirect mechanism is involved (Tontonoz 2008).

In a canonical pathway, GR interacts with AP-1 transcription factors and represses their activity on inflammatory promoters. This ability of nuclear receptor to inhibit the activity of a promoter without directly binding to it is known as transrepression (Tontonoz 2008). More recent studies propose that ligand-dependent receptor sumoylation is involved in nuclear receptor transrepression (Pascual 2005). Glass et al. suggest that sumoylation directs PPARgamma to the promoters of inflammatory genes where it stabilizes NCoR-containing corepressor complexes and suppresses transcription (Tontonoz 2008).

Several strands of evidence reveal that PPARgamma ligands have anti-inflammatory effects *in vivo*. For instanec, mice heterozygous for the PPARgamma-null mutation (PPARgamma +/-) display enhanced susceptibility to experimentally induced arthritis and allergic encephalomyelitis (Natarajan 2003; Setoguchi 2001). PPARgamma ligands have

been shown to suppress the development of chemical-induced colitis in mice (Rousseaux 2005; Natarajan 2003; Su 2001; Su 1999). Moreover, treatment with PPARgamma ligands reduces the number of macrophages and the production of cytokine in adipose tissue of diabetic mice (Wellen 2005). Clinical data also indicate beneficial effects of TZDs in patients with chronic psoriasis (Bongartz 2005). However, the mechanism of these TZD effects remains to be elucidated. It is not yet clear whether these effects are secondary to changes in lipid metabolism/insulin sensitivity or the result of direct repression of inflammatory gene expression in macrophages (Tontonoz 2008).

Researchers have been interested in understanding the anti-inflammatory actions of PPARgamma. One particular explanation for the anti-inflammatory effects is that PPARgamma has the ability to alter the inflammatory phenotype of myeloid cells. Plenty of evidence in the literature supports that PPARgamma regulates polarization of monocyte differentiation between pro-inflammatory (M1) and alternative anti-inflammatory (M2) macrophage phenotypes. For example, Glass and colleagues showed that PPARgamma expression was induced by the TH2 cytokine IL-4 (Huang 1999). Chawla et al. demonstrated that the ability of PPARgamma to alter the balance between M1 and M2 macrophages involved the control of arginase I (Odegaard 2007). With its functions in M2 macrophages, PPARgamma is implicated in immune responses and metabolic diseases. For instance, analysis of human atherosclerotic lesions revealed that the expression of PPARgamma and M2 markers correlated positively. In addition, PPARgamma activation directed primary human monocytes into M2 differentiation. These data strongly suggested that PPARgamma

activation skews human monocytes toward an anti-inflammatory M2 phenotype (Bouhlel 2007). Consistent with these observations, the loss of PPARgamma resulted in enhanced level of M1 macrophages in adipose tissue and increased insulin resistance (Odegaard 2007). Fesus and colleagues also reported that PPARgamma activation during differentiation of human monocytes to macrophages reduced the capacity of macrophages to engulf apoptotic neutrophils (Majai 2007).

Chapter 3C. PPAR-GAMMA AND ATHEROSCLEROSIS

The development of atherosclerotic lesions is influenced by inflammatory signals in the vessel wall, and macrophages play a critical role in this process. PPARgamma is expressed in various myeloid, as well as vascular cell types, and has been implicated in inflammatory responses. Previous studies demonstrated that PPARgamma was highly expressed in macrophage foam cells of atherosclerotic lesions and treatment with rosiglitazone inhibited the development of atherosclerosis in LDL receptor-deficient male mice. Improved insulin sensitivity and reduced tissue expression of TNF-alpha were also reported in mice treated with rosiglitazone (Li 2000). Based on these findings, Li and colleagues suggested that PPARgamma ligands may exert anti-atherogenic effects in diabetic patients.

In addition to its functions in macrophages, PPARgamma has been shown to have effects in vascular smooth muscle cells (VSMCs) as well as endothelial cells. For instance, treatment with troglitazone inhibited not only TNF-alpha-induced VCAM-1 expression in

human umbilical vein endothelial cells, but also the homing of monocytes/macrophages in apoE-deficient mice (Pasceri 2000). Moreover, treatment with troglitazone repressed the expression of MMP9 and migration of VSMCs (Marx 1999; Marx 1998). Besides migration, the proliferation of VSMCs was also inhibited by treatment with PPARgamma ligands (Law 2000).

These studies which shed light on the role of PPARgamma in the atherosclerotic process are critical findings since more than one million diabetic patients, who are highly susceptible to atherosclerotic disease, are treated with TZDs (Chawla 2001; Reginato 1999). Early studies also provided evidence that TZDs improve atherosclerosis in patients with type II diabetes (Rocchi 1999).

To directly prove that macrophage PPARgamma is involved in the development of atherosclerosis, PPARgamma-deficient bone marrow was transplanted into LDLR-deficient mice. Loss of bone marrow PPARgamma increased atherosclerotic lesion development (Chawla 2001), suggesting that PPARgamma is atheroprotective. PPARgamma ligands were reported to reduce foam cell formation in LDLR-deficient mice *in vivo* (Li 2004). A few investigators proposed that this protective effect of PPARgamma was due to its ability to regulate LXR-alpha expression and cholesterol efflux in macrophages (Akiyama 2002; Chawla 2001). In general, PPARgamma removes modified lipoproteins from the artery wall by shuttling the lipid into the reverse cholesterol transport pathway (Tontonoz 2008).

Collectively, these studies suggested that PPARgamma activation ameliorated atherosclerosis in murine models and TZDs may be protective against cardiovascular disease

in humans. Haffner and colleagues examined if rosiglitazone affects serum levels of C-reactive protein (CRP), interleukin-6 (IL-6) and metalloproteinase-9 (MMP-9), which are markers of systemic inflammation with implication in cardiovascular disease, in diabetic patients. The results of their studies indicated that rosiglitazone may have beneficial effects on overall cardiovascular risk by significantly reducing serum levels of MMP-9 and CRP in patients with type II diabetes (Haffner 2002). Other studies, on the contrary, suggested that treatment with TZDs led to increased risk of cardiovascular disease (Nissen 2007). Nissen and colleagues conducted searches of the published literature, the Web site of the Food and Drug Administration, as well as a clinical-trials registry maintained by the drug manufacturer (GlaxoSmithKline). The results of their meta-analysis revealed that rosiglitazone was associated with increased risk of myocardial infarction as well as increased risk of death from cardiovascular causes (Nissen 2007). Thus, Nissen et al. concluded that patients and providers should be aware of the potential adverse cardiovascular effects of treatment with rosiglitazone for type II diabetes. These conflicting results demanded more research to be done to elucidate the consequences of long-term systemic PPARgamma activation.

Chapter 4

A REVIEW OF GPR132

Chapter 4A. DISCOVERY OF GPR132 AND ITS POTENTIAL ROLE IN CANCER

The G protein-coupled receptor 132 (GPR 132), is also known as G2A (for G2 accumulation). This receptor was first identified by Owen Witte's group as a DNA damage and stress inducible GPCR that blocked cell cycle. Weng et al. demonstrated that Gpr132 overexpression attenuated the transformation potential of BCR-ABL and other oncogenes, resulting in accumulation of cells at G2/M (Weng 1998). Based on these findings, Gpr132 was proposed to have "tumor suppressive" function by arresting cell cycle at the G2 checkpoint. Using micro-positron emission tomography (micro-PET), Le and colleagues examined leukemia development in mice transplanted with BCR-ABL expressing bone marrow cells. The results showed that Gpr132 deficiency accelerated BCR-ABL-induced leukemogenesis (Le 2002). However, other studies in fibroblastic cell lines revealed that Gpr132 overexpression led to actin stress fiber formation via Gα13-dependent activation of RhoA, and repressed contact inhibition of fibroblast growth (Kabarowski 2000; Zohn 2000). It was important to note that no inhibitory effect of Gpr132 overexpression on fibroblast proliferation was reported in these studies. Disproving previous discovery that Gpr132 overexpression led to accumulation of NIH 3T3 cells in the G2/M phase of cell cycle, these findings supported Gpr132 as an oncogenic G protein-coupled receptor (Kabarowski 2000; Zohn 2000). Nevertheless, these studies with fibroblasts may not be biologically relevant

because Gpr132 is predominantly expressed in activated macrophages, T and B lymphocytes, not in fibroblasts.

Chapter 4B. GPR132 MECHANISMS OF ACTION

Initial studies proposed that Gpr132 was a receptor for lyso-PC. Kabarowski et al. demonstrated that Gpr132 activation by lyso-PC increased the intracellular Ca2+ level, activated ERK mitogen-activated protein kinase, and altered migratory responses of T lymphocytes (Kabarowski 2001). However, subsequent work refuted this claim that lyso-PC was a ligand binding directly to Gpr132 (Witte 2005). Instead of binding directly to the receptor, lyso-PC stabilized the surface expression of Gpr132, controlling the balance between cell surface and internal distribution of Gpr132 (Frasch 2007; Wang 2005). Frasch et al. suggested that insertion of cone shaped lyso-phospholipids into the membrane led to looser packing of membrane lipids, and this perturbation might cause dimerization or oligomerization, therefore changing the functions of Gpr132 (Frasch 2007). However, recent work by Khan and colleagues demonstrated that the lyso-PC mix caused internalization of Gpr132 and G-protein coupled signaling, rather than stabilizing Gpr132 on the cell surface (Khan 2010). This discrepancy may be due to differences in the experimental details, such as the method of presenting the lipids.

To further examine the role of Gpr132 in response to lyso-PCs, researchers performed studies employing an antibody against Gpr132, which blocked neutrophil cytoplasmic Ca2+ flux, ROS production, as well as bacterial clearance (Frasch 2007; Yan 2004).

Chapter 4C. GPR132 AS A PROTON-SENSING RECEPTOR

The tumor micro-environment is acidic because of glycolytic cancer cell metabolism, hypoxia, and deficient blood perfusion (Yang 2012; Cairns 2006; Gatenby 2004; Vaupel 1989). Acidic pH has pleiotropic effects on the proliferation, migration, invasion, and therapeutic response of cancer cells, as well as the function of immune cells, vascular cells, and other stromal cells. First, chronic acidosis had been suggested to facilitate cancer cell clonal evolution by inducing chromosomal instability and gene mutations (Xiao 2003; Morita 1992). Second, acidosis contributed to metastasis by degrading the extracellular matrix (Brisson 2012; Rozhin 1994). However, there were studies showing that acidosis can be cytotoxic, inhibiting cancer cell proliferation and inducing stress response, as well as apoptosis (Smallbone 2010; Putney 2003; Williams 1999; Ohtsubo 1997).

Compared to other family members, including GPR4, OGR1, and TDAG8, the pH-sensing function of Gpr132 is less defined (Justus 2013). Although the proton-sensing activity was detected in Gpr132-overexpressing cells, Radu et al. demonstrated that the receptor was dispensable for acid sensing in native lymphocytes (Radu 2005). The pH-sensing capability of Gpr132 was much weaker than other family members (Radu 2006).

The mechanism by which Gpr132-mediated signal transduction is regulated by the extracellular pH remains poorly understood. In a recent study, Lan et al. visualized the pH-dependent Gpr132 distribution change in living cells using a sortase A-mediated pulse labeling technology (Lan 2014). The results demonstrated that acidic pH inhibited Gpr132 internalization from cell surfaces into intracellular compartments, and this inhibition was

relieved at neutral pH. Moreover, acidic pH redistributed internalized Gpr132 onto the cell surface (Lan 2014).

Chapter 4D. GPR132 AND INFLAMMATION

Gpr132 is expressed in a broad range of immunoregulatory cell-types, including cells of the innate immune system (macrophages, dendritic cells, neutrophils) and cells of the adaptive immune system (T and B lymphocytes) (Frasch 2007; Parks 2005).

Several lines of evidence suggested that Gp132 played a role in chemotactic action in macrophages and T cells. Genetic studies with primary macrophages from Gpr132 deficient mice, as well as experiments with macrophages/T cells expressing Gpr132 siRNA molecules or overexpressing the Gpr132 receptor, demonstrated that stimulation of macrophage and T lymphocyte chemotaxis by LPC was mediated by Gpr132 (Wang 2005; Yang 2005; Radu 2004; Kabarowski 2001). Collectively, these findings showed that Gpr132 regulated the recruitment of macrophages and T lymphocytes into inflammatory foci rich in LPC.

Parks and colleagues proposed that Gpr132 had pro-atherogenic effects because Gpr132 deficiency suppressed the development of atherosclerosis in LDLR-/- mice. They explained that the suppressed development of atherosclerosis was due to the attenuation of inflammatory cell recruitment resulted from the loss of Gpr132-mediated chemotactic responses of monocytes/macrophages to LPC in the vascular wall (Parks 2006). In contrast, subsequent experiments demonstrated that the chemotactic function of Gpr132 in bone marrow-derived monocytes did not modulate the development of atherosclerosis in LDLR-/-

mice (Parks 2009). Thus, researchers explored other functions of Gpr132 that may affect atherosclerosis.

Gpr132 deficient LDLR-/- mice displayed elevated levels of plasma HDL, suggesting that modulation of lipoprotein metabolism might contribute to the athero-protective effect of Gpr132 deficiency (Parks 2006). Similarly, studies with bone marrow transplantation indicated that deletion of Gpr132 in resident tissues alone was sufficient to increase plasma HDL levels and inhibit atherosclerosis in LDLR-/- mice (Parks 2009). Parks et al. also reported that Gp132 deficiency was associated with an increased secretion of apolipoprotein A1 (ApoA1), the principal constituent of all HDL particles. Based on these findings, Parks as well as other investigators proposed a model in which Gpr132 localized to hepatocyte endosomes functioned as a "brake" on ApoA1 secretion, regulating plasma HDL levels (Kabarowski 2009; Parks 2009).

RESULTS

1. MACROPHAGE PPAR-GAMMA DELETION ENHANCES TUMOR GROWTH IN VIVO

We generated macrophage PPARgamma knockout mice (mf-g-KO) by breeding PPARgamma flox mice with Tie2Cre or Lysozyme-Cre (LyzCre). Tie2Cre deleted PPARgamma in hematopoietic cells and endothelial cells as previously described[39,40]. LyzCre deleted PPARgamma specifically in the myeloid lineage[41]. We compared PPAR$\gamma^{flox/flox}$;$Cre^{+/-}$ KO mice with PPAR$\gamma^{flox/flox}$;$Cre^{-/-}$ littermate controls.

To determine the effects of macrophage PPARgamma deletion on breast cancer development, we performed mammary fat pad orthotopic injections of C57BL/6J-compatible mouse breast cancer cells EO771 in female mice, and followed tumor growth by measuring tumor size. Compared to the littermate controls, both Tie2Cre-induced and LyzCre-induced mf-g-KO mice showed enhanced tumor development as indicated by earlier onset and larger tumor volume (**Fig. 1a-b**). These results indicate that the pro-tumor effect observed was largely caused by PPARgamma deletion in myeloid cells such as macrophages. Staining for Ki67 and phospho histone H3 (PH3) in the tumor sections showed increased cell proliferation in mf-g-KO mice (**Fig. 1c-d**). These findings suggest that macrophage PPARgamma inhibits tumor growth *in vivo*.

2. PPAR-GAMMA DELETION PROMOTES MACROPHAGE INFILTRATION INTO TUMORS

We collected tumor tissues, bone marrow cells and spleen cells from tumor-bearing mf-g-KO or control mice, and compared gene expression. The results showed a higher expression of pro-inflammatory genes in these PPARγ-deficient cells and tissues, including COX-2, MMP9 and MCP-1 (**Fig. 1e-g**). Macrophage infiltration into tumors is a strong indicator for cancer malignancy and poor prognosis (Ruffell 2015; Komohara 2014; Zhang 2012). TAM quantification by immunofluorescence staining revealed enhanced tumor macrophage recruitment in both Tie2-g-KO and Lyz-g-KO mice compared with control mice (**Fig. 1h and Fig. S9**). Consistent with the reports that PPARgamma agonists inhibit angiogenesis (Scoditti 2010; Keshamouni 2005; Goetze 2002), we found that the number of blood vessels in tumor sections was increased in Tie2-g-KO mice but unaltered in Lyz-g-KO mice (**Fig. S1**), further indicating that PPARγ deficiency in macrophage alone is sufficient to augment tumor growth independent of changes in angiogenesis. Together, these findings suggest that macrophage PPARgamma deletion changes both the number and property of TAMs to establish a pro-inflammatory tumor environment.

3. PPAR-GAMMA-DEFICIENT MACROPHAGES PROMOTE CANCER CELL PROLIFERATION IN VITRO

To determine if PPARgamma-deficient macrophages regulate cancer cell behavior in the absence of other components in the tumor microenvironment such as fibroblasts and extracellular matrix, we performed macrophage and cancer cell co-culture experiments *in vitro* (**Fig. 2a**). Mouse macrophages were differentiated from the progenitors in bone marrow or spleen and then co-cultured with a luciferase-labelled subline of the MDA-MB-231 human breast cancer cell line (1833 cells). Specific quantification of tumor cell proliferation was achieved by luciferase output as only the cancer cells, but not the macrophages, were tagged with a luciferase reporter. The results showed that tumor cell proliferation was significantly augmented by PPARgamma-deficient macrophages compared with WT control macrophages (**Fig. 2b**). Consistent with this observation, co-culture with PPARgamma-deficient macrophages also led to an increased tumor cell colony formation (**Fig. 2c**). Since mouse macrophages and human cancer cells were from different species, mRNA expression in these two cell types in the co-culture setting could be distinguished by species-specific QPCR primers. We found that co-culture with PPARgamma-deficient macrophages resulted in higher expression of proliferation markers and lower expression of apoptosis markers in cancer cells compared with WT control macrophages (**Fig. 2d-e**).

