

Exploring the metabolic role of GPR30 in mice

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Academic Abstract

Recent studies showed that GPR30, a seven-transmembrane G protein-coupled receptor, is a novel estrogen receptor (ER) that mediates some biological events elicited by estrogen in several types of cancer cells. However, its physiological or pathological role *in vivo* is unclear. For the first project of my dissertation, I investigated the physiological role(s) of GPR30 in energy metabolism by using transgenic mouse model as well as immortalized cell lines and primary stromal cells.

We discovered for the first time that GPR30 knockout (GPRKO) female mice were protected from high-fat diet (HFD)-induced obesity, glucose intolerance, and insulin resistance. The decreased body weight gain in GPRKO female mice is due to the reduction in body fat mass. These effects occurred in the absence of significant changes in food intake, intestinal fat absorption, or triglyceride metabolism. However, GPR30 had no significant metabolic effects in male mice fed the HFD and both sexes of mice fed a chow diet. Further, GPR30 expression levels in fat tissues of WT obese female mice greatly increased, whereas ER α/β expression was not altered. Deletion of GPR30 reduced adipogenic differentiation of adipose tissue-derived stromal cells. Conversely, activation of GPR30 enhanced adipogenic differentiation of 3T3-L1 preadipocytes.

For the second project, I explored whether estrogen acts through GPR30 to affect adiposity in female mice. For this study, I generated and examined three independent transgenic mouse models, aromatase (Ar) knockout (ArKO) mice, GPRKO, and GPR30 and Ar double knockout (DKO) mice. We discovered that GPR30 deficiency had limited effects on energy metabolism in

mice fed a standard chow diet (STD). However, deletion of GPR30 promoted metabolic flexibility in both genders fed a HFD regardless of the presence of estrogen, suggesting that GPR30 may not solely act as an ER. Consistent with our previous findings, GPRKO mice had higher body temperature, indicating that GPR30 deficiency may promote thermogenesis and energy metabolism, resulting in the reduced fat depots and enhanced metabolic flexibility. For the third project, I further explored whether GPR30 is involved in regulating browning of adipose tissue and thermogenesis in mice. The results show that the expression of UCP-1, the key regulator of thermogenic browning, was higher in the adipose tissue of HFD-fed GPRKO female mice as compared with that of WT mice. Consistently, deletion of GPR30 enhanced mitochondrial respiration in brown adipose tissue (BAT), suggesting that GPR30 deficiency at least partially suppressed the fat accumulation by promoting thermogenesis and dissipating energy. *Ex vivo*, the expression of thermogenic genes and UCP-1 protein level were upregulated in beige adipocytes differentiated from GPR30-deficient stromal vascular fraction (SVF) cells.

These findings provide evidence for the first time that deletion of GPR30 reduces adiposity, promotes white adipose beigeing and thermogenesis, therefore preventing the development of obesity in female mice exposed to excess energy. Further investigations elucidating the underlying mechanism by which GPR30 promotes obesity in females could provide a novel therapeutic target to fight obesity in females.

Keywords: GPR30, estrogen, obesity, energy metabolism, browning

General Audience Abstract

Estrogen can elicit pleiotropic genomic and rapid nongenomic cellular responses via a diversity of estrogen receptors (ERs). Unlike the genomic responses, where the classical nuclear ER α and ER β initiate gene transcription in estrogen target tissues, the nongenomic cellular responses to estrogen are believed to start at the plasma membrane, leading to rapid activation of second messenger-triggered cytoplasmic signal transduction cascades. The recently acknowledged ER, GPR30, was discovered in human breast cancer cells two decades ago and subsequently in many other cells. Since its discovery, it has been claimed that estrogen, ER antagonist fulvestrant, as well as some estrogenic compounds can directly bind to GPR30, and therefore initiate the rapid nongenomic cellular responses. We are interested to investigate the physiological role(s) of GPR30 in energy metabolism by using transgenic mouse model as well as immortalized cell lines and primary stromal cells.

We discovered for the first time that deletion of GPR30 protects female mice from high fat-diet (HFD)-induced obesity and the expression of GPR30 increased in fat tissues of wild type (WT) obese mice, while no alterations of classical ER α/β observed. Consistently, activation of GPR30 by the selective agonist G-1 promotes adipogenic differentiation of 3T3-L1 preadipocytes. ER α is known to exert a protective effect against excess fat accumulation whereas GPR30 may acts as an “obesity gene” and counteracts the classical ER α 's action in regulating fat metabolism. We speculated that there might be a “Yin-Yang” relationship between GPR30 and ER α regarding their actions in the development of obesity. Therefore, we generated three independent transgenic mouse models, GPR30 and aromatase (Ar) double knockout (DKO), GPR30 knockout (GPRKO), and Ar knockout (ArKO) to test our hypothesis that the excess fat accumulation in HFD-fed WT mice could be, or at least partially, caused by the enhanced estrogen-GPR30 signaling. Ar is the

key enzyme that catalyzes the biosynthesis of C18 estrogens from C19 androgens in men and postmenopausal women, thereby the ArKO and DKO mouse models allowed us to investigate the role of GPR30 in the absence of endogenous estrogen. We discovered that GPR30 deficiency had limited effects on energy metabolism in young mice fed a standard chow diet (STD). However, deletion of GPR30 promoted metabolic flexibility in both genders fed a HFD regardless of the presence of estrogen, suggesting GPR30 may not solely acts via the ligation of estrogen. Interestingly, consistent with our previous findings, GPRKO mice had higher body temperature, indicating that GPR30 deficiency may promote thermogenesis and energy metabolism, resulting in the reduced fat depots and enhanced metabolic flexibility. Hence, we explored that deletion of GPR30 exerted thermo-promoting effect via upregulation of the mitochondrial uncoupling protein-1 (UCP-1) and enhanced mitochondrial respiration in brown adipose tissue (BAT). Further, the expression of thermogenic genes were significantly higher in the stromal cells-differentiated beige adipocytes, suggesting that GPR30 deficiency suppressed fat accumulation by promoting thremogenic browning of white adipose tissue (WAT) and dissipating excess energy as heat.

In summary, my dissertation work provide valuable insight regarding the role of GPR30 in energy metabolism. Further investigations testing whether GPR30 acts as a pro-obesity gene would facilitate our understanding of obesity development and provide a novel therapeutic target to fight obesity.

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LIST OF ABBREVIATIONS

A

AA: antimycin A

ANOVA: analysis of variance

AR: adrenergic receptor

ATP: adenosine triphosphate

AUC: area under the curve

B

BAT: brown adipose tissue

β_3 AR: β_3 -adrenergic receptor

BW: body weight

C

cAMP: cyclic adenosine monophosphate

CREB: cAMP response element binding protein

D

DMEM: Dulbecco's modified eagle medium

Dio2: type II iodothyronine deiodinase

E

E2: 17 β -estradiol

ECAR: extracellular acidification rate

ER: estrogen receptor

F

FBG: fasting blood glucose

FBS: fetal bovine serum

FCCP: 2-[2-[4-(trifluoromethoxy) phenyl] hydrazinylidene]-propanedinitrile

G

GPRKO: GPR30 knockout

GTT: glucose tolerance test

H

HFD: high fat diet

I

IBMX: 3-isobutyl-1-methylxanthine

ICI: ICI182,780

Ip: intraperitoneal

ITT: insulin tolerance test

K

KO: knockout

N

NFBG: non-fasting blood glucose

O

OCR: oxygen consumption rate

P

Pgc-1 α : peroxisome proliferative activated receptor, gamma, co-activator 1, alpha