Bone marrow and spleen progenitor cells collected from PPARgamma KO mice were differentiated into macrophages *in vitro* and the expression level of PPARgamma was examined via QPCR. The results confirmed the PPARgamma KO macrophages had

significant deletion of PPARgamma (**Fig. 2k**) compared to the wild type controls.

In accordance to our *in vivo* observations (**Fig. 1**), PPARgamma-deficient macrophages exhibited elevated expression of pro-inflammatory genes such as COX-2, MCP-1 and MMP-9 (**Fig. 2f**). In addition, PPARgamma-deficient macrophages displayed higher levels of anti-apoptotic genes and lower levels of pro-apoptotic genes (**Fig. 2g**), indicating an augmented survival. Moreover, PPARgamma-deficient macrophages showed increased proliferation, measured by ATP content (**Fig. 2h**) or MTT assay (not shown). Our *in vitro* findings further support our *in vivo* observations that the increased number and pro-inflammatory property of PPARgamma-deficient macrophages are sufficient to promote tumor progression.

4. ROSIGLITAZONE ACTIVATION OF MACROPHAGE PPAR-GAMMA INHIBITS CANCER CELL PROLIFERATION IN VITRO

As a complementary approach to our loss-of-function genetic approach, we next performed gain-of-function pharmacological experiment to assess the effect of rosiglitazone activation of macrophage PPARgamma on cancer cells. Mouse macrophages were pre-treated with rosiglitazone or vehicle control; rosiglitazone was removed by medium change before human cancer cells were seeded for co-culture (**Fig. 2a**). The results showed that cancer cell growth was significantly inhibited when co-cultured with rosiglitazone-treated WT macrophages compared with vehicle-treated WT macrophages (**Fig. 2i**). Importantly, this rosiglitazone effect was macrophage-PPARgamma-dependent because tumor cell proliferation was increased equally when co-cultured with PPARgamma-deficient macrophages regardless of rosiglitazone or vehicle treatment (**Fig. 2i**). Together, these findings indicate that activation of macrophage PPARgamma by either endogenous or synthetic agonists suppresses tumor growth.

5. MACROPHAGE PPAR-GAMMA IS A KEY MEDIATOR OF THE ANTI-TUMOR EFFECT OF ROSIGLITAZONE IN VIVO

To assess the functional significance of macrophage PPARgamma in the pharmacological effects of rosiglitazone, we treated mf-g-KO mice and littermate controls with rosiglitazone or vehicle control starting 4 days after cancer cell injection. The results show that the ability of rosiglitazone to suppress breast cancer growth was significantly attenuated in both Tie2Cre- and LyzCre-induced mf-g-KO mice (**Fig. 2j**). This indicates that macrophage is an essential cell type that is required for the anti-tumor function of rosiglitazone.

6. MACROPHAGE PPAR-GAMMA REPRESSES GPR132 EXPRESSION

To understand how PPARgamma alters the transcription program in macrophages to control cancer cell proliferation, we next set out to identify the key PPARgamma target genes. Our experiments reveal that tumor cell proliferation could be significantly enhanced by co-culture with PPARgamma-deficient macrophages but not by the conditioned medium from PPARgamma-deficient macrophages (**Fig. 3a-b**), indicating that physical contact between macrophages and cancer cells is required and thus the key tumor-modulating PPARgamma target gene in macrophages likely encodes a membrane protein. By searching published microarray databases (Hevener 2007; Welch 2003), we selected several candidate membrane proteins that might be regulated by PPARgamma in macrophages. Some of the

candidates including Notch1/2, IL-3Ra (CD123) and CXCR7 have been implicated in cancer. For instance, aberrant level of Notch1 was detected in breast cancer tissues, and Notch1 hyper-activation was shown to correlate with poorer outcome for breast cancer patients (Battle 2014; Cao 2014; Mittal 2014; Ercan 2012; Simmons 2012; Reedijk 2005; Parr 2004). Moreover, evidence from the literature suggested that activation of Notch1 enhanced EMT (Xie 2012; Leong 2007). Notch inhibition also increased the effectiveness of current therapies (Brennan 2013). Notch2, on the other hand, was suggested to have opposite effects compared to Notch1 (Chu 2011). High level of Notch2 had been shown to correlate with higher chance of survival (Xu 2015; Parr 2004). IL-3Ra was reported to be overexpressed on AML cells (Hauswirth 2007; Florian 2006; Muñoz 2001; Jordan 2000). And overexpression of IL-3Ra was clinically correlated with a lower survival rate in AML patients (Graf 2004). Pharmacological studies demonstrated that IL-3 fused with diphtheria toxin induced cytotoxicity to both AML leukemia stem cells and blasts (Black 2003; Feuring-Buske 2002). In addition, monoclonal antibodies against IL-3Ra impaired AML in mice by inhibiting the homing and self-renewal of AML LSC cells (Jin 2009). Overall, IL-3Ra provided a promising cell surface target for AML patients with high IL-3Ra expression. CXCR7 was involved in various biological processes, such as cell survival, adhesion, and mobility (Burns 2006). And overexpression of CXCR7 was observed in various tumors, including breast cancer (Iwakiri 2009; Wang 2008; Miao 2007). Several studies suggested that the expression of CXCR7 could be an indicator of the metastatic potential of breast cancer (Hassan 2009; Cabioglu 2007) as CXCR7 enhanced migration and metastasis (Inaguma 2015; Luker 2012).

CXCR7 was also shown to promote breast cancer growth by activating STAT3 signaling (Wani 2014). Targeting CXCR7 could lead to inhibition of breast cancer growth and metastasis (Li 2015).

Upon examining the expression of candidates in our macrophage cultures, we found that G protein-coupled receptor 132 (Gpr132, also known as G2A) was consistently and significantly upregulated in PPARgamma-deficient macrophages compared with WT control macrophages (see below), whereas the expression of 11 other candidates was unaltered (**Fig. S2**). Therefore, we decided to further investigate whether Gpr132 is a functional PPARgamma target gene in macrophages. Another reason for studying Gpr132 is that the role of this receptor in breast cancer is unclear. The functions of this receptor in macrophages and the effects it has on cancer cell growth remain to be elucidated.

Gpr132 has been previously described as a stress-inducible seven-pass transmembrane receptor that functions at the G2/M checkpoint of the cell cycle (Weng 1998), which modulates immune cell function (Kabarowski 2009; Yang 2005; Radu 2004). We found that Gpr132 was predominantly expressed in the hematopoietic cell types/tissues and highly expressed in macrophages, but largely absent in other tissues or tumor cells (**Fig. 3c-d**), indicating that it may play an important role in macrophage function. Gpr132 expression was significantly higher in PPARgamma-deficient macrophages compared with control macrophages, either in macrophage cultures alone or in macrophages co-cultured with cancer cells (**Fig. 3e-f**). In line with this observation, PPARgamma activation by rosiglitazone reduced Gpr132 expression in WT macrophages but not PPARgamma-deficient macrophages

(**Fig. 3g**). These findings suggest that PPARgamma represses Gpr132 expression.

7. PPAR-GAMMA BINDS TO GPR132 PROMOTER AND REPRESSES ITS TRANSCRIPTIONAL ACTIVITY

To determine whether Gpr132 is a direct PPARgamma transcriptional target, we investigated if PPARgamma can bind to the Gpr132 promoter and regulate its transcription. Gpr132 promoter regions (0.5 kb and 1 kb) were cloned into a luciferase reporter vector. Transient transfection and reporter assays reveal that luciferase output from both 0.5 Kb and 1 Kb Gpr132 promoter was reduced by the co-transfection of PPARgamma and further diminished by rosiglitazone treatment (**Fig. 3h**). These results indicate that PPARgamma represses Gpr132 promoter via critical element(s) within the 500 base pairs upstream of Gpr132 transcription start site. Indeed, we identified a PPAR response element (PPRE) half site in this region (-188: CATCCGAGCA**AGGTCA**GAC). Chromatin-immunoprecipitation (ChIP) assay showed that PPARgamma could bind to the endogenous Gpr132 proximal promoter in macrophages but not an upstream negative control region (**Fig. 3i**); moreover, rosiglitazone treatment led to a decreased level of H3K9Ac active transcription histone mark at the Gpr132 transcriptional start site (**Fig. 3j**). These mechanistic studies reveal that PPARgamma directly represses Gpr132 transcription in macrophages.

8. GPR132 IS REPRESSED BY PPAR-GAMMA IN HUMAN MACROPHAGES AND CORRELATES WITH HUMAN BREAST CANCER

Gpr132 expression in human macrophages derived from human peripheral blood mononuclear cells (hPBMN) was also blunted by rosiglitazone (**Fig. 3k**). This indicates that PPARgamma repression of Gpr132 is evolutionally conserved and our findings in mice may translate to human physiology and disease. To explore the significance of Gpr132 in human breast cancer, we analyzed the RNA-Seq and clinical data of breast invasive carcinoma (BRCA) from The Cancer Genome Atlas (TCGA) database. Because Gpr132 is highly expressed in mouse and human macrophages (**Fig. 3k**) but absent or expressed at extremely low level in mouse and human breast cancer cells used respectively in our *in vivo* models and *in vitro* experiments (**Fig. 3d**), Gpr132 expression in tumors mainly originates from hematopoietic cells in the microenvironment such as macrophages. Thus, we proposed that the effects observed here were due to the actions of Gpr132 in macrophages rather than cancer cells. However, we could not rule out the possibilities that other breast tumors express Gpr132 as well. Compared with normal breast samples, the majority of breast cancer lesions displayed significantly higher Gpr132 expression (**Fig. 3l**); this may be a contribution by hematopoietic cells in the microenvironment as well as the tumor cells. In addition, compared with ER-positive breast cancers, the more aggressive ER-negative breast cancers also exhibited higher Gpr132 expression (**Fig. 3m**). Moreover, linear regression analyses showed that higher Gpr132 expression was significantly correlated with higher expression of

pro-inflammatory markers including CCL2 (MCP-1), MMP9 and PTGS2 (COX-2) in breast cancer lesions (**Fig. 3n**). These findings further suggest that macrophage Gpr132 may promote inflammation and tumor progression.

9. MACROPHAGE GPR132 FACILITATES CANCER CELL PROLIFERATION IN VITRO

We next examined the function of macrophage Gpr132 in regulating cancer cells using our *in vitro* co-culture system. Gpr132 knockdown in macrophages significantly reduced cancer cell growth (**Fig. 4a-c**). Conversely, Gpr132 over-expression in macrophages increased cancer cell growth (**Fig. 4d-g**). We then compared macrophages derived from the bone marrow or spleen of Gpr132-KO mice vs. littermate WT control mice. Gene expression analyses reveal that Gpr132-KO macrophages displayed the opposite phenotype from PPARγ-deficient macrophages, with lower pro-inflammatory genes (**Fig. 4g**), higher pro-apoptotic genes and lower anti-apoptotic genes (**Fig. 4h**) compared with WT macrophages. *In vitro* macrophage-tumor cell co-culture experiments showed that Gpr132-KO macrophages exhibited a significantly reduced ability to promote cancer cell colony formation and growth (**Fig. 4i-j**). These results indicate that Gpr132 enhances inflammation and macrophage survival, and the upregulated Gpr132 in PPARγ-deficient macrophages may confer their tumor-promoting effects.

Bone marrow and spleen progenitor cells collected from Gpr132 KO mice were differentiated into macrophages *in vitro* and the expression level of Gpr132 was examined via QPCR. The results confirmed the Gpr132 KO macrophages had significant deletion of Gpr132 compared to wild type controls (**Fig. 4q**).

10. GPR132 KNOCKOUT MICE SUPPORT LESS TUMOR GROWTH IN VIVO

To examine the effects of Gpr132 deletion in the tumor environment on cancer growth *in vivo*, we injected EO771 mouse breast cancer cells into the mammary fat pad of Gpr132-KO mice and WT littermate controls. In this system, Gpr132 was deleted in macrophages as well as other Gpr132-expressing tissues such as bone marrow, spleen and thymus, but not in the injected cancer cells which had essentially no Gpr132 expression (**Fig. 3c-d**). Previous study show that Gpr132-KO mice display a normal pattern of T and B lineage differentiation, appearing healthy and indistinguishable from WT littermates throughout young adulthood, but develop progressive secondary lymphoid organ enlargement associated with abnormal expansion of both T and B lymphocytes that become pathological when older than one year of age (Le 2001). Therefore, our experiments were initiated in young mice and terminated before Gpr132-KO mice aged to prevent any potential effects of lymphoid defects on cancer growth. Compared with WT and Gpr132 heterozygous (Het) controls, Gpr132-KO mice exhibited significantly diminished tumor growth (**Fig. 4k**). Compared with WT controls, Gpr132-Het mice also showed attenuated tumor growth at later stage (**Fig. 4k**), indicating that Gpr132 regulation is dosage-sensitive. Together, our *in vitro* and *in vivo* results indicate that macrophage Gpr132 promotes tumor growth, suggesting that Gpr132 inhibition may impede cancer progression.

11.MACROPHAGE GPR132 MEDIATES PPAR-GAMMA REGULATION AND ROSIGLITAZONE EFFECTS

To further examine whether Gpr132 is a functional PPARgamma target in macrophage that is required for PPARgamma cancer regulation, we conducted pharmacological and genetic experiments. As a pharmacological gain-of-function strategy, we treated the macrophage-cancer cell co-cultures or tumor-grafted mice with rosiglitazone or vehicle control. Pre-treating Gpr132-KO macrophages with rosiglitazone before cancer cell seeding no longer showed any inhibition of cancer cell proliferation in the co-cultures (**Fig. 4l, S3**). Consistent with this *in vitro* observation, the anti-tumor effect of rosiglitazone *in vivo* was also abolished in Gpr132-KO mice (**Fig. 4m**). These pharmacological findings support that PPARgamma repression of Gpr132 in macrophages is a significant contributor to the anti-tumor effects of rosiglitazone.

As a genetic loss-of-function strategy, we bred Gpr132-KO mice with mf-g-KO mice to generate mf-g/Gpr132 double KO (DKO) mice. Mammary fat pad tumor graft experiments demonstrated that Gpr132 deletion in the DKO mice impaired the ability of macrophage PPARgamma deficiency to exacerbate tumor growth because DKO mice showed similar tumor volume as Gpr132-KO mice (**Fig. 4n**). This genetic rescue further supports that Gpr132 is an essential mediator of macrophage PPARgamma regulation of breast cancer progression.

12. PHARMACOLOGICAL GPR132 INHIBITION IMPEDES TUMOR GROWTH

To further explore Gpr132 as a potential cancer therapeutic target, we next examined whether acute pharmacological inhibition of Gpr132 could attenuate breast cancer progression. Because macrophage precursors reside in hematopoietic tissues such as blood, bone marrow and spleen that can be efficiently targeted by siRNA (Larson 2007), we chose to employ siRNA-mediated Gpr132 knockdown. We treated female mice with si-Gpr132 or si-Ctrl at 10μg/mouse twice/week for 18 days, 3 days before and 15 days after cancer cell graft. The results showed that si-Gpr132 significantly reduced tumor volume compared with si-Ctrl (**Fig. 4o**). Body weight was unaltered by either si-Ctrl or si-Gpr132 (not shown), indicating a lack of overt toxicity by either siRNA delivery or Gpr132 knockdown. These results support Gpr132 inhibition as a novel anti-cancer strategy.

DISCUSSION

Given the pleotropic and important roles of PPARgamma in physiology and disease, as well as the wide-spread usage of TZD drugs for the treatment of insulin resistance and type II diabetes, it is of paramount importance to elucidate the mechanisms for how PPARgamma and TZDs affect cancer. Here we have uncovered a crucial yet previously unrecognized role of macrophage PPARgamma in suppressing cancer progression and mediating the anti-tumor effects of rosiglitazone (**Fig. 4p**). Mechanistically, PPARgamma activation in macrophages tunes down inflammatory programs by repressing the transcription of a novel target gene Gpr132, which is a pro-inflammatory membrane receptor (**Fig. 4p**). Consequently, tumor growth is inhibited when macrophage Gpr132 level is low by either Gpr132 deletion/inhibition or PPARgamma activation via rosiglitazone; whereas tumor growth is exacerbated when macrophage Gpr132 level is high as the result of macrophage PPARgamma deficiency. Importantly, Gpr132 deletion abolishes the cancer regulation by macrophage PPARgamma or rosiglitazone, indicating that Gpr132 is an essential mediator of PPARgamma functions in macrophages and tumor progression. These findings reveal PPARgamma and Gpr132 as fundamental key players in TAM, providing new mechanisms how macrophages interact with tumor cells to promote cancer malignancy.

Cancer cells form an intimate relationship with TAMs to proliferate and survive. Targeting the infiltrating macrophages to alter their number and properties can lead to a significant inhibition of cancer malignancy. Our data suggest that this can be achieved by

either PPARgamma activation or Gpr132 inhibition in the macrophage. By elucidating the mechanisms that macrophages use to promote cancer and inflammation, effective diagnostic tools as well as innovative anti-tumor and anti-inflammatory therapeutics can be designed. For example, macrophage levels of PPARgamma and Gpr132 may predict not only tumor aggressiveness but also the pharmacological responses to rosiglitazone or Gpr132 inhibitors. Our findings may explain why rosiglitazone exerts anti-tumor effects in certain cancers but not others – cancers with abundant PPARgamma-positive macrophages may be sensitive whereas cancers with limited macrophages or PPARgamma-negative macrophages may be resistant. Moreover, the positive association of Gpr132 with inflammation and breast cancer in human (**Fig. 3l-n**), the repression of Gpr132 expression by rosiglitazone in human macrophage (**Fig. 3k**) and the anti-tumor effects of pharmacological Gpr132 inhibition (**Fig. 4o-p**) highlight the exciting potential of Gpr132 blockade as a new therapeutic.