PKA: protein kinase A

PKC: protein kinase C

PI3K: phosphatidylinositol 3 kinase

PPAR γ : peroxisome proliferator-activated receptor gamma

Prdm16: PR domain containing 16

R

Rote: rotenone

RQ: relative quantification

S

Sirt1: NAD-dependent protein deacetylase sirtuin-1

STD: standard chow diet

SVF: stromal vascular fraction

T

T2D: type 2 diabetes

T3: triiodothyronine

U

UCP-1: uncoupling protein 1

W

WAT: white adipose tissue

WT: wild-type

Chapter One

Introduction

Background

Overweight and obesity are growing health problems worldwide. In the U.S., 34.9% of adults aged 20 years or older and 16.9% of children are considered to be obese in 2012 (1). Obesity is an established risk factor for the pathogenesis of various chronic diseases such as type 2 diabetes (T2D), cardiovascular disease (CVD), and cancer (2). It has been established that estrogen plays a significant role in maintaining fat homeostasis in both women and rodent models (3-6), a role that is believed to be mediated primarily via its receptor ER α (7), whereas ER β function in fat metabolism is inconclusive (7-10). GPR30, a seven transmembrane-domain G-protein-coupled receptor (11) discovered and cloned in 1997 (11-16), was recognized as a novel, specific ER with a high affinity for 17 β -estradiol (E2) (17,18). It is expressed in numerous tissues including reproductive systems, adipose tissue, heart, intestine, ovary, central nervous system, pancreatic islets, neurons, inflammatory cells, and bone tissue (19-26). It has been shown that GPR30 induces signaling via activation of G α s or G α i (17,27), strongly suggesting that the plasma membrane is the action site of this receptor. Intriguingly, GPR30 has been localized either to the plasma membrane (17,28,29) or in intracellular compartments, such as the endoplasmic reticulum and the Golgi complex (18,29-34), suggesting that GPR30 may be an atypical GPCR. Indeed, a study shows that GPR30 is activated intracellularly, which then diffuses across cell membranes and initiates cellular signaling (18,33). Thus, a role for GPR30 as a membrane-based ER remains controversial, and the exact mechanism by which GPR30 acts in response to estrogen remains elusive.

Both human and rodent adipose tissue expresses ER α , ER β , and GPR30, suggesting that estrogen signaling could occur through both classical ERs and GPR30 in adipose tissue. While ERs have been well investigated regarding their roles in mediating estrogen effects on fat metabolism and metabolic diseases, little is known about metabolic action of GPR30 as well as the possible complex interactions among the three ERs in different cell systems. The generation of GPR30 knockout (GPRKO) mice facilitates our understanding of the function of GPR30. It was showed that GPRKO reduced body weight (BW) and bone growth in female, but not male mice, fed a standard chow diet (STD) (35). In contrast, data from another recent study show that BW and abdominal adiposity were increased in both GPRKO male and female mice fed the STD (36). Interestingly, Davis et al. reported that only male, but not female GPRKO mice displayed the increased fat mass as compared to their WT littermates fed a STD (37). However, other studies found no significant effect of GPR30 on BW of both female and male mice (38,39). The reasons for these disparate results are not clear. However, most these studies were not designed for investigating the roles of GPR30 in obesity development. As female mice in these studies were used at their young ages and fed a STD, they remain lean without apparent metabolic abnormalities, which therefore are not sufficient to reveal the role of GPR30 in obesity development in females that is typically induced by high calorie intake. Thus, I propose to explore the metabolic role(s) of GPR30 in the development of obesity using both *in vivo* and *in vitro* approaches and the outcome of my research may potentially help to develop therapeutic strategies targeting GPR30 to combat obesity.

Significance

As aforementioned, overweight and obesity are growing health problems worldwide. In the U.S., 34.9% of adults aged 20 years or older and 16.9% of children are considered to be obese in 2012 (1). Although the pathophysiological mechanism under obesity remains largely unknown, differentiation of preadipocytes into mature adipocytes is crucial in driving the expansion of adipose tissue mass that leads to obesity (40). My first study found for the first time that GPR30 deficiency reduces adiposity in response to high-energy intake. Next, we generated three transgenic mouse models to study whether the obesity-promoting action of GPR30 depends on the activation by estrogen. Our results provide invaluable findings that deletion of GPR30 has certain capability to improve the metabolic flexibility in female mice, including glucose homeostasis and thermogenesis. Continuously, we discovered that GPR30 deficiency is involved in regulating browning of white adipose tissue, thereby suppressing fat accumulation when challenged with excess calorie intake. Together, these findings provide novel aspects to understand the metabolic role of GPR30 in energy metabolism and may be instrumental in the development of novel therapeutic strategies of obesity.

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Chapter Two
Literature Review

Abstract

Estrogen can elicit pleiotropic genomic and rapid nongenomic cellular responses via a diversity of estrogen receptors (ERs). Unlike genomic responses, where the classical nuclear ER α and ER β act as transcription factors following estrogen binding to regulate gene transcription in estrogen target tissues, the nongenomic cellular responses to estrogen are believed to start at the plasma membrane, leading to rapid activation of second messengers-triggered cytoplasmic signal transduction cascades. The recently acknowledged ER, GPR30, was discovered in human breast cancer cells two decades ago and subsequently in many other cells. Since its discovery, it has been claimed that estrogen, ER antagonist fulvestrant, as well as some estrogenic compounds can directly bind to GPR30, and therefore initiate the nongenomic cellular responses. Various recently developed genetic tools as well as chemical ligands greatly facilitated research aimed at determining the physiological roles of GPR30 in different tissues. However, there is still lack of evidence that GPR30 plays a significant role in mediating estrogen action *in vivo*. This review summarizes the current knowledge of GPR30 with emphasis on the research findings elucidating the role of GPR30 in health and disease. Understanding the role of GPR30 in estrogen signaling will provide significant opportunities for the development of new therapeutic strategies to strengthen the benefits of estrogen in metabolism while limiting the potential side effects.

Keywords: GPR30, estrogen receptor

1 Introduction

Estrogen, more specifically, 17 β -estradiol (E2), is a female sex hormone, which is essential for not only the development of the female reproductive organs but also the secondary sex characteristics (1). In addition, this hormone plays a critical role in the development and function of the male reproductive tract (2). Moreover, E2 has important physiological roles in almost every part of the body, including the nervous system (3), immune system (4,5), skeletal tissue (6,7), musculature (8-11), as well as the endocrine system (12,13). Our understanding of the widespread physiological roles of E2 is complicated by the discovery of multiple types of estrogen receptors (ERs) and their various downstream cellular signaling pathways that span from seconds to hours (14,15). In this review, we provide a brief overview of estrogen signaling and describe the characteristics of its receptors, emphasizing on GPR30, presumably a novel G-protein-coupled ER. We focus on discussing studies that elucidated the potential physiological roles of GPR30 in regards to its estrogen binding properties and in mediating physiological actions of E2 *in vivo*.

2 Estrogen Receptors

Steroid hormones are synthesized in the ovaries (E2, progesterone), testes (androgens, testosterone), and adrenal glands (cortisol, androgens). E2 is a critical steroid hormone that was originally believed (in the 1960's) to exert its physiological effects through a nuclear ER, later termed as ER α , which was identified in the rat uterus (16-18). About three decades later, the first ER α knockout mouse model was generated (19). The second ER, ER β , was identified in the rat prostate in 1996 (20). ERs are ligand-regulated nuclear transcriptional factors that are believed to mediate a wide array of biological actions of E2. These two ERs are highly homologous in their central DNA-binding domains (97%), but their carboxyl-terminal ligand-binding domains only

share approximately 56% sequence homology (21). In addition, the amino-terminal domains of these two receptors display little sequence homology (22). Regardless, both receptors bind E2 and E2 analogs with similar affinities and recognize identical DNA sequences.

Besides these classical nuclear ERs, which can initiate transcriptional events in the promoter regions of target genes, E2 was also reported to engage in rapid nongenomic events via rapid activation of several cellular signaling cascades (23,24). Several studies have shown that E2 triggers a variety of intracellular signaling events, including mobilization of intracellular calcium (25), production of cyclic adenosine monophosphate (cAMP) (26), and activation of mitogen-activated protein kinases p38 (27,28) and extracellular signal-regulated kinase 1/2 (ERK 1/2) (29). The underlying mechanism for E2 exerting these rapid cellular events appears to be complex that may involve ERs, the variants of ER α , and unknown E2 receptors (24,30). Cellular signal transduction can occur as a result of E2 activating G proteins, which then lead to the modulation of downstream cellular pathways (31-34). Thus, a potential role for G-protein coupled receptors (GPCRs), which utilize E2 as ligand, has been proposed as an important route through which E2 exerts cellular functions.