In summary, the significance of our findings resides in the following aspects: 1) it reveals macrophage as an important cell type that contributes to PPARgamma suppression of cancer and the anti-tumor effects of rosiglitazone; 2) it identifies Gpr132 as a novel PPARgamma direct target gene in macrophages that mediates PPARgamma functions; 3) it uncovers Gpr132 as a pro-inflammatory and pro-tumor factor in macrophages, and thus a novel therapeutic target. Ultimately, these new knowledge will enhance our understanding of macrophage regulation, cancer microenvironment as well as PPARgamma and Gpr132 biology, which may translate to a better intervention of diseases such as cancer, diabetes and

inflammatory disorders. These findings also lead us to believe that cancer patients may respond differently to PPARgamma ligands due to differential expression of Gpr132 and/ or mutations in PPARgamma.

Some clinical data highlighted the safety of TZD drugs, which had low toxicity profile, and showed that the oral administration of TZDs was well tolerated without adverse effects (Esteva 2013). *In vitro* work and *in vivo* mouse models demonstrated that TZD drugs synergized with other chemotherapeutic agents to reduce cancer cell proliferation and induce apoptosis (Frohlich 2015; Feng 2011; Cao 2009; Girnun 2007; Lee 2006; Yang 2003). These cumulative findings support the potential use of TZDs in combined therapies for cancer patients, especially the ones with diabetes. One of the future directions could be to investigate the effects of combined therapies using our PPARgamma conditional knockout mouse models. Complementing the previous studies which examined the effects of combined therapies on cancer cells, our conditional knockout mouse models allow us to study if the effects of combined therapies are dependent on PPARgamma in stromal cells, particularly the myeloid lineages. These future experiments are important because a comprehensive understanding of how the combined drugs affect the cancer cells and the stromal cells helps determine whether cancer patients will benefit from the combined treatment.

Moreover, further studies are needed to investigate the detailed mechanism of Gpr132 and how it promotes breast tumor cell proliferation, including the downstream signaling

pathways involved. Also, it will be interesting to examine if Gpr132 has effects on metastasis. Based on the literature, acidic pH has pleiotropic effects on the proliferation, migration, invasion, and therapeutic response of cancer cells, as well as the function of immune cells, vascular cells, and other stromal cells. Being sensitive to changes in pH, Gpr132 had also been shown to positively correlate with MCP-1, MMP-9 and COX-2 in breast cancer lesions by our linear regression analyses. These observations suggest that Gpr132 may play a role in cancer metastasis.

ADDITIONAL EXPERIMENTS

A. CONDITIONED MEDIUM EXPERIMENT

To determine whether the key mediator of PPARgamma' effects on cancer cells was a secreted molecule, I cultured luciferase-labelled human breast cancer cells with conditioned medium collected from mouse macrophage cultures. Bone marrow and spleen progenitor cells were collected from PPARgamma KO as well as WT mice, and differentiated into macrophages *in vitro* for 6 days. I replaced the old medium in the tissue culture plates with serum-free medium, and collect the conditioned medium the next day. The conditioned medium contained proteins, cytokines, or growth factors released by the macrophages that may promote the growth of cancer cells. The results showed that human and mouse breast cancer cells cultured with conditioned medium collected from PPARgamma KO or WT macrophage cultures grew at similar rates **(Fig. S4**), suggesting that the key mediator of PPARgamma' effects on cancer cells was not a secreted molecule. These observations indicated that physical contact between macrophages and cancer cells was important for macrophage PPARgamma's anti-tumor effects.

B. TROUBLESHOOTING THE CHROMATIN-IMMUNOPRECIPITATION PROTOCOL

Before performing the ChIP protocol to examine the interaction between PPARgamma and the promoter region of Gpr132 gene, I tested the sonication protocol and the specificity of the primers to eliminate potential confounds. Running the sonicated DNA fragments on a gel indicated that the sonication process worked as the DNA was broken down into fragments with fragment size of 100-1000bp (Fig. S5a). Running the PCR products on a gel also demonstrated the specificity of the primers. A clear single band indicated that the primers recognized only specific regions of Gpr132 promoter (Fig. S5b).

C. TESTING THE ROLE OF PPAR-GAMMA IN METASTASIS

Intracardiac inoculation of tumor cells directly into the arterial blood supply of mice was a commonly used model to understand the molecular mechanisms controlling the establishment and growth of tumors in metastatic sites. In order to examine if PPARgamma deletion enhanced metastasis, I injected B16 melanoma cells, which express luciferase, into the left ventricle of the hearts of PPARgamma KO mice and wild type controls, and monitored tumor metastasis via bioluminescence imaging (BLI). The results showed an increased cancer spread in PPARgamma Tie2Cre KO mice (Fig. S6a). Similarly, an elevated level of luciferase signal was observed in PPARgamma LyCre KO mice (Fig. S6b), suggesting that PPARgamma in the myeloid lineages protected against cancer metastasis.

D. DOXYCYCLINE-INDUCIBLE MAMMARY TUMOR MODEL

I obtained a mouse model in the FVB genetic background, which developed mammary tumor upon doxycycline treatment (MMTV-*rtTA*/tet-O-*PyMT*-*IRES*-*Luc* mice). These MMTV-rtTA mice have expression of the reverse tetracycline-controlled transactivator (rtTA) protein directed primarily to the breast epithelia of the mammary ductal system by the mouse mammary tumor virus (MMTV) promoter. These mice are a Tet-On tool that allows conditional, dox-inducible expression of oncogene PyMT primarily in mammary gland epithelial cells (Podsypanina 2008).

To examine the effects of PPARgamma in the Tie2 lineage on breast cancer development, I bred PPARgamma flox/flox Tie2Cre mice with MMTV-*rtTA*/tet-O-*PyMT* mice to obtain PPARgamma KO and WT mice carrying the oncogene for experiments. I then induced tumor development by feeding the mice doxycycline (2mg/ml). The results demonstrated that there was no significant difference in tumor burden between PPARgamma KO and WT mice (**Fig. S8**). The data were inconclusive due to the huge variations among the mice. One possible confound was the amount of doxycycline each mouse consumed. I dissolved doxycycline into the drinking water but I could not control the amount of water each mouse drank.

I also backcross the FVB mice to B6 background since B6 mice were more sensitive to metabolic changes, including PPARgamma deficiency. However, I did not observe any tumor formation after treating the mice with doxycycline for 6-8 weeks. It might take more doxycycline or longer treatment to induce tumor development in these B6 mice.

Figure 1a.

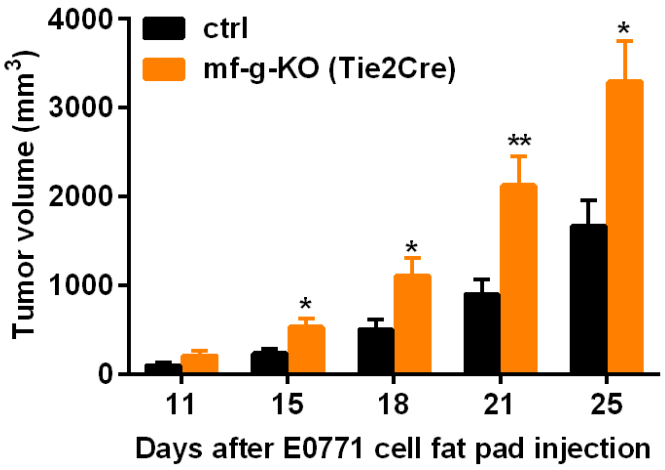

Figure 1b.

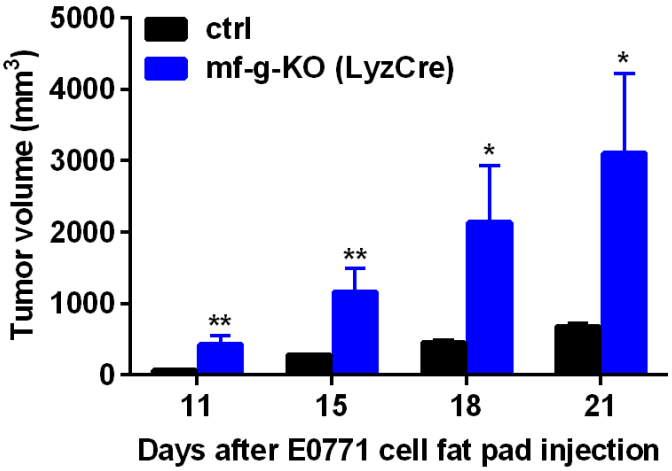

Figure 1. Macrophage PPARgamma deletion enhances mammary tumor growth *in vivo*.

a. Tie2Cre-induced mf-g-KO mice (n=26) showed enhanced tumor growth compared to control mice (n=16) as indicated by earlier onset and larger tumor volume. EO771 mouse mammary tumor cells were injected into the mammary fat pad of 6-8 weeks old female mice.

b. LyzCre-induced mf-g-KO mice (n=6) showed augmented tumor growth compared to control mice (n=6) as indicated by earlier onset and larger tumor volume.

Figure 1c.

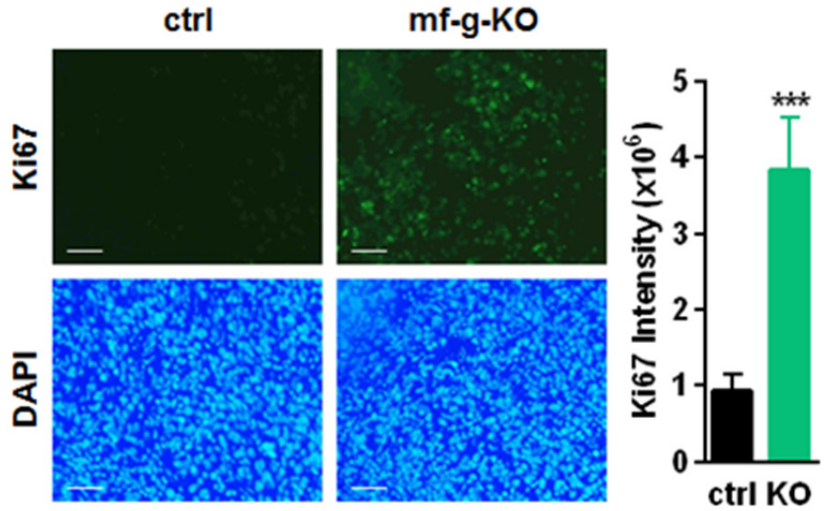

Figure 1d.

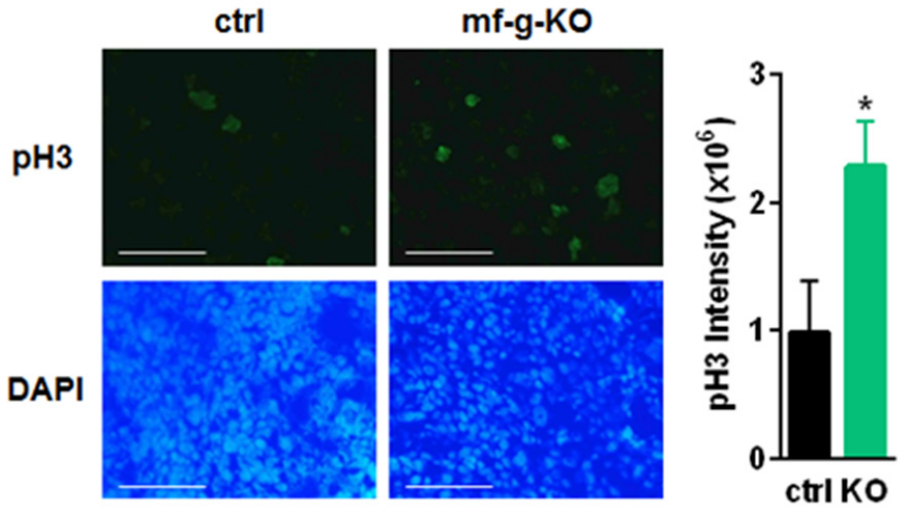

Figure 1. Macrophage PPARgamma deletion enhances mammary tumor growth *in vivo*.

c-d. Quantification of cell proliferation markers Ki67 (**c**) and phosphor histone H3 (PH3) (**d**) in tumor sections showed increased cell proliferation in mf-g-KO mice (n=4).

Figure 1e-g.

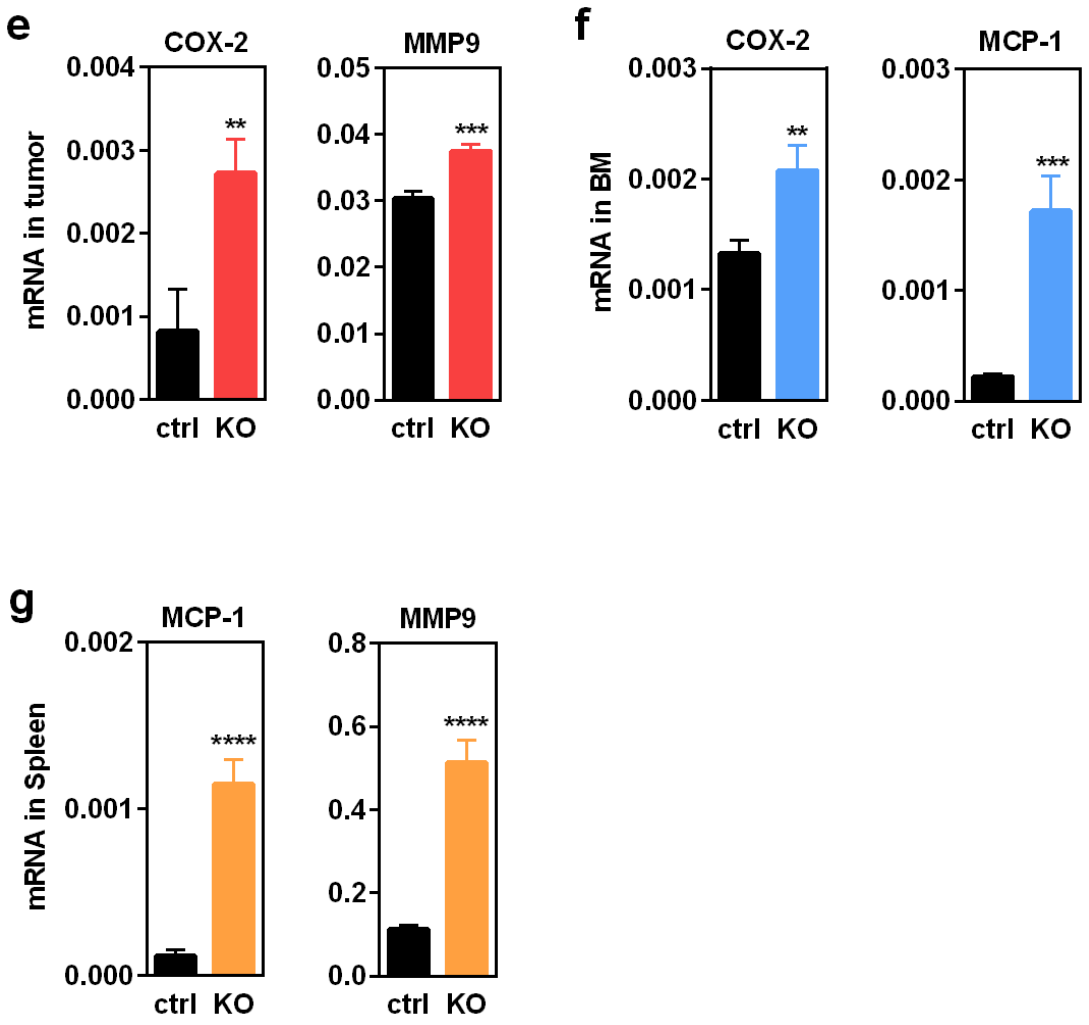

Figure 1. Macrophage PPARgamma deletion enhances mammary tumor growth *in vivo*.

e-g. RT-QPCR analyses showed an increased expression of pro-inflammatory genes in tumor tissues (**e**), bone marrow (BM) (**f**) and spleen (**g**) from mf-g-KO mice (n=3).

Figure 1h.

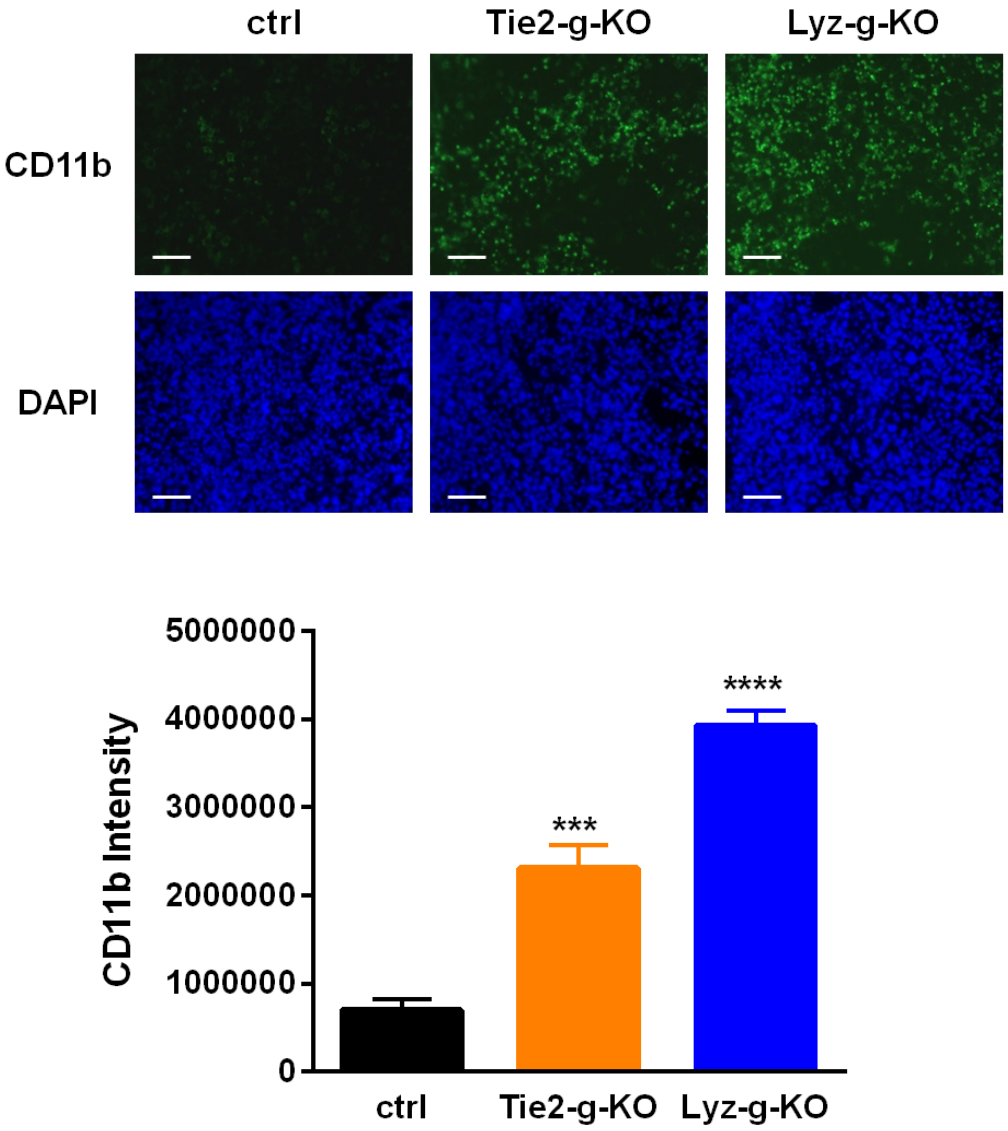

Figure 1. Macrophage PPARgamma deletion enhances mammary tumor growth *in vivo*.

h. Immunofluorescence staining of tumor sections for macrophage marker CD11b showed an enhanced macrophage recruitment in the tumors from both Tie2Cre- and LyzCre-induced mf-g-KO mice compared with control mice (n=4).

Error bars, SD; *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$; n.s. non-significant.

Figure 2a.

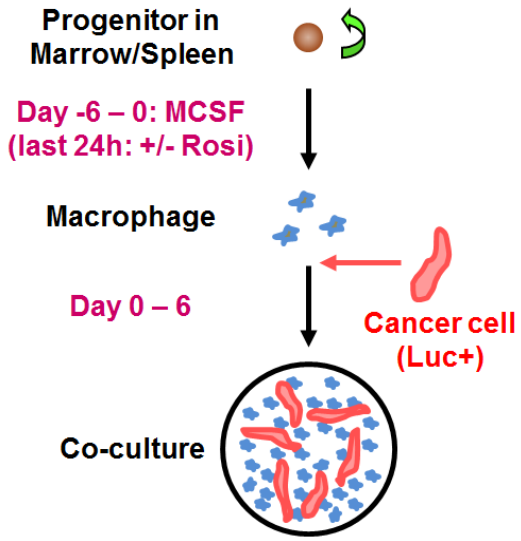

Figure 2. Macrophage PPARgamma deletion exacerbates breast cancer cell proliferation and attenuates the anti-tumor effect of rosiglitazone.

a. A diagram of mouse macrophage and human breast cancer cell co-culture. Progenitors in bone marrow or spleen were differentiated into macrophages with M-CSF for 6 days before the seeding of luciferase-labelled 1833 human breast cancer cells to the cultures. For rosiglitazone (Rosi) pre-treatment, macrophages were treated with 1 μM Rosi or vehicle control for the last 24hrs of macrophage differentiation; after medium were removed and cells were washed, cancer cells were added to the macrophage cultures in fresh medium without Rosi or vehicle.

Figure 2b.

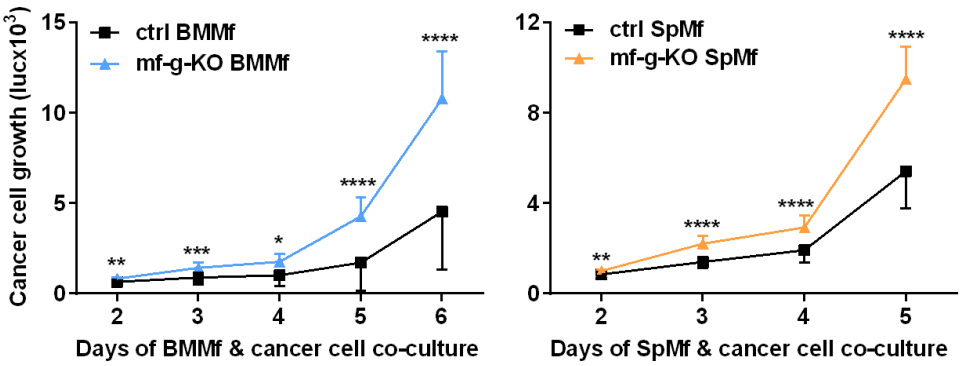

Figure 2c.

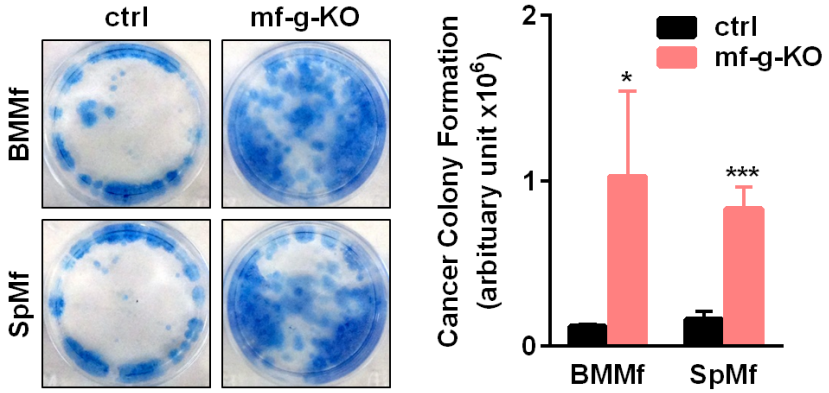

Figure 2. Macrophage PPARgamma deletion exacerbates breast cancer cell proliferation and attenuates the anti-tumor effect of rosiglitazone.

b. Cancer cell proliferation was increased when co-cultured with PPARgamma-deficient macrophages derived from bone marrow (left) or spleen (right) of mf-g-KO mice compared with WT control macrophages (n=3). Cancer cell growth was quantified by luciferase signal for 2-6 days.

c. PPARgamma-deficient macrophages promoted tumor cell colony formation in the co-cultures (n=3). Tumor cells were cultured for 11-12 days for the colonies to form. Left, representative images of crystal violet staining. Right, quantification of colony formation.

Figure 2d-e.

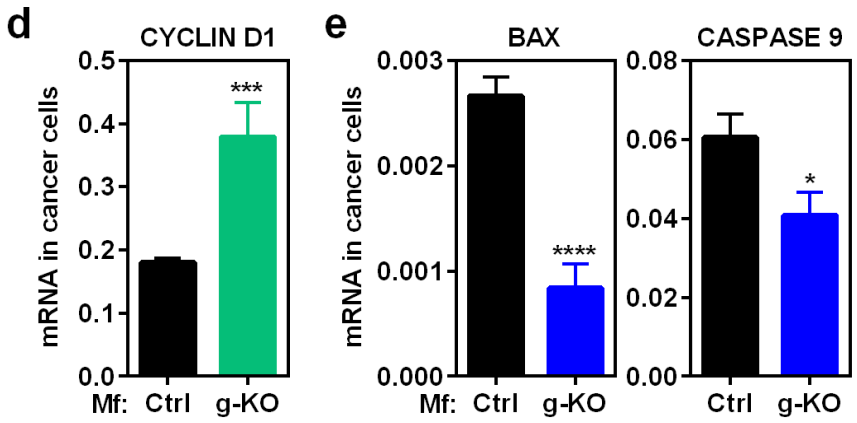

Figure 2f.

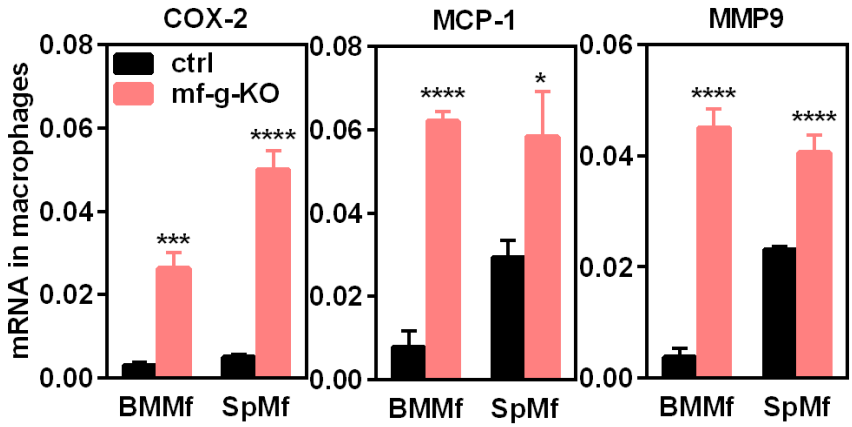

Figure 2. Macrophage PPARgamma deletion exacerbates breast cancer cell proliferation and attenuates the anti-tumor effect of rosiglitazone.

d-e. Co-culture with PPARgamma-deficient macrophages resulted in higher expression of proliferation markers (**d**) and lower expression of apoptosis markers (**e**) in breast cancer cells (n=3). Human gene expression in cancer cells was quantified by RT-QPCR and human-specific primers.

f. PPARgamma-deficient macrophages exhibited higher expression of pro-inflammatory genes (n=3). BMMf, bone marrow macrophage; SpMf, spleen macrophage.

Figure 2g-h.

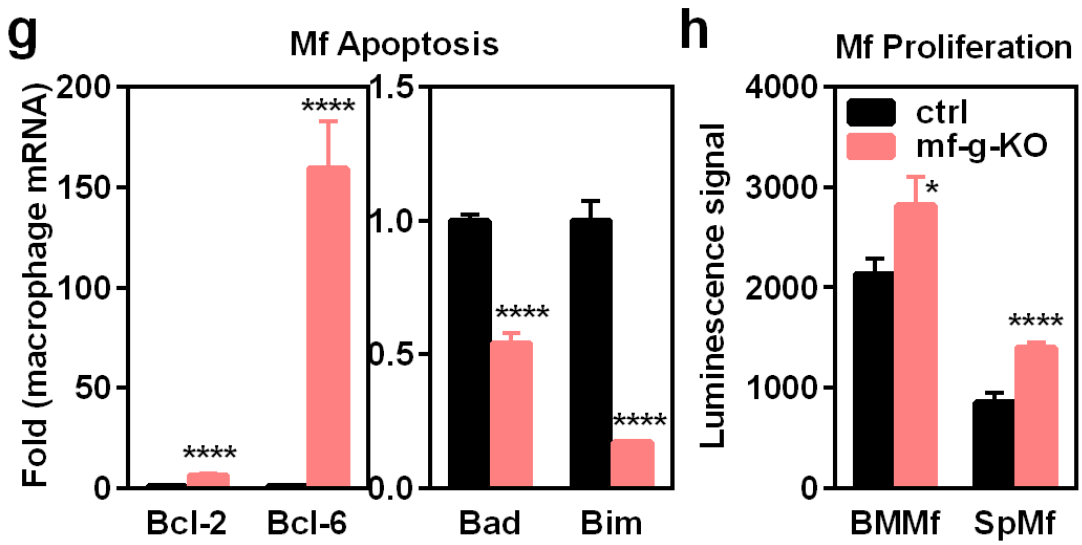

Figure 2. Macrophage PPARgamma deletion exacerbates breast cancer cell proliferation and attenuates the anti-tumor effect of rosiglitazone.

g. PPARgamma-deficient macrophages displayed higher levels of anti-apoptotic genes (left) and lower levels of pro-apoptotic genes (right) (n=3).

h. PPARgamma-deficient macrophages showed increased proliferation (n=3). The number of metabolically active cells was determined by ATP content using the CellTiter-Glo® Assay.

Figure 2i.

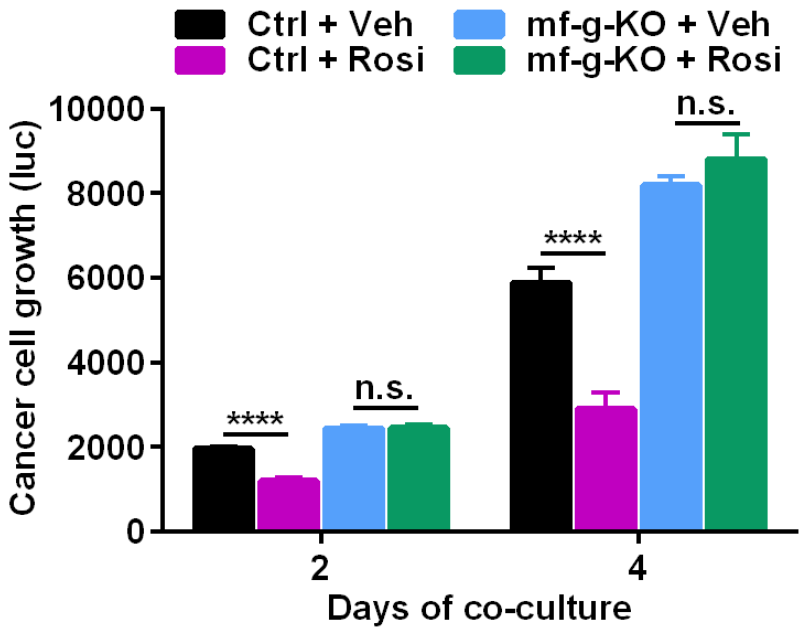

Figure 2. Macrophage PPARgamma deletion exacerbates breast cancer cell proliferation and attenuates the anti-tumor effect of rosiglitazone.

i. Co-culture with Rosi pre-treated macrophages inhibited breast cancer cell growth compared with vehicle (Veh) pre-treated macrophages in a macrophage-PPARgamma-dependent manner (n=3).

Figure 2j.

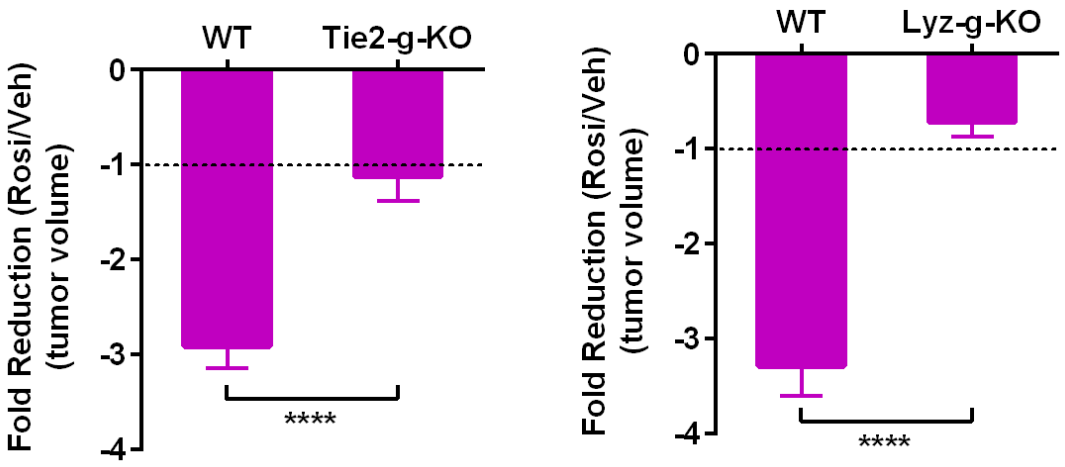

Figure 2. Macrophage PPARgamma deletion exacerbates breast cancer cell proliferation and attenuates the anti-tumor effect of rosiglitazone.

j. The ability of Rosi to suppress tumor growth *in vivo* was significantly attenuated in mf-g-KO mice (n=6). Left: Tie2-g-KO. Right, Lyz-g-KO. Four days after EO771 cell mammary fat pad injection, mf-g-KO mice or control mice were treated with Veh or Rosi (10mg/kg) every two days for one week before tumor volume measurement.

Error bars, SD; *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$; n.s. non-significant.

Figure 2k.