3 Discovery of GPR30

As early as the 1960s-1970s, several studies reported the rapid cellular effects of E2 on cAMP synthesis (35) and calcium mobilization (36). These acute effects evoked by E2 are transmitted through enzymatic pathway and ion channels via the activation of membrane-associated ERs that may not involve transcription, which are thereby referred as nongenomic or extra-nuclear signaling pathway (37,38). In 1997, a novel seven transmembrane-domain GPCR, named GPR30, was first identified and cloned (39), which showed high sequence homology to the interleukin 8 receptor

and the angiotensin II receptor type 1 (40,41). Therefore, it was initially speculated that the endogenous ligand activating GPR30 is a chemokine or peptide (40,42). However, chemokines and/or peptides failed to evoke responses in GPR30 transfected cells (40,42), suggesting that GPR30 might be an orphan GPCR without cognate endogenous ligands. In 2004, Maggiolini et al. performed gene expression analysis of SKBr3 cells lacking ERs. The results indicated that the proto-oncogene c-fos was upregulated in response to E2. Interestingly, the upregulation of c-fos by E2 was blocked when the endogenous GPR30 expression was silenced (43). In another study that used breast cancer cell lines, GPR30 expression was positively correlated with ER α expression, suggesting these two receptors might be regulated by the same regulatory mechanism or transcription factors (39). The orphan fate of GPR30 reached a turning point in 2005 (44). Two independent research groups provided data demonstrating that E2 directly binds to GPR30, which thus acts as a membrane-bound ER (33,34). In 2007, the physiological role of GPR30 *in vivo* was first examined in rats (45). The results showed that administration of E2 induced GPR30 expression and attenuated hepatic injury via protein kinase A (PKA)-mediated mechanism in rats. Consistently, knockdown of GPR30 but not ER attenuated the E2-dependent activation of PKA in hepatocytes isolated from rats. The characteristics of all three known ERs summarized in Table 1.

With the discovery of GPR30 as a novel ER, a growing number of evidence has emerged to describe the rapid action of E2 via GPR30 (15,33,34,46,47). A search in PubMed in April 2019 with the keywords “estrogen and nongenomic” yielded 760 publications since 1984, with 42% (318) published during the past decade. This area has attracted a surge of interest recently and represents the most active area in the field of E2 research.

Table 1. Characteristics of ERs (48-56).

<i>ER characteristics</i>	ERα	ERβ	GPR30
<i>Category</i>	Nuclear steroid hormone receptor superfamily		G-protein coupled receptor superfamily
<i>Location</i>	nucleus	nucleus	Membrane-associated
<i>Size</i>	595 aa	530 aa	375 aa
<i>Numbers of isoforms</i>	3	5	1
<i>Chromosome region</i>	6q25.1	14q23.2	7p22.3
<i>Structure</i>	DNA-binding domain, ligand-binding domain, N-terminal domain		7 transmembrane α -helical regions, 4 extracellular and 4 cytosolic segments
<i>Distribution in tissues</i>	Hypothalamus, hippocampus, testes, ovary, endometrium, uterus, prostate, kidney, liver, breast, epididymis, muscle, adipose tissue	Testes, ovary, prostate, vascular endothelium, bladder, colon, adrenal gland, pancreas, muscle, adipose tissue	Central and peripheral nervous system, uterus, ovary, mammary glands, testes, pancreas, kidney, liver, adrenal and pituitary glands, cardiovascular system, adipose tissue

3.1 GPR30 expression in tissue

Expression of GPR30 protein is not only restricted to E2 responsive tissues, as originally speculated. It is expressed in many other tissues in humans (39-42,57,58) and rodents (59-65), such as the brain (including the hypothalamus), placenta, lung, liver, prostate, ovary, pancreatic islets, adipose tissue, vasculature, muscle, skeleton, as well as immune cells (66,67). Interestingly, it appears that the expression pattern of GPR30 is age-, species-, gender- or tissue-dependent. For example, the mRNA expression of GPR30 in skeletal muscle tended to be higher in premenopausal women compared to postmenopausal women (68). In mouse skeletal muscle, GPR30 mRNA abundance is almost 4-fold greater in females than that in males (59), with greater expression of GPR30 mRNA in female soleus than in extensor digitorum longus muscle (EDL) (69). GPR30 is also highly expressed in human bone tissues, and thus it may mediate the action of E2 on preserving bone density (70), suggesting a potential therapeutic strategy to prevent or alleviate menopausal osteoporosis by targeting GPR30. Moreover, a high density of GPR30 was observed in the brain of hamster, including hypothalamus, thalamus, cerebellum, and amygdala and the expression pattern of GPR30 behaved in a sexually dimorphic fashion in both young (postnatal 7 days) and adult (postnatal 60 days) animals (71). The gene expression of GPR30 was significantly higher in adult female hypothalamus than that of adult male, whereas the opposite expression pattern was observed in thalamus in adult hamster. Similarly, the expression pattern of GPR30 mRNA displayed contrary trend in cerebellum and amygdala areas in young hamster between male and female (71). However, it is presently unclear whether GPR30 shows a similar expression pattern in humans. Interestingly, GPR30 expression is developmentally regulated. In the mammary gland, GPR30 expression is lower in the elongating ducts during puberty and then increase through periods of sexual maturity (15). In the cartilage of the human growth plate, GPR30 expression

decreases as puberty progresses in both genders (72). Studies have shown that GPR30 expression level in mammary ductal epithelia is dependent on estrous-cycle (15), and consistently, the highest GPR30 mRNA expression level was found on day 3 of estrous cycle and then declined to the lowest level on day 12 in equine endometrium (73). Results from another study examining GPR30 expression in hamster ovarian cells during estrous cycle exhibited similar pattern that GPR30 mRNA and protein abundance reached the peak levels on day 3 of estrous cycle and decreased on day 4. These findings are very important, as they provide a basis to investigate the physiological roles of GPR30 including cancer development, immune regulation, and reproductive, cardiovascular, as well as metabolic functions (67,74,75).

3.2 GPR30 localization in cells

GPR30 is a seven transmembrane GPCR and therefore it is presumed to be located on the plasma membrane (76) as are most GPCRs (33,77). Indeed, it has been shown that GPR30 induces signaling via activation of G α s or G α i (15,33), strongly suggesting that this receptor is associated with the plasma membrane. Interestingly, however, several studies provide evidence showing that a larger fraction of total cellular GPR30 is localized in intracellular compartments. Revankar and colleagues used fluorescent E2 derivatives (E2-Alexas) to visualize the extra- and intracellular binding properties of GPR30 in COS-7 (monkey kidney fibroblast) cells. Surprisingly, the confocal images revealed that E2-Alexas failed to label the plasma membrane but predominantly bound to the endoplasmic reticulum (34). In addition, E2-Alexas or antibody stained GPR30 is also colocalized in the Golgi apparatus and nuclear membrane in all other GPR30 expressing cancer cell lines (34). Similarly, the predominant intracellular staining pattern of GPR30 was observed in human umbilical vein endothelial cells (78), vascular smooth muscle cells (79), and

insulinoma and pancreatic islet cells (80,81). However, some other studies reported that GPR30 is strongly localized to the plasma membrane of uterine epithelia (82), myometrium (83), renal epithelia (84-87), hippocampal neurons (77,88), though an intracellular expression of GPR30 has also been reported in neurons (65). Therefore, the cellular distribution of GPR30 remains controversial, which seems tissue-dependent. Interestingly, two studies indicated that GPR30 is activated intracellularly, which then diffuses across cell membranes and initiates cellular signaling (34,89). These results indicate that GPR30 is an atypical GPCR, and its intracellular location may dynamically change in response to specific environmental cues and also could be tissue-dependent. Thus, a role for GPR30 as a plasma membrane-based ER is still controversial, and the exact mechanism by which GPR30 acts in response to E2 remains elusive.

3.3 GPR30 ligands

As discussed above, two groups utilizing fluorescent derivative of E2 displayed the binding of E2 to GPR30 (34,90). Measurement of steroid binding to membrane-associated receptors is challenging because of the lipophilic nature of steroids and relatively low levels of membrane proteins that cause high background binding. Nevertheless, results from ligand binding assays demonstrated that GPR30 is a specific receptor for E2 with estimated binding affinities of 3-6 nM, (33,34), which is however much lower as compared with its binding affinities for classical ERs that are in the range of 0.1-1.0 nM (91).

In addition to E2, compounds with estrogenic activity can be found in a large variety of natural sources such as plants (e.g., soy) and fungi (92). With the rapid development of synthetic estrogenic substances, it is not surprising that a large number of estrogenic compounds have been shown to interact with GPR30. Tamoxifen, for instance, is a well-known selective ER modulator

and was recently found to act as a GPR30 agonist (34,93). Interestingly, stimulation with 4-hydroxytamoxifen, the active metabolite of tamoxifen, failed to activate PI3K in ER α positive cells but did activate PI3K in GPR30 expressing cells (90). Another widely used selective ER α / β antagonist, ICI182,780 (ICI), was also shown to bind to GPR30 (33) and activate this receptor (94). Consistently, treatment of breast cancer cell line, MCF-7 cells, with ICI resulted in rapid intracellular calcium mobilization and ERK1/2 phosphorylation (95), confirming the effect of ICI as a GPR30 agonist. Consistently, another recent study demonstrated that raloxifene, a selective ER modulator, also elicited cellular response via GPR30 in ER α -deficient cells (96). In addition, numerous synthetic estrogenic compounds have been shown to bind and/or activate GPR30, including zearalalone, nonphenol, kepone, p, p'-DDT, o, p'-DDE, 2, 2', 5', -PCB-4-OH (97), atrazine (98,99), and bisphenol A (100,101). Finally, several lines of research have demonstrated the agonistic actions of some plant-derived polyphenolic compounds towards GPR30, including genistein (43,97,102), quercetin (43), equol (103), resveratrol (104), oleuropein, hydroxytyrosol (105), and daidzein (106). However, it should be noted that the results from these studies were obtained exclusively from *in-vitro*-based assays using cancer cells or clonal cells with artificially over-expressed GPR30, and whether and how they exert estrogenic effects as well as the target tissue *in vivo* are still unknown. Hence advancing the field of GPR30 research using these estrogenic compounds is fraught with complications. Fortunately, a highly selective GPR30 agonist, G-1 (GPR30-specific compound-1, structure shown as Fig. 1A), was synthesized in 2006 (107) and further studies of GPR30 action are greatly facilitated by this compound.

G-1 showed high binding affinity for GPR30 ($K_d = 10$ nM) without binding to ER α / β at concentrations as high as 10 μ M (108). Three years later, a subsequent study identified a highly selective GPR30 antagonist, G15 (structure shown as Fig. 1B), with a similar structure as G-1 but

lacking the ethanone moiety (109), which displayed a minimal binding to ER α/β ($K_d > 10 \mu\text{M}$) (110). As a result of minimized steric conflicts that limit binding within the classical ER α ligand binding pocket, a derivative, G36, was generated to restore the steric bulk of G-1 and maintain antagonistic effect of GPR30 while restoring the ER counter selectivity (111). Since these three synthetic compounds have been available, they have been used in over 200 studies to evaluate GPR30 actions in a variety of cellular and animal models. In addition, two novel GPR30 specific agonists, GPER-L1 and GPER-L2, were synthesized in 2012 with binding affinities of $\sim 100 \text{ nM}$ (112). Intriguingly, the widely used ER α specific agonist, propyl pyrazole triol (PPT), has been reported to act as GPR30 agonist at concentrations as low as 10 – 100 nM. On the contrary, the ER β specific agonist, diarylpropionitrile (DPN), showed no effect on GPR30 at concentrations up to 10 μM (96). Therefore, the results from studies regarding the use of these compounds aimed at modulating the ERs actions should be interpreted carefully with respect to the concentrations of these compounds.

3.4 Evidence of E2-independent effects of GPR30

As aforementioned, the estimated binding affinities of E2 to GPR30 (3 – 6 nM) (33,34) are considerably higher as compared with its binding affinities for classical ERs (0.1 – 1 nM) (91). This raises an interesting question as to whether GPR30 plays a significant role in mediating various E2 effects *in vivo*, given that circulating E2 levels in young female rodent is only about 7.3 – 734.2 pM (113-115), depending on the stage of estrous cycle. Indeed, conflicting results regarding the GPR30-mediated signaling events in response to E2 continue to emerge, which warrant further investigations as to whether GPR30 plays a physiological role as an ER *in vivo*. Selective activation of GPR30 by administration of G-1 did not stimulate estrogenic effects in the

uterus and mammary gland of mice [38], which are the E2 target organs where both ER α and GPR30 are expressed [28]. In addition, ICI, a specific ER antagonist but GPR30 agonist, hindered E2-induced rapid cellular responses in MCF-7 cells expressing ERs and GPR30. Knockdown of GPR30 in these cells had no impacts on E2-induced cAMP production (116). Others demonstrated that transient expression of GPR30 in MCF-7 cells resulted in a reduction of cell growth in the absence of E2 (117). Based on these results, GPR30 may not signal in response to the stimulation of E2 at physiologically relevant levels. Intriguingly, the existence of membrane ERs (mERs) (118-121), though with a limited amount at about 3-10% of the classical nuclear ERs (31,116), further complicated the rapid nongenomic signaling events mediated by E2. Interestingly, G-1 was shown to induce the phosphorylation of ERK1/2 in GPR30-negative HEK293 cells stably transfected with ER α -36 (122), a novel variant of human ER α -66 (123). Moreover, knockdown of ER α -36 in MDA-MB-231 and SKBr3 cells suppressed the phosphorylation of ERK1/2 and intracellular calcium mobilization stimulated by G-1, suggesting that G-1 also recognizes ER α -36, and therefore it may not be specific for GPR30. The use of ICI, ER α antagonist but GPR30 agonist, and GPR30 antagonist G-15, in the mouse hippocampal cell lines mHippoE-14 and mHippoE-18 demonstrated that acute E2 treatment protected hippocampal cells from glutamate-induced neurotoxicity and the protective action requires both mER α and GPR30 (124). In ovariectomized female mice, it was shown that infusion of E2 into the dorsal hippocampus activated ER α and ER β , leading to ERK1/2 signaling and the enhanced object recognition and spatial memory. However, infusion of G-1 but not E2 activated GPR30, which triggered a different cell-signaling mechanism to facilitate hippocampal memory in female mice (125). These results suggest that GPR30 in the dorsal hippocampus might not act as an ER. In GPR30 overexpressed COS-7 and CHO cells, E2 only showed specific saturated binding to ER α , but not to GPR30 (126). Taken together, it remains

to be determined whether GPR30 function as a true ER that mediates endogenous E2 effects *in vivo* as well as the primary target tissues, although it has been classified as a novel ER.

4 GPR30 in health and disease

With the increasing spectrum of research on GPR30 *in vitro*, many critical questions remain: what is the physiological role of GPR30? Does GPR30 really serve as a novel GPER and act independently or collaborate with the classical ERs? Will drugs targeting GPR30 be more effective than those targeting ER α / β for treatment of disease? Although GPR30 was officially named as a GPER by the International Union of Basic and Clinical Pharmacology in 2007 (127), deciphering the physiology role(s) of GPR30 as a novel ER in health and disease remains challenging, which is due to the complex nature of estrogen signaling, e.g. directly or indirectly binding of the estrogen-ER complex to DNA, regulation of gene expression, or modulation of cellular signaling cascades, respectively. While these aspects are beyond the scope of this review, different mechanisms of estrogen signaling are summarized in Figure 2 (see (94,128,129) for more detailed reviews on this topic). In the following section, recent studies regarding the physiological roles of GPR30 in different tissues and disease are discussed.