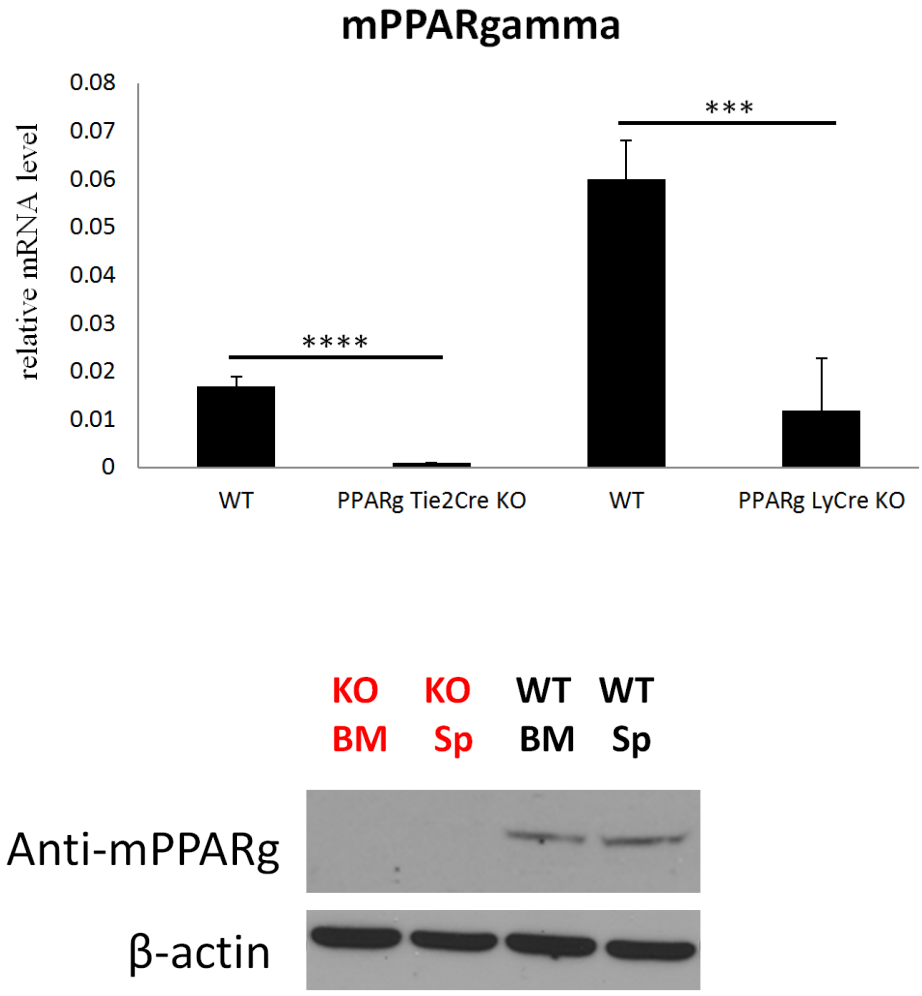

Figure 2. Macrophage PPARgamma deletion exacerbates breast cancer cell proliferation and attenuates the anti-tumor effect of rosiglitazone.

k. PPARgamma mRNA was reduced by about 80-94 % in PPARgamma KO macrophages. This deletion was also observed at the protein level.

Figure 3a.

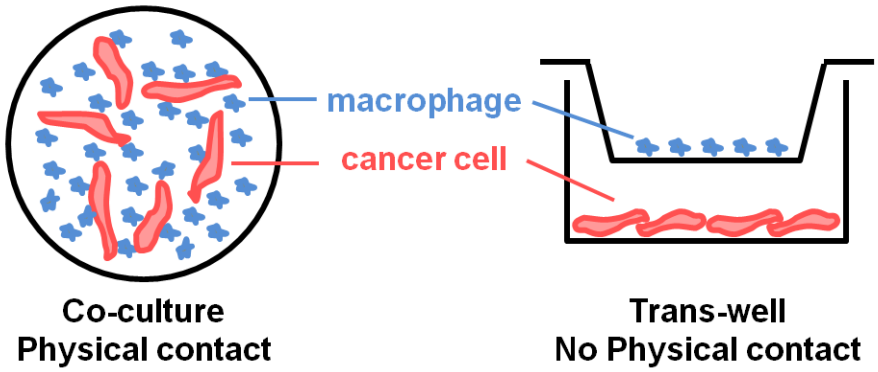

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

Physical contact is required for the pro-tumor effects of PPARgamma-deficient macrophages.

a. A schematic diagram of the co-culture vs. trans-well systems.

Figure 3b.

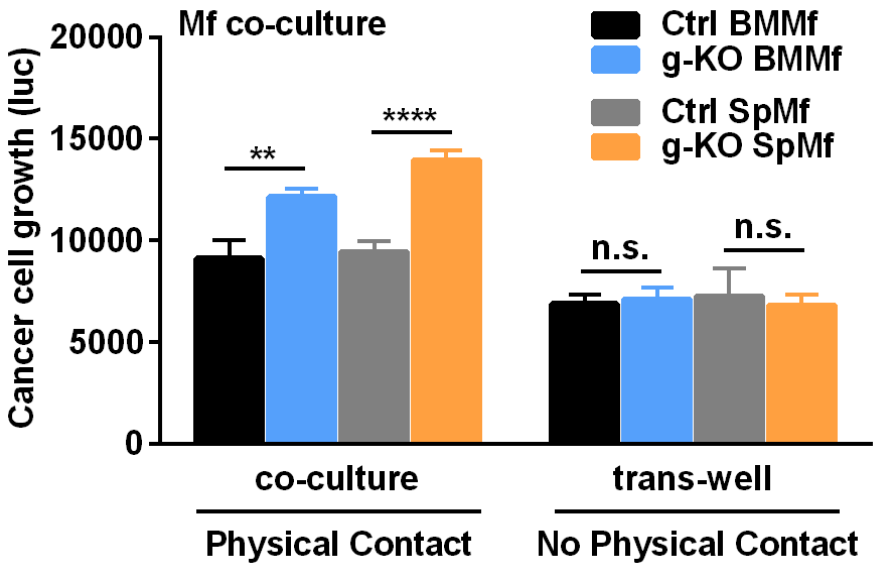

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

Physical contact is required for the pro-tumor effects of PPARgamma-deficient macrophages.

b. Tumor cell proliferation was enhanced by co-culture with PPARgamma-deficient macrophages but not by their conditioned medium delivered via trans-well (n=3).

Figure 3c.

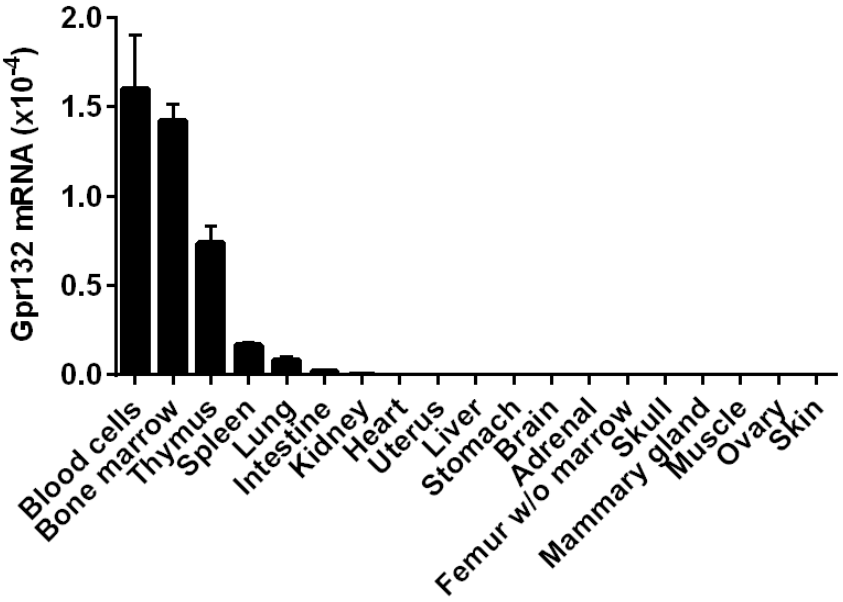

Figure 3d.

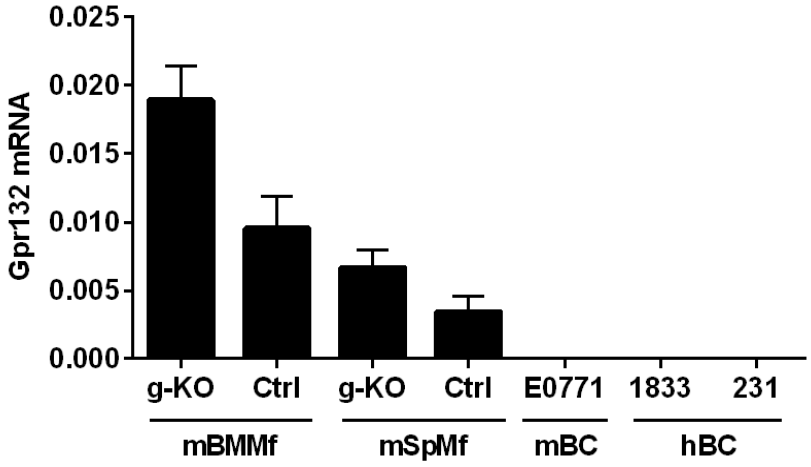

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

c. Gpr132 was predominantly expressed in the hematopoietic cell types and tissues (n=3).

d. Gpr132 was expressed in macrophages but largely absent in breast cancer cells. mBMMf, mouse bone marrow macrophage; mSpmf, mouse spleen macrophage; mBC, mouse breast cancer cells; hBC, human breast cancer cells.

Figure 3e.

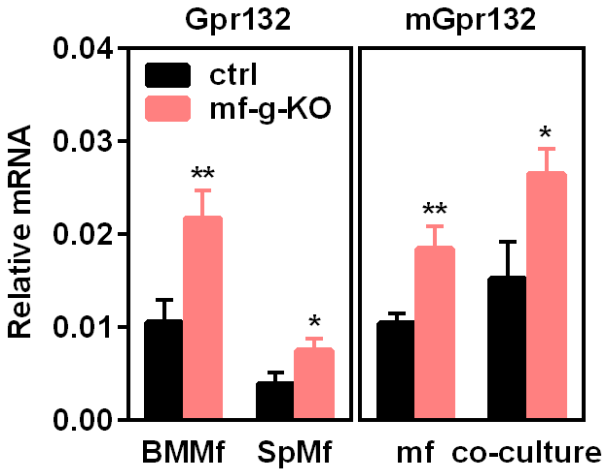

Figure 3f.

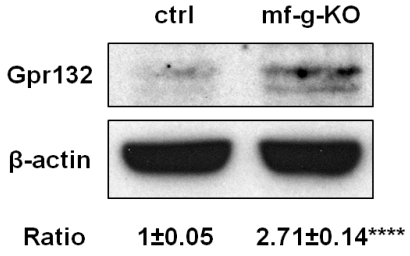

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

e. Gpr132 mRNA levels were significantly higher in PPARgamma-deficient macrophages compared with control macrophages, either in macrophage cultures alone or in macrophages co-cultured with human breast cancer cells (n=3).

f. Gpr132 protein expression was significantly higher in PPARgamma-deficient macrophages (n=3).

Figure 3g.

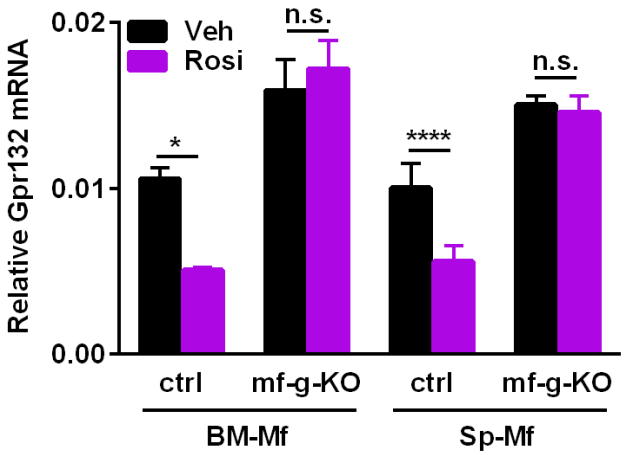

Figure 3h.

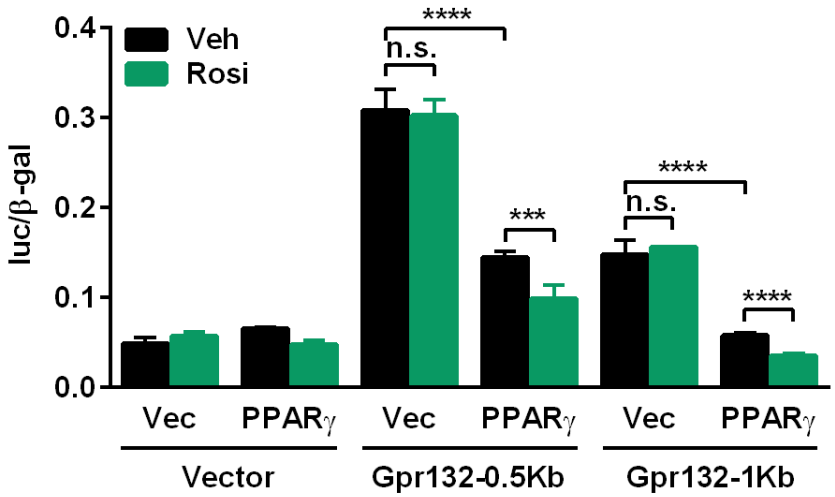

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

g. PPARgamma activation by rosiglitazone reduced Gpr132 mRNA in WT macrophages but not in PPARgamma-deficient macrophages (n=3).

h. Transcriptional output from both 0.5 Kb and 1 Kb Gpr132 promoters was reduced by the co-transfection of PPARgamma and further diminished by rosiglitazone (n=3). HEK293 cells were transfected with PPARgamma and its heterodimer partner retinoic X receptor α

(RXRα), together with a luciferase reporter driven by 0.5 Kb or 1 Kb Gpr132 promoter, and compared with vector-transfected controls. Next day, cells were treated with rosiglitazone or vehicle control for 24 hours before harvest and reporter analyses.

Figure 3i-j.

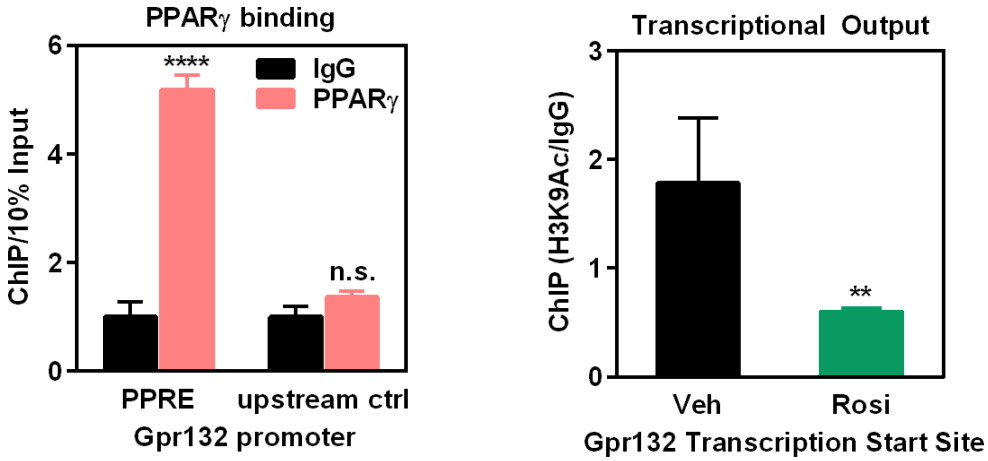

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

i. ChIP assay of PPARgamma binding to the endogenous Gpr132 promoter in macrophages. PPRE region in the Gpr132 promoter was pull-down with anti-PPARgamma antibody or an IgG control antibody in RAW264.7 mouse macrophages and detected by QPCR (n=3). An upstream Gpr132 promoter region served as a negative control.

j. ChIP assay of H3K9Ac active transcription histone mark at the Gpr132 transcription start site showed that rosiglitazone represses the transcriptional activity from Gpr132 promoter (n=3). RAW264.7 mouse macrophages were treated with 1μM rosiglitazone or vehicle control for 4 hours before harvest.

Figure 3k.

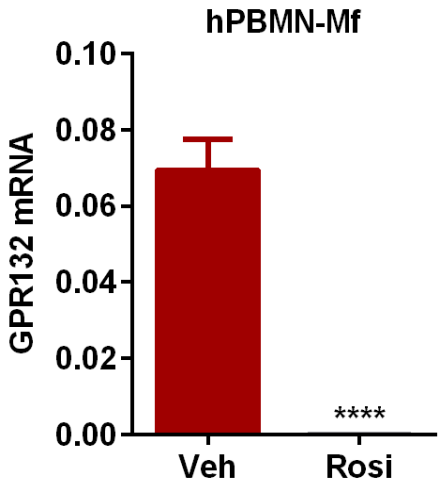

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

k. Human Gpr132 expression in hPBMN-derived macrophages was blunted by rosiglitazone treatment (n=3). Macrophages were treated with 1μM rosiglitazone or vehicle for 4 hours.

Figure 3l.

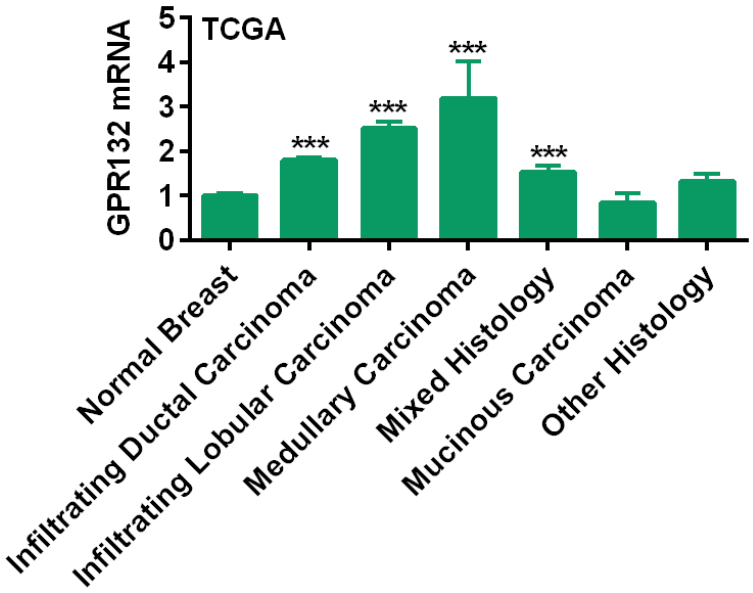

Figure 3m.