4.1 GPR30 in reproductive system

Since GPR30 is believed to be a GPER, its action in the reproductive system attracted a lot of attention. Early studies investigating the action of GPR30 were performed in various cancer cells derived from reproductive tissues, including breast (33,46), ovary (98,99,130,131), endometrium (93,132), testis (133,134), prostate (135), as well as thyroid tissues (102,136). Upon stimulation with E2, estrogenic compounds (e.g. genistein, hydroxytamoxifen) or GPR30 selective agonist G-

1, GPR30 enhanced cancer cell proliferation in the classical-ERs-negative breast cancer cells (33) and in the thyroid (102), endometrial (93), and ovarian cancer cells (131). On the contrary, in human bladder urothelial cells, activation of GPR30 by E2 or G-1 suppressed cell proliferation via down-regulating the activation protein-1 (AP-1) (137), which is one of the major regulators of cell proliferation (138). Similarly, in human prostate cancer cell PC-3, activation of GPR30 by G-1 induced cell-cycle arrest at the G2 phase resulted from the sustained activation of ERK1/2 and c-jun/c-fos-dependent upregulation of cyclin-dependent kinase inhibitor-1A (p21) (139). In human, activation of GPR30 by G-1 enhances contractile responses to oxytocin in the myometrium during labor (140). In addition, GPR30 together with ER α regulates the proliferative and/or apoptotic pathways involved in spermatogenesis via the EGFR/ERK/c-jun pathway in male rodent reproductive development (141,142). In contrast to these *in vitro* findings, mice with complete [28, 48] or partial deletion [49] of GPR30 displayed no alteration in the development of reproductive organs, which is also in clear contrast to the established phenotype of mice lacking ER α or E2. This result suggests that GPR30 may be not involved in mediating estrogenic actions of E2 in reproductive organs. Interestingly, studies from ovariectomized mice indicated that activation of GPR30 inhibited E2-induced uterine epithelial cell proliferation, which was associated with the reduced E2-stimulated ER α phosphorylation [50]. Therefore, GPR30 inhibition of E2-stimulated cell proliferation may be mediated via suppressing the phosphorylation of ER α , which is important for various E2-stimulated transcriptional events [51]. These findings suggest the complex roles of GPR30 in reproductive system and further investigation is required.

4.2 GPR30 in nervous system

Estrogen has many beneficial effects on the brain, including improving cognitive performance (143), reducing neuronal loss caused by cardiac arrest and stroke (3), increasing neuronal connectivity (144), and preventing or slowing age-related cognitive decline (145). Although these protective effects of E2 are largely attributed to the classical ER α / β , increasing evidence demonstrates that GPR30 also plays potential role(s) in E2-mediated neurological functions. As stated before, GPR30 is expressed throughout the central and peripheral nervous system of male and female rodents and humans (53), including hypothalamic-pituitary axis, hippocampal formation, and brain stem autonomic nuclei (62), as well as the spinal cord and dorsal root ganglia (63). Acute administration of E2 or GPR30 selective agonists STX or G-1, improves neuron survival rate by 40-45% compared to control in ovariectomized female rats (146). Similarly, administration of G-1 exerts significant neuron protective effect against global cerebral ischemia in a rat model (147). In addition, treatment of E2 or G-1 enhanced the activation of the pro-survival kinases Akt and ERK1/2, while antisense knockdown of GPR30 abrogated these signaling events (147). Therefore, GPR30 could be a novel target in treatment of neurodegenerative diseases, such as Alzheimer's disease and stroke (148). In addition to these neuron protective effects, it was found that E2 acts through a putative membrane-associated ER on hypothalamic proopiomelanocortin neurons, which then activates the G α q/phospholipase C/PKC/PKA pathway to promote the hypothalamic control of energy homeostasis in guinea pigs and mice (149). However, the identity of this proposed mER is unclear, which could be ER α / β or GPR30, as its mediated E2 effect in neurons was inhibited by ER antagonist ICI, which was found to activate GPR30. Interestingly, studies using ER α KO mice demonstrated that ER α is required for the E2-

mediated positive feedback control of hypothalamic gonadotropin release (150). Altogether, although the results from limited studies using chemical agonists of GPR30 consistently demonstrate a neuronal protective effect of GPR30, it remains to be determined whether GPR30 mediates this beneficial action of endogenous E2. In addition, the sole role and precise sites in neurons responsible for its neuronal protective effect needs to be elucidated, which may be achieved by using tissue-specific knockout animal models.

4.3 GPR30 and glucose metabolism

While ERs have been known to play a role in mediating E2 effects on glucose metabolism and metabolic diseases, the metabolic action of GPR30 remains to be determined. The generation of GPRKO mice facilitates our understanding of the function of GPR30. Martensson, U. E., et al. showed for the first time that GPRKO female mice displayed hyperglycemia, impaired glucose tolerance, and reduced body weight and bone growth whereas GPRKO male mice were metabolically normal (151), thus demonstrating a gender-dependent effect of GPR30, which may be involved in regulating glucose homeostasis in female mice. The potential anti-diabetic effect of GPR30 was revealed from studying ER α / β double knockout (DKO) mice treated with streptozotocin (STZ) (152), which removed E2/ER α / β signaling, thereby allowing to determine only GPR30-mediated role of E2. The results indicated that deletion of both ER α and ER β did not increase the morbidity of diabetes compared with single KO of ER α or ER β in STZ-induced insulin-deficient mice. In addition, ovariectomized mice had increased incidence of STZ-induced diabetes in ER α / β DKO mice, and the effect was attenuated by replacement of E2 (152). Similar to these findings, GPR30 agonist G-1 directly protects mouse and human islets and MIN6 cells against H₂O₂-induced apoptosis, and E2 still promoted pancreatic β -cells survival in ER α / β DKO

mice exposed to STZ (153). Taken together, their results showed that in the absence of the classical ER α/β , E2 still exerted the antidiabetic effects, suggesting the possible involvement of GPR30 in islet function. As discussed above, GPR30 is expressed in pancreatic islets (154-158), muscle and whole adipose tissue in humans and rodents (153,159), as well as in human liver (40,46,160-162), which are the key organs for insulin-dependent glucose metabolism (163-167). Deletion of GPR30 results in a reduced insulin secretion from pancreas suggesting that GPR30 indeed plays a critical role in maintaining the metabolic functions via regulating insulin secretion in mice and humans (152,155,168). Furthermore, the protective effect of E2 on pancreatic β -cells can be mimicked by GPR30 agonist, genistein (169). In contrast to the discussed study above showing that GPRKO female mice displayed hyperglycemia and glucose intolerance (151), we recently found that deletion of GPR30 protected female mice from high-fat diet (HFD)-induced obesity, glucose intolerance, and insulin resistance (170). After 15 weeks of HFD feeding, their blood glucose levels gradually diverged with the GPRKO displaying significantly lower fasting and non-fasting blood glucose levels as compared with WT. Consistently, female GPRKO mice were more glucose tolerant than WT mice while their insulin sensitivity was not different (170). The reason for these discrepancies are not clear. It should be noted that our study used GPR30 mice in 129 background in contrast to C57BL/6 GPR30 mice as used on other studies. Other factors, such as the genetic knockdown or knockout strategy, the breeding strategy, and the environment can have unexpected influence on the phenotypes as well (171). Of the note, certain maternal and/or experimental diets contain significant amount of phytoestrogens (i.e. soy protein or alfalfa meal) (172), which could modulate the estrogenic activity and therefore could profoundly alter the related outcome of a study, given the well-documented various effects of dietary phytoestrogens in rodent models (173,174).