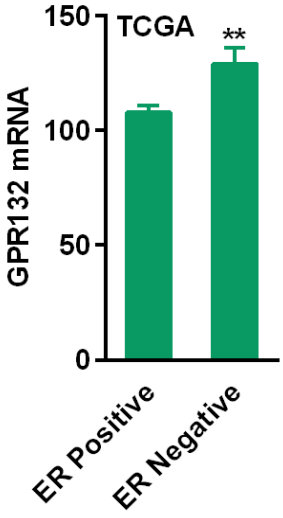

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

l. TCGA BRCA data analysis showed that compared with normal breast samples, breast cancer lesions displayed higher Gpr132 expression. Normal Breast (n=111); Infiltrating Ductal Carcinoma (n=750); Infiltrating Lobular Carcinoma (n=168); Medullary Carcinoma

(n=5); Mixed Histology (n=29); Mucinous Carcinoma (n=14); Other Histology (n=44). Error bars, SE.

m. TCGA BRCA data analysis showed that compared with ER-positive breast cancers (n=746), ER-negative breast cancers (n=221) exhibited higher Gpr132 expression. Error bars, SE.

Figure 3n.

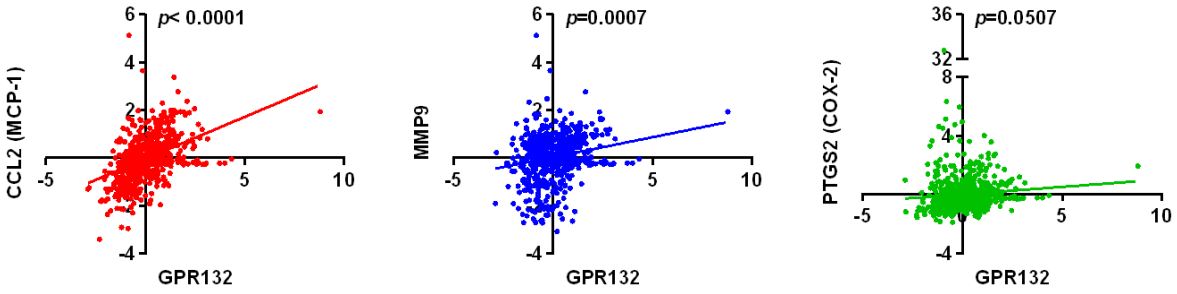

Figure 3. PPARgamma represses Gpr132 transcription in macrophages.

n. Linear regression analyses of TCGA BRCA data showed that Gpr132 expression was positively correlated with the expression of CCL2 (MCP-1), MMP9 and PTGS2 (COX-2) in breast cancer lesions (n=805).

Error bars, SD (**a-k**) or SE (**l-m**); *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001; n.s. non-significant.

Figure 4a-c.

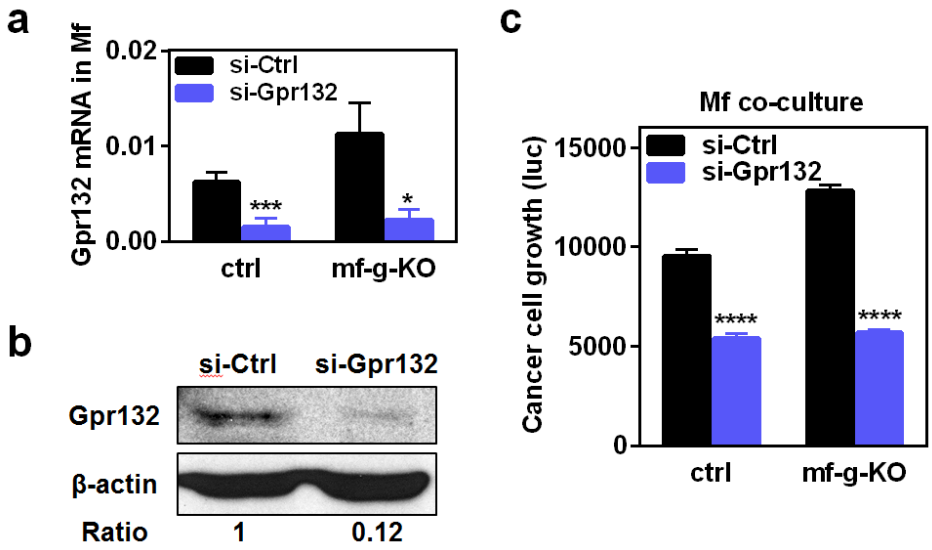

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

a-b. Gpr132 knockdown decreased Gpr132 mRNA (**a**) and protein (**b**) in macrophages (n=3).

c. In co-cultures, Gpr132 knockdown in macrophages reduced cancer cell growth (n=3).

Figure 4d-f.

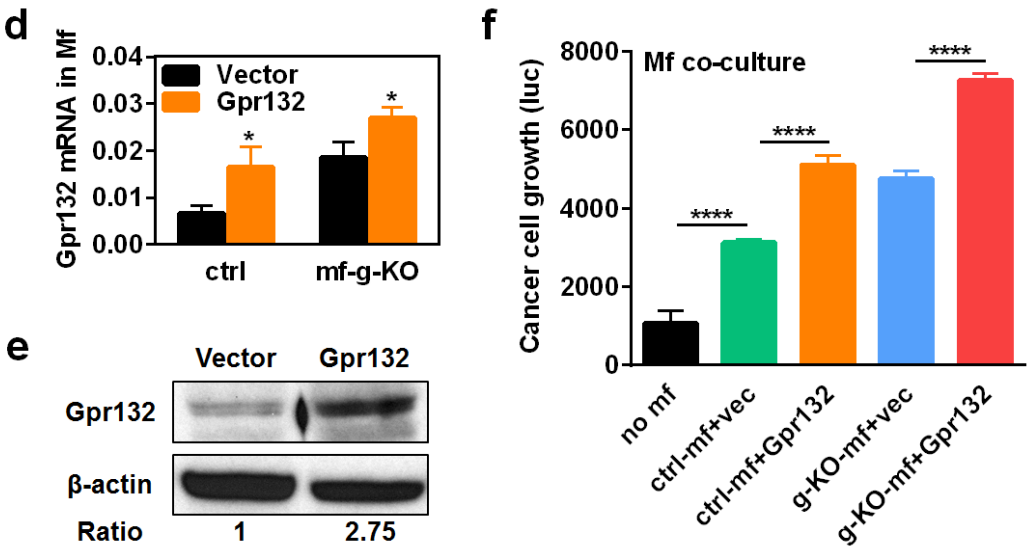

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

d-e. Gpr132 over-expression increased both mRNA (**d**) and protein (**e**) in macrophages (n=3).

f. In co-cultures, Gpr132 over-expression in macrophages enhanced cancer cell growth (n=3). Cancer cell alone without macrophages (no mf) served as a negative control.

Figure 4g.

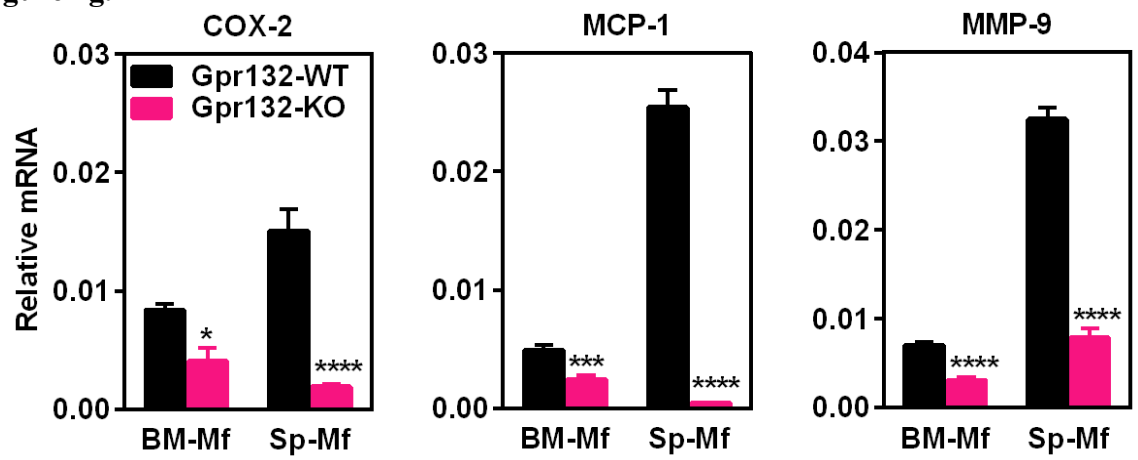

Figure 4h.

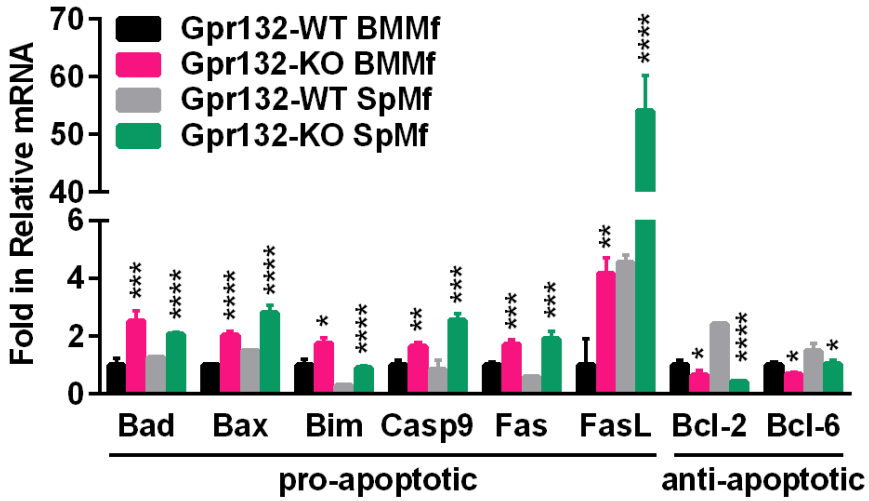

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

g. Gpr132-KO macrophages exhibited lower expression of pro-inflammatory genes compared with WT controls (n=3).

h. Gpr132-KO macrophages displayed higher levels of pro-apoptotic genes and lower levels of anti-apoptotic genes (n=3).

Figure 4i.

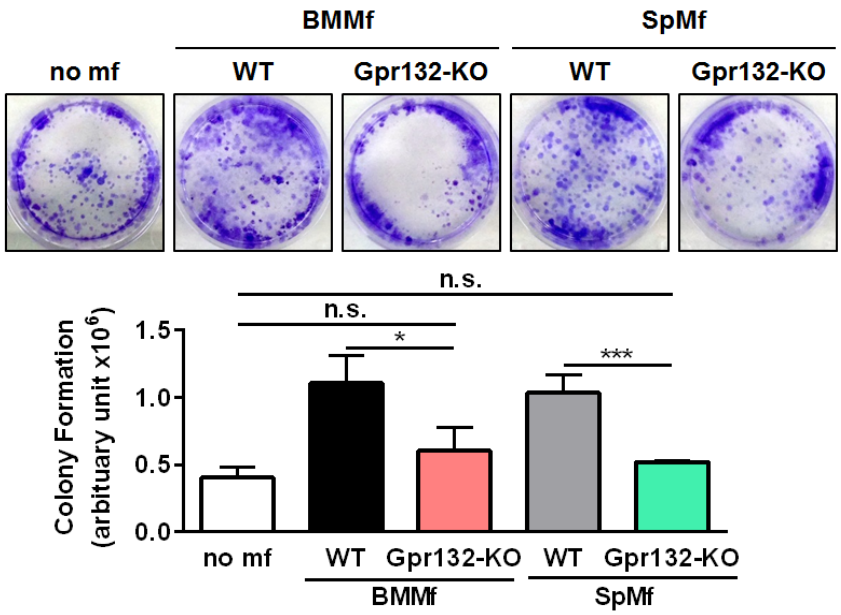

Figure 4j.

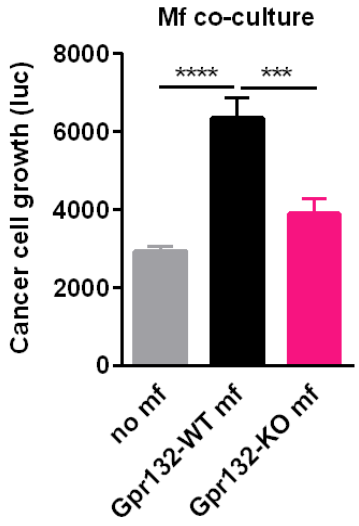

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

i-j. *In vitro* co-cultures showed that Gpr132 deletion in macrophages significantly reduced the ability of macrophages to promote cancer cell colony formation (**i**) and proliferation (**j**) (n=3).

Figure 4k.

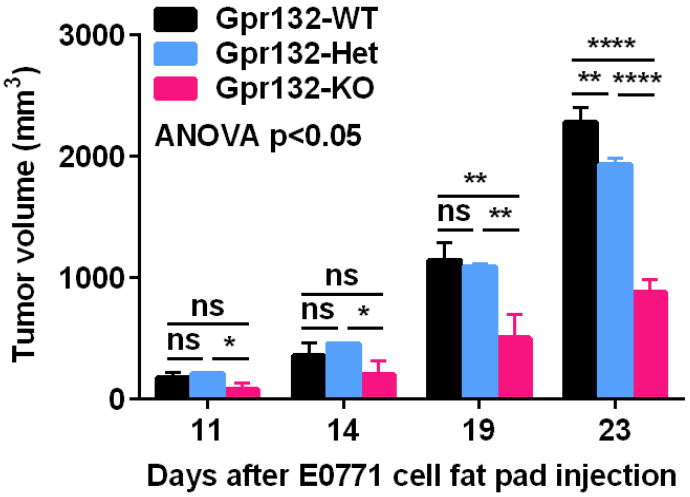

Figure 4l.

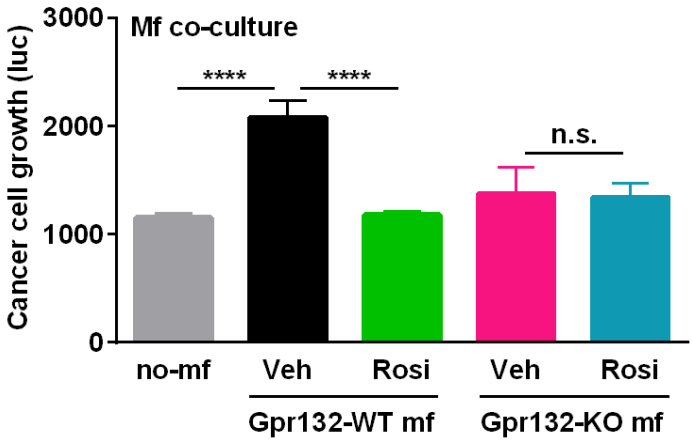

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

k. *In vivo* mammary fat pad tumor grafts showed that tumor growth was significantly diminished in Gpr132-KO mice compared with WT or Gpr132-Het mice (n=6).

l. In *in vitro* co-cultures, Rosi pre-treated WT macrophages but not Rosi pre-treated Gpr132-KO macrophages was able to inhibit cancer cell growth (n=3).

Figure 4m.

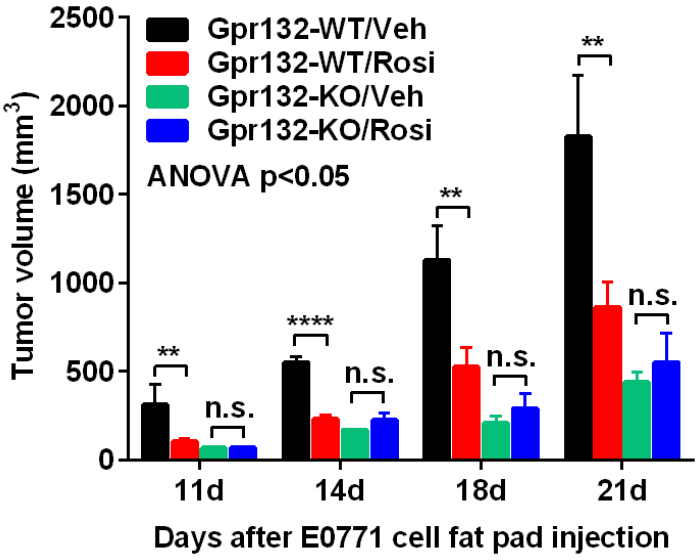

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

m. The ability of Rosi to suppress tumor growth *in vivo* was abolished in Gpr132-KO mice (n=6). Four days after EO771 cell mammary fat pad injection, Gpr132-KO or WT mice were treated with Veh or Rosi (10mg/kg) every two days.

Figure 4n.

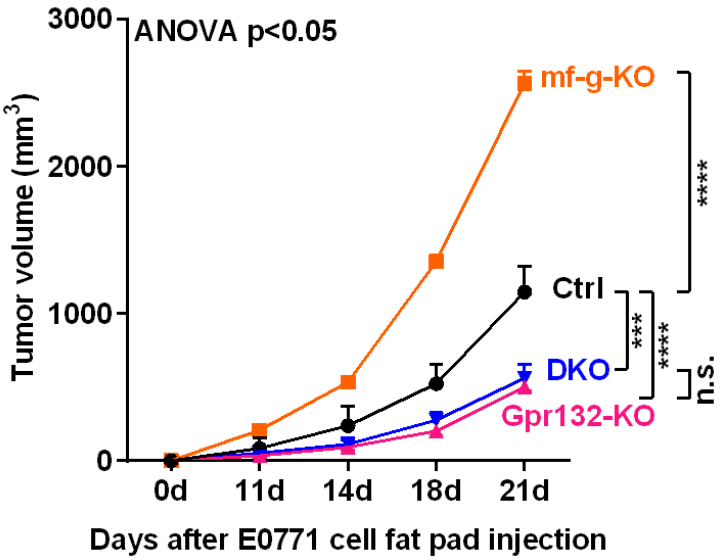

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

n. The ability of macrophage PPARgamma deletion to exacerbate tumor growth *in vivo* was abolished in Gpr132-KO mice (n=4). DKO, mf-g/Gpr132 double KO.