4.4 GPR30 and adiposity

While the classical ERs have been well investigated regarding their roles in mediating E2 effects on fat metabolism and metabolic diseases, little is known about metabolic action of GPR30 as well as the possible complex interactions among the three ERs in different cell types. E2 and STX, a synthesized nonsteroidal compound acting as a GPR30 selective agonist (175), rapidly attenuated the baclofen response in hypothalamic arcuate POMC neurons in WT, ER α KO, ER β KO, and ER α/β DKO mice, and prevented excessive body weight gain in ovariectomized guinea pigs, suggesting a potential role of GPR30 in energy metabolism in females (149). Multiple studies have investigated the role of GPR30 on regulating body weight and fat deposits. The first such study reported an increase of body weights and visceral adiposity in GPRKO animals as compared with WT in both male and female mice (176). Interestingly, Davis et al. reported that only male, but not female, GPRKO mice displayed the increased fat mass as compared to their WT littermates fed a standard chow diet (STD) [53]. However, two other studies found no significant effect of GPR30 on body weight of both female and male mice [49, 54]. Data from a recent study showed an increment of body weights in both male and female GPRKO mice caused by increased fat mass with enlarged adipocytes when fed a phytoestrogen free low fat diet (177). However, Martensson and colleagues reported contrary results that female GPRKO mice exhibited slightly lower body weights as compared with WT, whereas no such differences were observed in male GPRKO mice (59). Consistent with this finding, we recently performed relatively long-term study with detailed analyses of body weight and body composition of female GPRKO mice either maintained on a STD or exposed to a phytoestrogen-free HFD (170). There were no differences in their body weight, fat mass, and all other measured metabolic phenotypes between WT and GPRKO either

male or female mice on a STD. However, after 23 weeks of HFD feeding, female GPRKO mice gained 61% of their starting body weight while WT female mice increased by 85% with no difference of their energy intake. The NMR-based body composition analysis showed that fat mass was similar before HFD feeding and became significantly different after 5 weeks exposed to HFD. At 20 weeks, the fat mass of WT was 1.8 folds of that in GPRKO mice with only slightly higher lean body mass in GPRKO animals, suggesting that the difference in body weight was primarily due to their fat mass difference. Interestingly, no such differences were observed between WT and GPRKO male mice fed a HFD. In addition, we examined the weights of various fat depots and organs. The inguinal, gonadal, and perirenal fat pads from GPRKO mice weighted less than those in WT mice, while the pancreas from GPRKO mice was slightly heavier than WT. All the other measured organs weights are similar, suggesting the reduced fat mass in GPRKO female mice was not due to decreased body growth. Our H&E staining of fat sections revealed that GPRKO female mice had smaller adipocytes as compared to WT female mice fed a HFD. While the reasons for these disparities with respect of GPR30 modulation of body weight gain from past studies are not clear, which could be due to the different methods generating the transgenic animals as recently reviewed (75), and the variations of the diet compositions, duration, and environment, but overall they indicate that GPR30 might play an important role regulating lipid metabolism and controlling adiposity.

While how exactly GPR30 regulates lipid metabolism is still unclear, our data (170) demonstrate that the effect of GPR30 on fat mass in HFD-fed female mice was not due to a secondary action by which its deletion altered circulating E2 levels or expression of ER α , which is believed to play a major role in mediating estrogenic effects on energy homeostasis (178). Both human and rodent white adipose tissue expresses ER α , ER β , and GPR30, suggesting that E2

signaling could occur through both ERs and GPR30. Interestingly, it was reported that GPR30 and ER α inhibit each other's actions in several types of cells (179-181). These data suggest that there may be a “yin-yang” relationship between GPR30 and ER α in regulating energy metabolism in response to E2 (as hypothesized in Fig. 3.). In that regard, activation of ER α by E2 inhibits adiposity, whereas activation of GPR30 might promote obesity.

5 Conclusions and future directions

Although several studies showed that E2 directly binds to GPR30 and exerts estrogenic effects, it has been officially acknowledged as a GPER, whether it is an endogenous GPER that mediates E2 effects remains unclear (60,126,182). Some argued that most cellular signaling studies investigating the activation of GPR30 by E2 or G-1 were only performed in cells overexpressing GPR30 (34) or transfected with GFP-tagged GPR30 (183). It has been shown that E2 fails to bind GPR30 in a saturable fashion in COS-7 cells transfected with human GPR30 (126). In addition, MDA-MB231 and Hec50 cells that express endogenous GPR30 did not show saturable binding to E2 either (126). Consistent with these results, in primary endothelial cells from ER α/β DKO mice, E2 failed to specifically bind to GPR30 and activate cAMP, ERK1/2, or PI3K signaling as observed in clonal cancer cells (116). The reason for these disparate results on the role of GPR30 in E2 signaling is unclear. Many of these studies were obtained using cell-based experiments, in which cells were manipulated with overexpression of GPR30, which may result in ectopic expression of GPR30 in the cells. Even using the cells that endogenously express GPR30, cellular experiment results cannot recapitulate estrogen functions in whole body. Thus, with the appearance of the first transgenic GPR30 mutant mouse model, it became a powerful tool to investigate the physiology role(s) of GPR30. To use these models, it has to carefully take into

account that the deletion or silence of the target gene is not always exclusively represent the phenotype of a mutant animal. Other factors, such as the genetic knockdown or knockout strategy, the breeding strategy, and the environment can have unexpected influence on the phenotypes as well (171).

The pace of research into the physiological roles of GPR30 has been accelerating over the past decade, and potential functions of GPR30 have been recognized in almost every system of the body. Increasing evidence suggest that GPR30 plays a role in body weight regulation and metabolism. However, the clear effects of GPR30 in regulating body weight and fat mass needs further investigation. On the other hand, while current research results suggest that GPR30 might serve as a critical downstream target of the E2 signaling pathways *in vitro*, and data from animal studies do not exclude GPR30 as a GPER in mediating estrogenic responses, the convincing evidence that E2 acts through GPR30 to elicit significant physiological events *in vivo* is still lacking. In addition, whether GPR30 counteracts ER α in female adiposity is an intriguing question that needs to be addressed in the future research. Finally, studies determining GPR30 biology in humans are scant. Future research should also be aimed at resolving this critical issue.

Figures

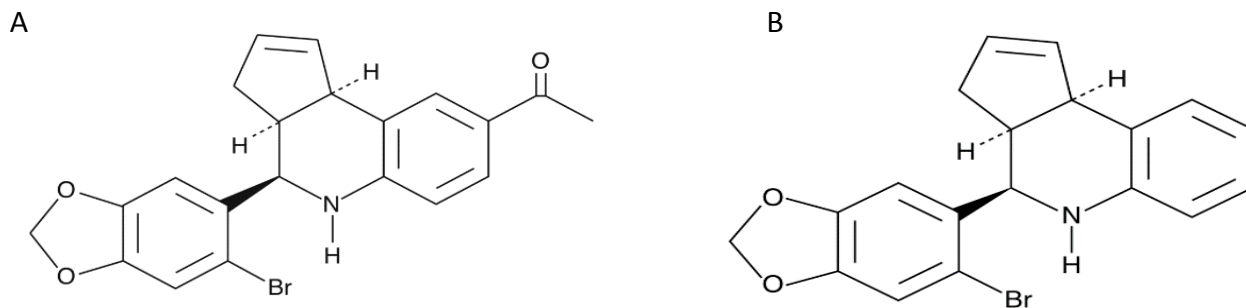


Figure 1. Molecular structures of GPR30 specific agonist G-1 (A) and antagonist G-15 (B).

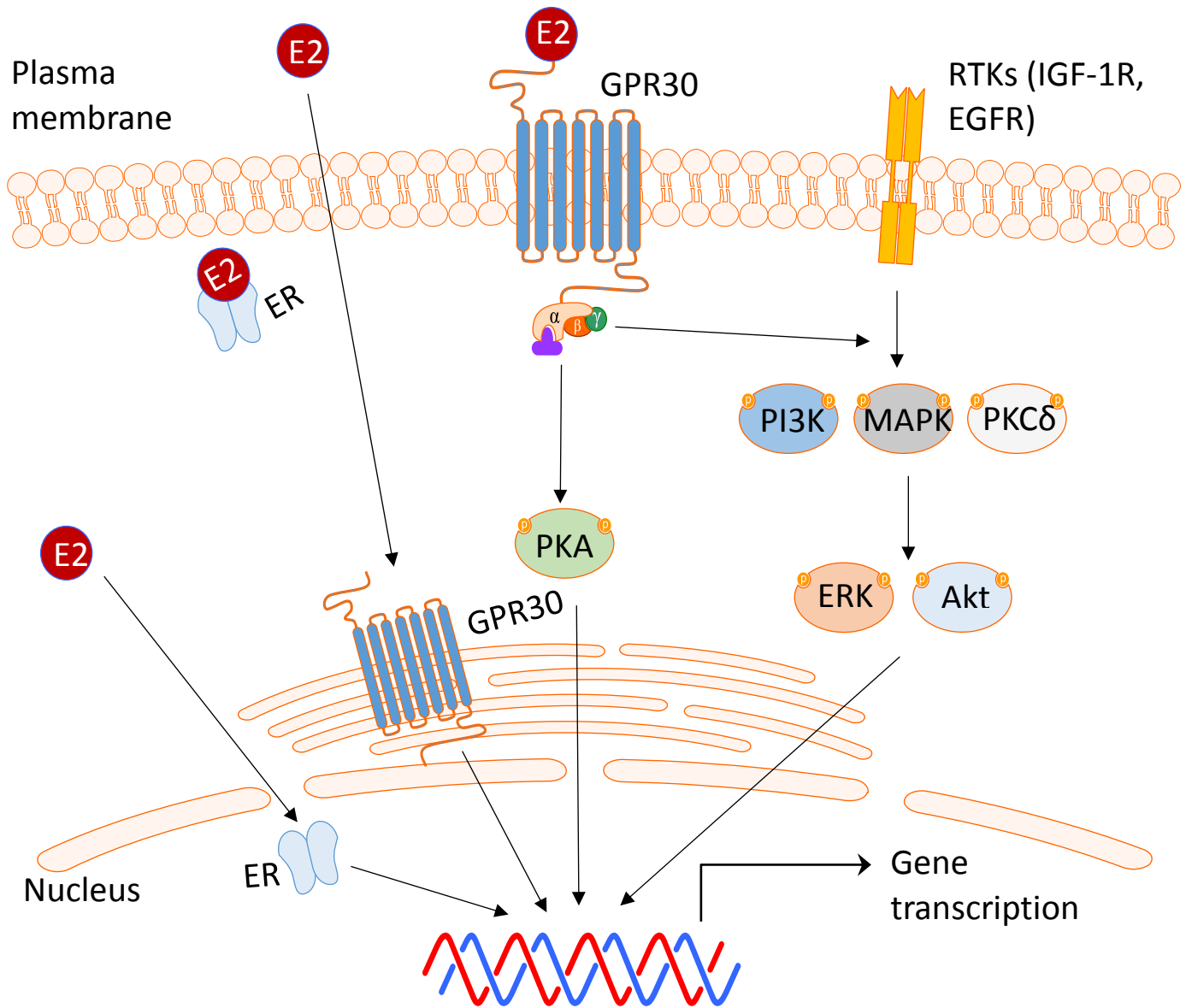


Figure 2. Schematic overview of E2 signaling pathways.

RTK, receptor tyrosine kinases; IGF-1R, insulin-like growth factor 1 receptor; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinases; PKC δ , protein kinase C-delta; Akt, protein kinase B.

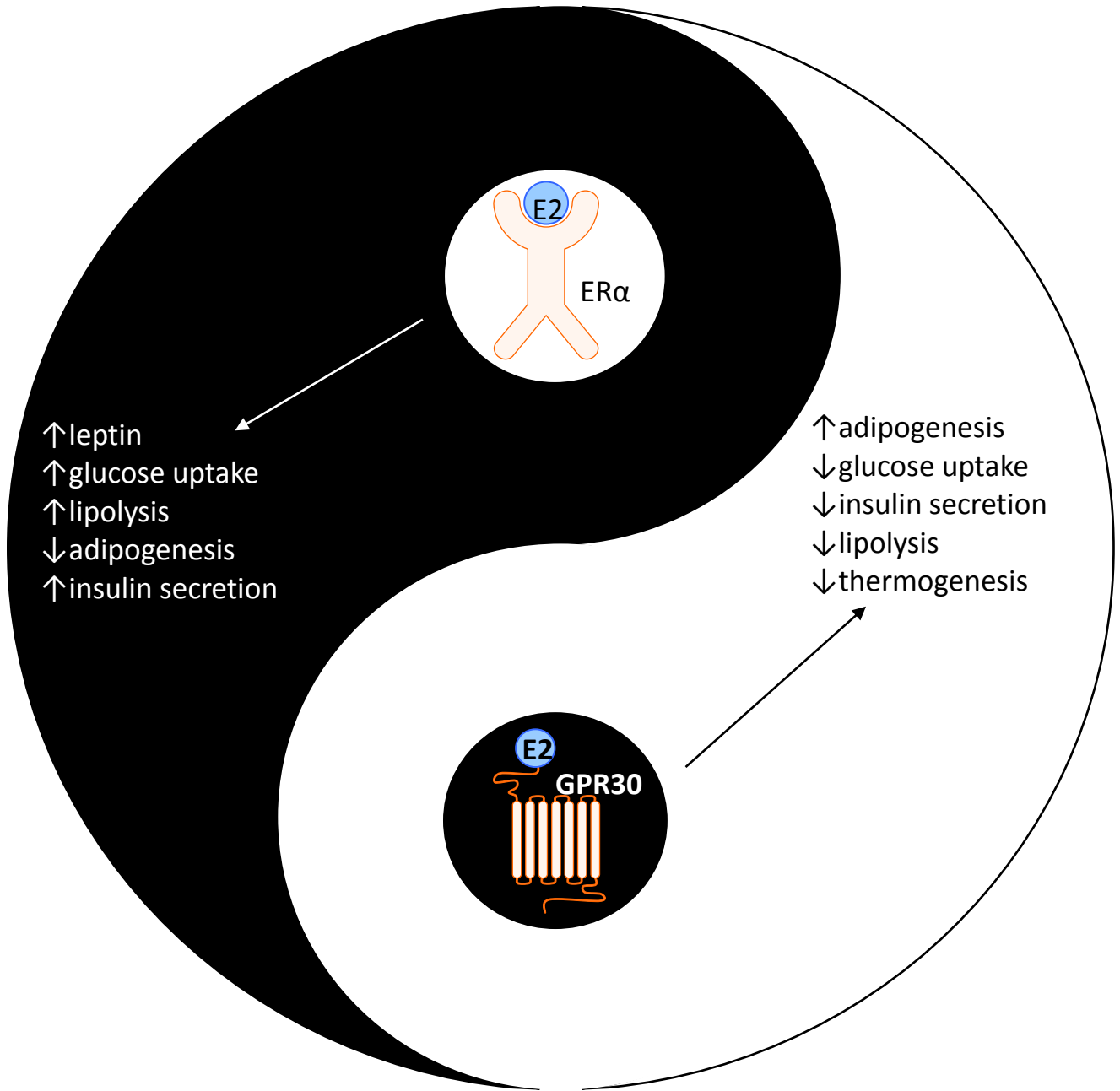


Figure 3. Yin-Yang relationship between $ER\alpha$ and GPR30.