Figure 4o.

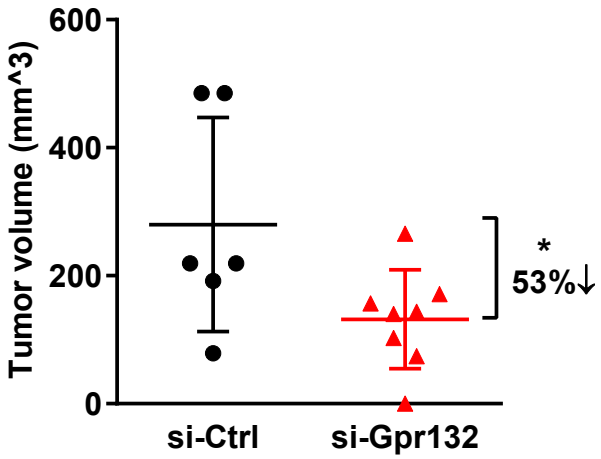

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

o. Pharmacological Gpr132 inhibition impeded mammary tumor growth. Female mice were treated with si-Gpr132 (n=8) or si-Ctrl (n=6) for 18 days via intravenous injection at 10μg/mouse twice/week, 3 days before and 15 days after EO771 cell mammary fat pad injection.

Error bars, SD; *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$; n.s. non-significant.

Figure 4p.

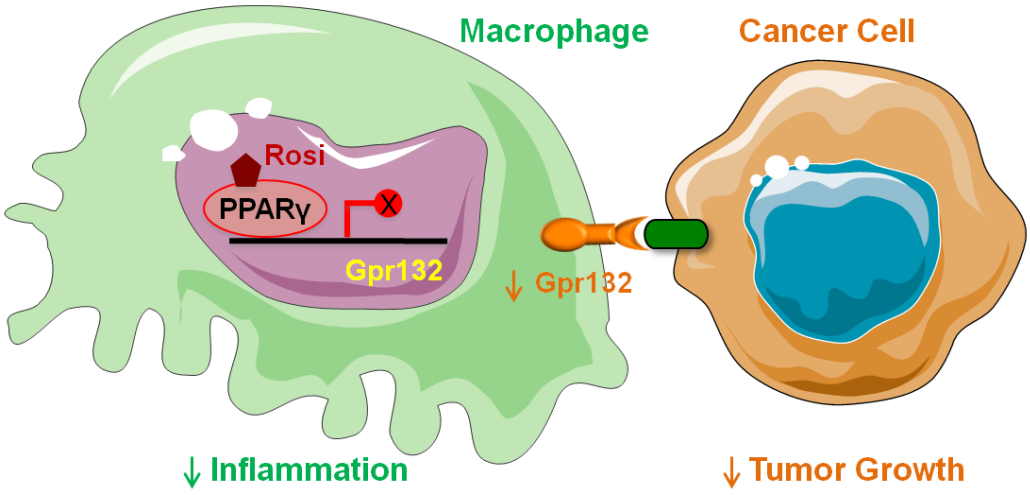

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

p. A simplified model for how macrophage PPARgamma inhibits inflammation and tumor growth by repressing the transcription of macrophage Gpr132, a novel pro-inflammatory and pro-tumor membrane receptor. Pharmacological PPARgamma activation or Gpr132 blockade attenuates breast cancer progression. Moreover, both macrophage PPARgamma and Gpr132 are key mediators of the anti-tumor effects of the clinically used TZD drug rosiglitazone.

Figure 4q.

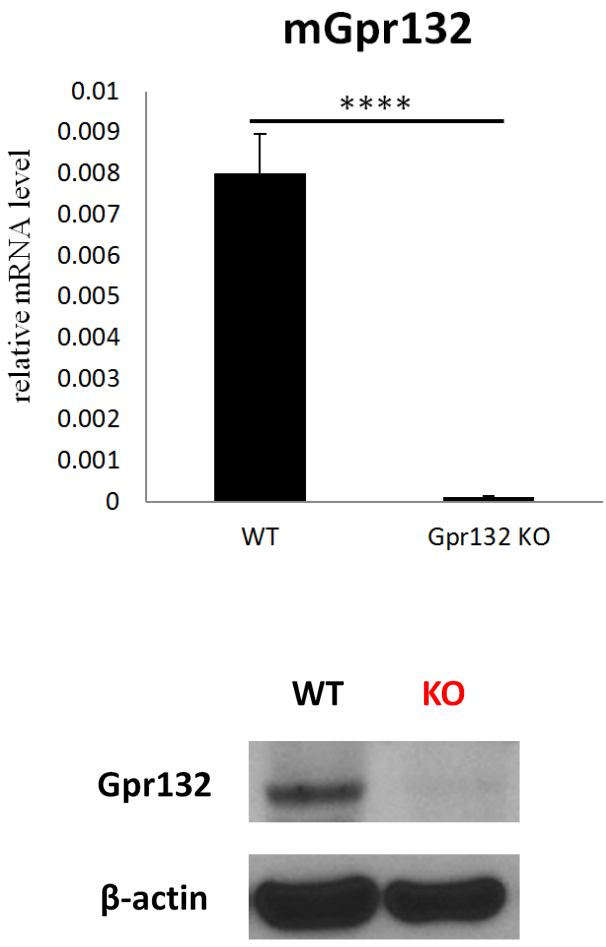

Figure 4. Macrophage Gpr132 promotes tumor growth and mediates the anti-tumor effect of rosiglitazone

q. Expression of Gpr132 was reduced by about 99% in Gpr132 KO macrophages.

Figure S1a.

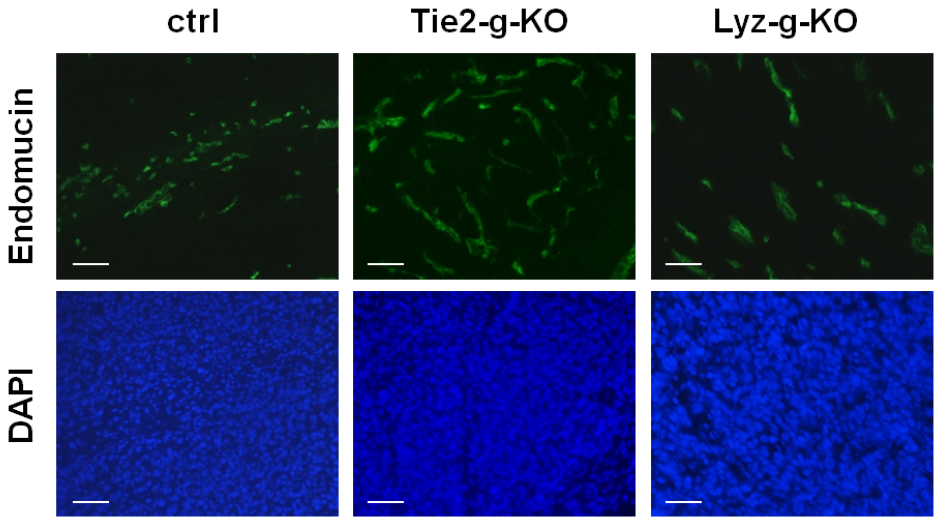

Figure S1b.

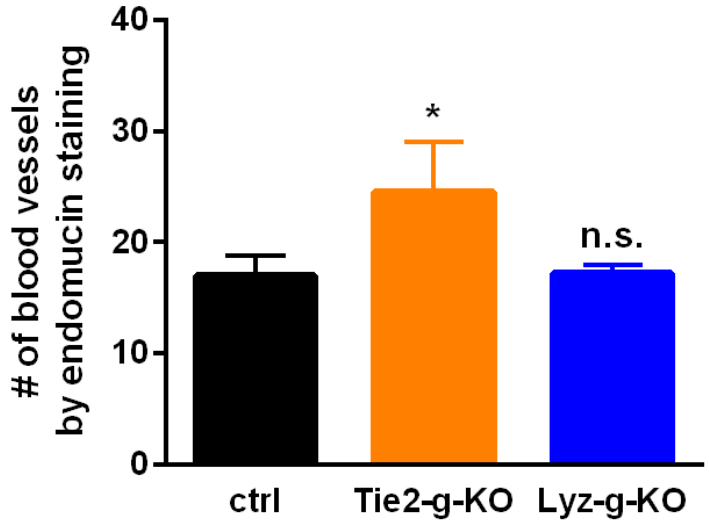

Figure S1. Angiogenesis was increased in Tie2Cre-PPARgamma-KO mice but not in LyzCre-PPARgamma-KO mice

a. Tumor sections were analyzed by immunofluorescence staining for an endothelial marker endomucin (n=4). Representative images of staining; scale bars, 25μm.

b. Quantification of the number of blood vessels. Error bars, SD.

Figure S2.

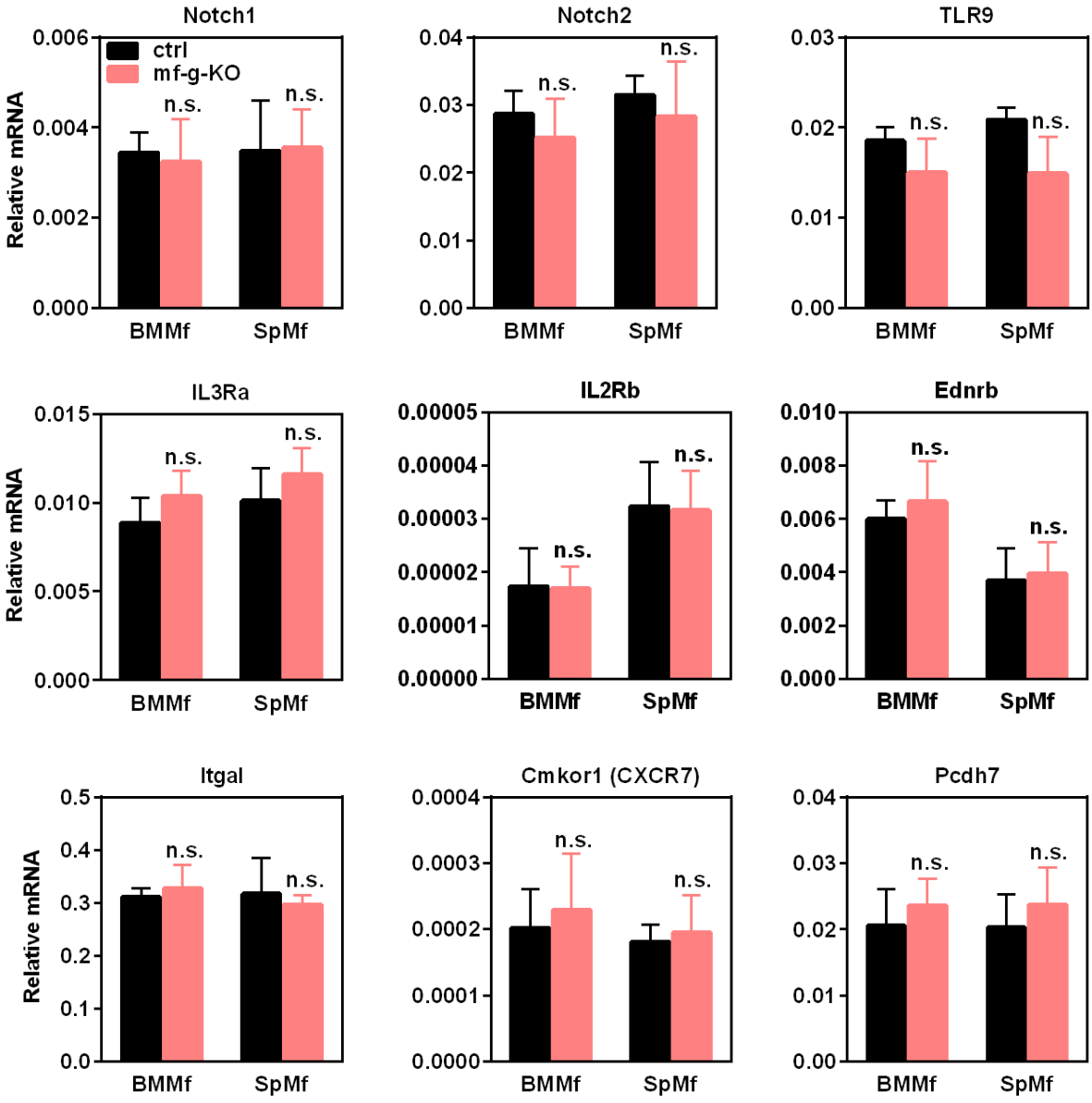

Figure S2. Expression of other candidate genes was unaltered in PPARgamma-deficient macrophages

Candidate genes that encode membrane proteins and are potentially regulated by macrophage PPARgamma were selected from published microarray databases. Their expression in bone

marrow macrophages (BMMf) or spleen macrophages (SpMf) derived from mf-g-KO mice or littermate control mice were quantified by RT-QPCR (n=3). LGR5 and CCR4 expression was also examined, but the expression was too low to detect. Error bars, SD.

Figure S3.

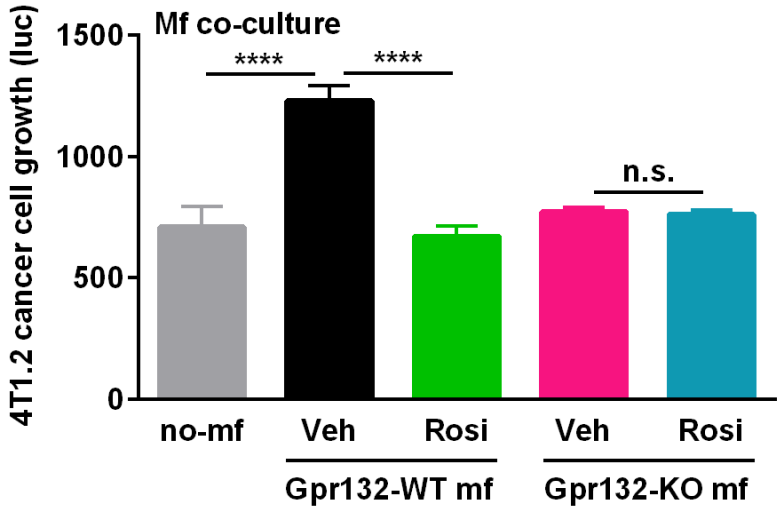

Figure S3. Gpr132-null macrophages were refractory to the anti-tumor effects of rosiglitazone pre-treatment

Rosiglitazone pre-treatment of WT macrophages, but not Gpr132-KO macrophages, inhibited the growth of luciferase-labeled 4T1.2 mouse mammary tumor cells in the co-cultures (n=3). Error bars, SD.

Figure S4.

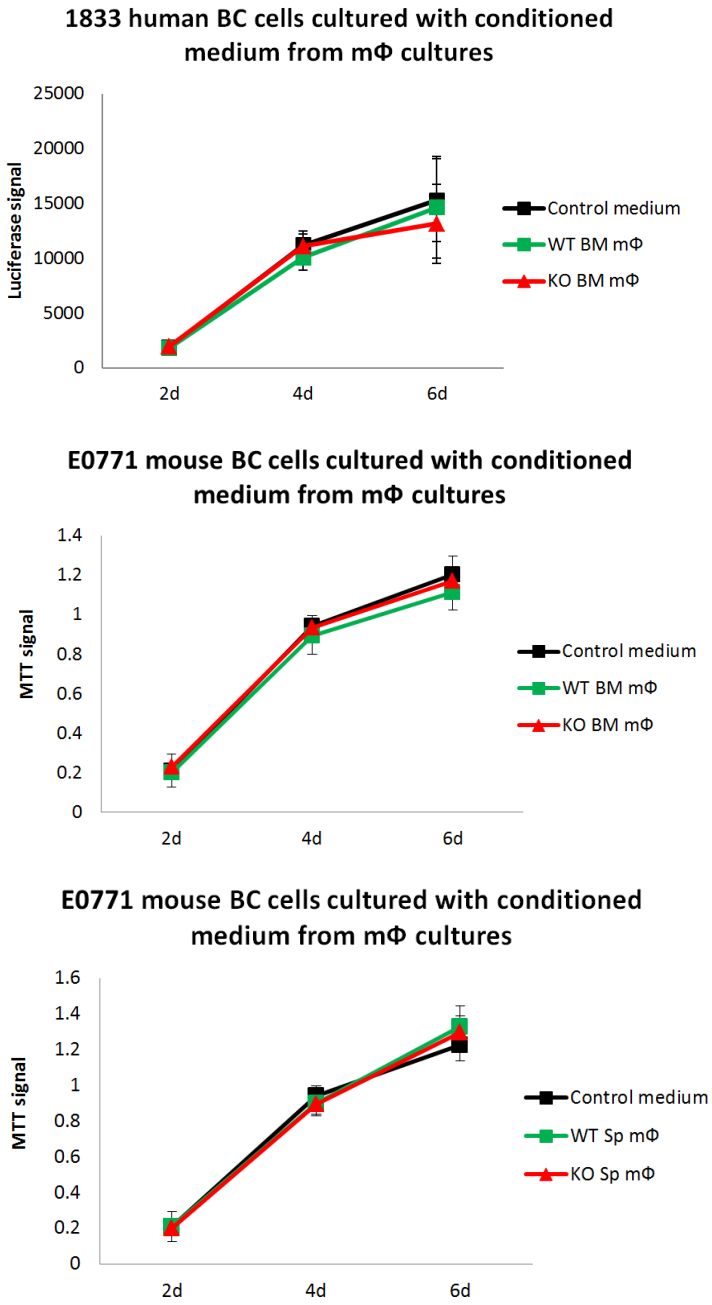

Figure S4. Conditioned medium experiments showed that the key mediator of PPARgamma' effects on cancer cells was not a secreted molecule. Growth of 1833 human

breast cancer cells was assessed by luciferase assay while growth of E0771 mouse breast cancer cells was assessed by MTT assays (n=3).

Figure S5a-b.

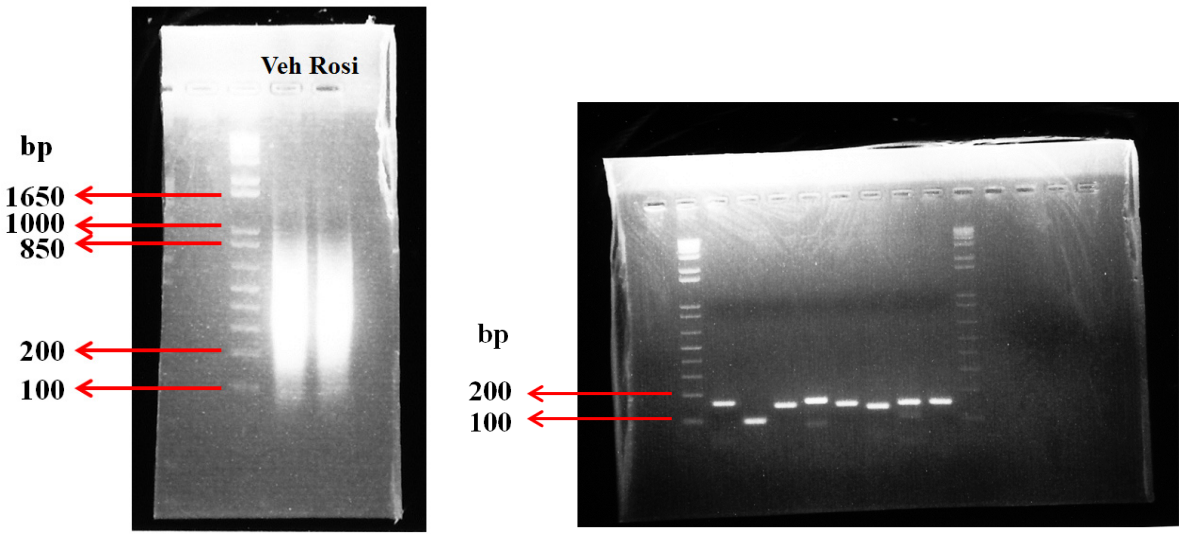

Figure S5. Troubleshooting the ChIP protocol

a. Test efficacy of sonication process.

b. Primers were able to detect specific regions of Gpr132 promoter.

Figure S6a.

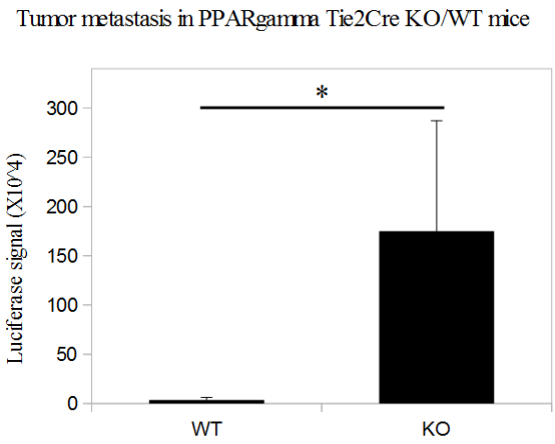

Figure S6b.

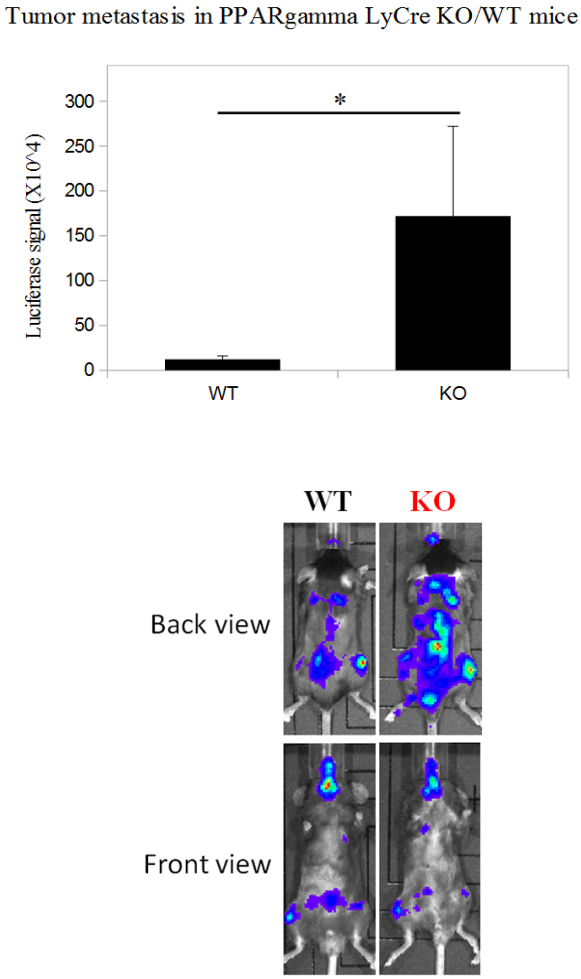

Figure S6. PPARgamma deficiency enhanced cancer metastasis

a. Cardiac injection of 5X10^5 B16 melanoma cells per mouse was performed on PPARgamma Tie2Cre KO mice and wild type controls. The quantification of luciferase signals 20 days after cancer cell injection were shown.

b. Cardiac injection of 5X10^5 B16 melanoma cells per mouse was performed on PPARgamma LyCre KO mice and wild type controls. The quantification of luciferase signals 20 days after cancer cell injection were shown.

Figure S7.

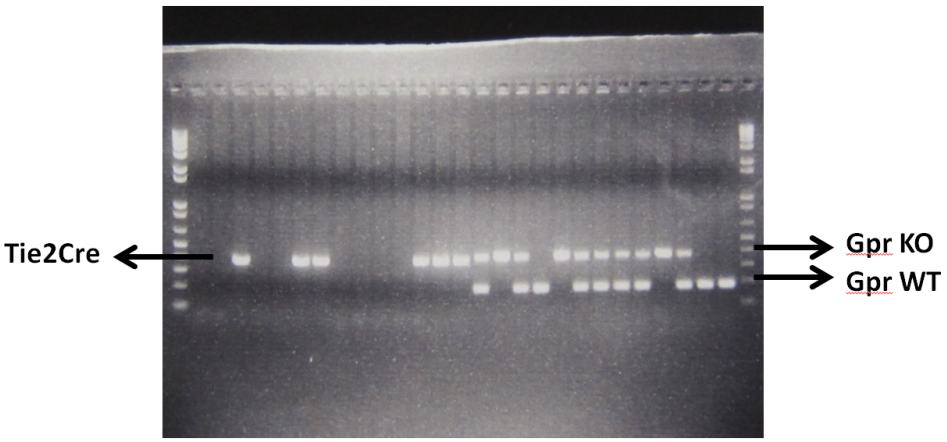

Figure S7. Confirming the genotype of mice by PCR

The band indicating either Tie2Cre or LyCre was present in all PPARgamma KO mice and absent in all wild type controls. All of the PPARgamma mice were confirmed to be PPARgamma flox/flox.

Only the Gpr132 WT band was detected in WT mice. Only the Gpr132 KO band was detected in Gpr132 KO mice. The heterozygous mice had both the WT and KO band.

Figure S8.

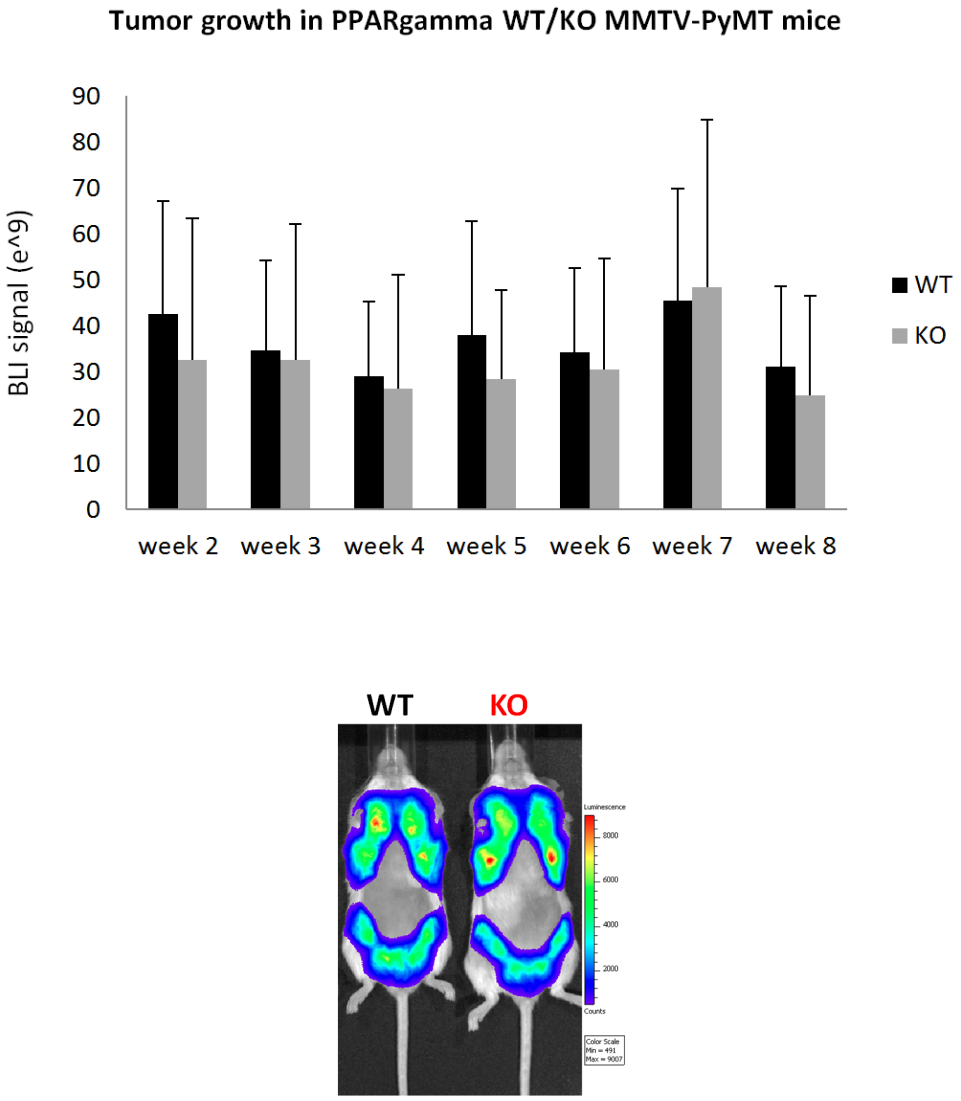

Figure S8. Doxycycline-inducible mammary tumor model showed that there was no difference in tumor burden between PPARgamma KO and WT mice.

Figure S9.

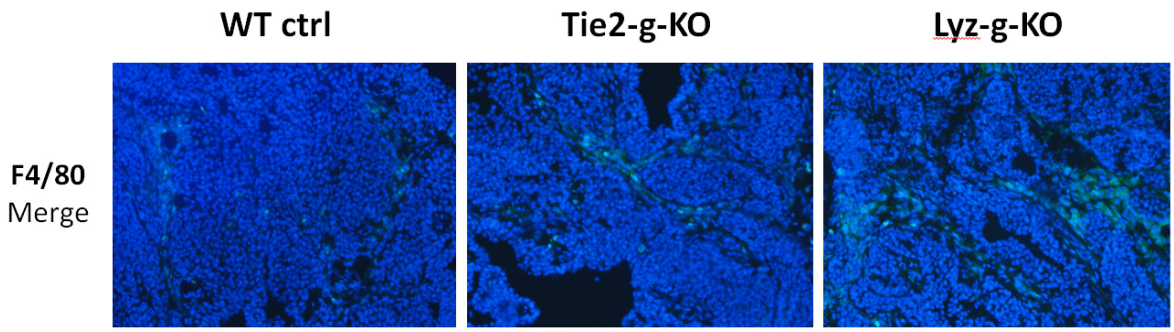

Figure S9. Staining for F4/80 demonstrated an enhanced macrophage recruitment in the tumors from PPARgamma KO mice.

Immunofluorescence staining of tumor sections for macrophage marker F4/80 showed an enhanced macrophage recruitment in the tumors from both Tie2Cre- and LyzCre-induced mf-g-KO mice compared with control mice (n=4).

MATERIALS AND METHODS

MICE

PPARgamma flox mice on a C57BL/6 background have been previously described (He 2003). Gpr132 knockout mice (Le 2001) on a C57BL/6 background were obtained from the Jackson Laboratory. Mice were fed standard chow ad libitum and kept on a 12-h light, 12-h dark cycle. PPARgamma flox mice were bred with Tie2-Cre (Kisanuki 2001) or Lysozyme-Cre (Clausen 1999) transgenic mice to generate mf-g-KO mice. Tie2cre-g-KO was bred with Gpr132-KO to obtain mf-g/Gpr132 double KO mice. All experiments were conducted using littermates. Sample size estimate was based on power analyses performed using SAS 9.3 TS X64_7PRO platform. All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee of UTSW.

ORTHOTOPIC FAT PAD INJECTION OF MOUSE BREAST CANCER CELLS

The EO771 cell line was derived from a spontaneous mammary tumor in a C57BL/6 mouse (Casey 1951). EO771 cells ($2.5x10^5$ or $5x10^5$) were injected into the mammary fat pad of 6-8 weeks old female mice. EO771 cell mixture was prepared with a 1:1 ratio in blank RPMI-1640 medium and matrigel (BD Biosciences). Every 2-3 days, tumor length and width were measured with a caliper and tumor volume was calculated using the formula V = (L x W x W) / 2, where V is tumor volume, L is tumor length, and W is tumor width.

IMMUNOHISTOCHEMISTRY

Tumor tissues were isolated from tumor-bearing mice three weeks after cancer cell injection. Tumors were frozen in OCT compound (Tissue-Tek), cryo-sectioned, and fixed with acetone before staining with antibodies. The tumor sections were blocked with 2% BSA, and then incubated with FITC anti-mouse CD11b antibody (BD Pharmingen; 1:50 dilution), rat monoclonal anti-endomucin antibody (Santa Cruz Biotechnologies; 1:50 dilution), Ki67 rabbit antibody (Cell Signaling; 1:400 dilution), or Phospho-Histone H3 (Ser10) antibody (Cell Signaling; 1:200 dilution). After washing with PBS, the sections were incubated with goat anti-rat IgG-FITC antibody or goat anti-rabbit IgG-FITC antibody (Santa Cruz Biotechnologies; 1:100 dilutions) for detection. After washing with PBS, cover slips were mounted with the Vectashield medium containing DAPI (Vector Laboratories Burlingame, CA, USA).

MACROPHAGE AND CANCER CELL CULTURES

For bone marrow-derived macrophage (BMMf) and spleen-derived macrophage (SpMf), mouse bone marrow or splenocyte were collected with serum-free DMEM (Invitrogen). After passing through a 40 μm cell strainer, the cells were cultured in macrophage differentiation medium (DMEM containing 10% fetal bovine serum (FBS) and 20ng/ml M-CSF) for 6 days. Rosiglitazone (Cayman Chemical) treatment was conducted at 1 μM. Gpr132 overexpression was performed with lentiviral transduction. The luciferase-labeled MDA-MB-231 human

breast cancer cell sub-line (1833) (Kang 2003) was generously provided by Dr. Joan Massagué (Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center). The luciferase-labeled 4T1.2 mouse breast cancer cell subline (Lelekakis 1999) was generously provided by Drs. Robin Anderson (Peter MacCallum Cancer Centre) and Yibing Kang (Princeton University).

CO-CULTURE OF MACROPHAGES AND CANCER CELLS

Mouse bone marrow and spleen cells were plated in 96-well plate and differentiated into macrophages with 20ng/ml M-CSF for 9 days. Luciferase-labeled 1833 cells or 4T1.2 cells were then added to the culture dish. At the end point of experiment, cell lysates were collected for luciferase assay to assess cancer cell growth. For the pre-treatment, macrophages were cultured with 1 μM rosiglitazone for 24 hrs; the medium was removed and the macrophages were washed before cancer cell seeding.

GENE EXPRESSION ANALYSES

Tissue samples were snap frozen in liquid nitrogen and stored at −80°C. RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. RNA was first treated with RNase-free DNase I using the DNA-free kit (Ambion) to remove all genomic DNA, and then reverse-transcribed into cDNA using an ABI High Capacity cDNA RT Kit (Invitrogen). The cDNA was analyzed using real-time quantitative PCR (SYBR Green, Invitrogen) with an Applied Biosystems 7700 Sequence Detection System. Each reaction was performed in

triplicate in a 384-well format. The expression of mouse gene was normalized by mouse L19. The expression of human gene was normalized with human GAPDH. Anti-Gpr132 antibody (Sigma) was used for western blot detection of Gpr132 protein.

TCGA DATA ANALYSIS

RNA-Seq and clinical data of breast invasive carcinoma (BRCA) were downloaded from The Cancer Genome Atlas (TCGA) data portal (Cancer Genome Atlas 2012) and tested for associations. Gene expression for GPR132, CCL2 (MCP-1), MMP9 and PTGS2 (COX-2) were analyzed by linear regression.

STATISTICAL ANALYSES

All statistical analyses were performed with Student's t-Test and represented as mean ± standard deviation (SD) unless noted otherwise. All data are representative of at least three experiments. For *in vivo* experiments with >=3 groups, statistical analyses were performed with ANOVA followed by the post hoc Tukey pairwise comparisons. The p values were designated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$; n.s. non-significant ($p>0.05$).

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