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Chapter Three

GPR30 regulates diet-induced adiposity in female mice and adipogenesis *in vitro*

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Abstract

Recent studies showed that GPR30, a seven-transmembrane G-protein-coupled receptor, is a novel estrogen receptor (ER) that mediates some biological events elicited by estrogen in several types of cancer cells. However, its physiological or pathological role in vivo is unclear. Here, we show that GPR30 knockout (GPRKO) female mice were protected from high-fat diet (HFD)-induced obesity, blood glucose intolerance, and insulin resistance. The decreased body weight gain in GPRKO female mice is due to the reduction in body fat mass. These effects occurred in the absence of significant changes in food intake, intestinal fat absorption, triglyceride metabolism, or energy expenditure. However, GPR30 had no significant metabolic effects in male mice fed the HFD and both sexes of mice fed a chow diet. Further, GPR30 expression levels in fat tissues of WT obese female mice were greatly increased, whereas ER α and β expression was not altered. Deletion of GPR30 reduced adipogenic differentiation of adipose tissue-derived stromal cells. Conversely, activation of GPR30 enhanced adipogenic differentiation of 3T3-L1 preadipocytes. These findings provide evidence for the first time that GPR30 promotes adipogenesis and therefore the development of obesity in female mice exposed to excess fat energy.

Keywords: GPR30, high-fat diet, obese, mice

Introduction

GPR30 is a seven transmembrane G-protein-coupled receptor (GPCR) (1). It is expressed in numerous tissues including reproductive systems, adipose tissue, vasculature, intestine, ovary, central nerve system, pancreatic islets, neurons, inflammatory cells, and bone tissue (2). It has been shown that GPR30 induces signaling via activation of *Gas* or *Gai* (3,4), strongly suggesting that the plasma membrane is the action site of this receptor. Intriguingly, while GPR30 is expressed in the plasma membrane (4-6), a larger fraction of total cellular GPR30 can be located in intracellular compartments, including the endoplasmic reticulum and the Golgi complex (6-12), suggesting that GPR30 may be an atypical GPCR. Indeed, studies show that GPR30 is activated intracellularly, which then diffuses across cell membranes and initiates cellular signaling (10,11).

GPR30 is now recognized as a specific G-protein coupled estrogen receptor (ER) because it has a high affinity (nanomolar) for 17β -estradiol (E2) (4,11). However, the physiological or pathological role of GPR30 is still unclear. Data from in vitro studies has demonstrated that GPR30 mediates some rapid biological events elicited by E2 in several types of cells that ultimately lead to cell proliferation and migration (13-18). However, the biological relevance of these findings obtained from cultured cells is unclear. Indeed, recent studies showed that administration of G1, a specific GPR30 agonist (19), did not stimulate estrogenic effects in the uterus and mammary gland of mice (9). In contrast, studies from ovariectomized mice demonstrated that activation of GPR30 inhibits E2-induced uterine epithelial cell proliferation via inhibition of E2-stimulated ER α activity (20). These results indicate that GPR30 may act as a negative regulator for some ER-mediated physiological processes.

It has been established that E2 plays a significant role in fat metabolism in both humans and rodents (21-24). While classical ERs have been well investigated regarding their roles in

mediating E2 effects on fat metabolism and metabolic diseases, the metabolic action of GPR30 is still unclear. It was shown that GPR30 deficiency caused a number of metabolic alterations and reduced body weight (BW) and bone growth in female, but not male mice, fed a standard chow diet (STD) (25). On the contrary, another recent study showed that BW and abdominal adiposity were increased in both GPR30 knockout (GPRKO) male and female mice fed the STD (26). Interestingly, Davis et al reported that only male, but not female GPRKO mice displayed the significantly increased fat mass as compared to their wide-type (WT) littermates fed a STD (27). However, several other studies reported no significant effect of GPR30 on BW of either female or male mice (28,29). The reasons for these disparate results are not clear. However, most previous studies were not specifically designed for investigating the roles of GPR30 in obesity development in females. As female mice in these studies were used at their young ages and fed a STD during the experiments, they remain lean without apparent metabolic abnormalities, which therefore may be not sufficient to reveal the role of GPR30 in obesity development in females that is typically induced by high calorie intake. In this study, we investigated the metabolic effects of GPR30 in mice and its effect on adipogenesis in vitro.

Results

GPR30 deficient female mice are resistant to diet-induced obesity and glucose intolerance

We examined GPRKO and WT mice either maintained on a chow diet (STD) or fed a high-fat diet (HFD) to determine the metabolic effects of GPR30. There were no differences in BW, fat mass, and all other measured metabolic phenotypes between GPRKO and WT female mice on a STD through the experiment (data not shown). However, when female mice were fed a HFD, the BW

between the GPRKO and WT mice became significantly different after 12 wks. After 23 wks on HFD, the BW of WT female mice increased by 85%, whereas GPR30 KO females gained 61% of their starting BW ($p < 0.05$) (Figure 1A). The amount of food intake however was similar between female WT and KO mice (Figure 1B), indicating that the lower BW of KO mice was not due to reduced caloric intake. Data of NMR-based body composition analysis show that fat mass in WT and GPRKO females was similar before exposed to HFD, but became significantly different after 5 wks on HFD (Figure 1C). The fat mass in GPRKO female mice relative to WT mice became more diverged with advancing age. After exposure to HFD for 20 wks, fat mass in WT mice (8.97 g) was 1.8 fold of that in GPRKO mice (4.97 g) (Figure 1C), but GPRKO mice only had slightly higher lean body than that of the control mice (Figure 1D). Therefore, the difference in BW between WT and KO mice were primarily due to their fat mass difference. As this is the first time showing, to the best of our knowledge, that deletion of GPR30 reduces adiposity of HFD-fed female mice, we conducted second study with another cohort of female mice and obtained the similar results (data not shown). Interestingly, deletion of GPR30 had no effect on metabolic phenotypes in male mice fed a HFD (Supplemental Figure 1). Interestingly, there was no difference in GPR30 gene expression in white adipose tissue (WAT) between male and female mice (Supplemental Figure 2).

To determine if GPR30 affects glucose homeostasis, we measured NFBG at 0, 5, 9, 14, 19th wks and FBG concentrations at 7, 11, 16, 22nd wks. GPRKO mice fed HFD displayed significantly lower non-fasting blood glucose (NFBG) levels as compared with those in WT mice (Figure 2A). Fasting blood glucose (FBG) levels of HFD-fed GPRKO and WT mice gradually diverged after 15 wks and by 22nd wk, GPRKO females on a HFD were 14% lower in FBG concentrations as compared with WT females ($p < 0.05$; Figure 2B). Consistently, Female GPRKO

mice were more glucose tolerant than WT mice (Figure 2C). While whole body insulin sensitivity was not different between WT and GPRKO mice (Figure 2D), WT female mice fed a HFD had higher insulin (Figure 2E) and leptin (Figure 2F) levels than those in GPRKO female mice ($p < 0.05$; Figure 2E), which are typically associated with obesity and insulin resistance. HFD-fed GPRKO female mice were 52% lower in homeostatic model assessment of insulin resistance (HOMA-IR) than that of WT female mice ($p < 0.05$) (Figure 2G).

Deletion of GPR30 has no effect on fat metabolism or postprandial triglyceride clearance

To determine if GPR30 deficiency reduces intestinal fat absorption, thereby causing the decrease in fat deposit, we collected feces at 10th and 19th wk of HFD treatment and measured triglyceride content. No significant differences in fecal triglyceride levels were observed between WT and GPRKO female mice (Supplementary Figure 3A). In addition, we didn't found significant difference in fat tolerance between GPRKO and WT mice (Supplementary Figure 3B). Consistently, liver triglyceride contents were similar between WT and GPRKO mice (Supplementary Figure 1C) as well as the gene expression levels of transcription factors ChREBP and SREBP-1c (data not shown), which coordinate the expression of genes required for fatty acid synthesis (20, 21). These results indicate that deletion of GPR30 has no effect on postprandial hepatic triglyceride metabolism and plasma clearance of intragastrically loaded triglycerides. Both WT and GPRKO female mice fed HFD had similar fasting plasma total cholesterol (Supplementary Figure 3D) and non-esterified fatty acids (NEFA; Supplementary Figure 3E) levels, suggesting that lipolysis may not be altered by deletion of GPR30. Paradoxically, KO mice displayed higher triglyceride concentrations as compared with WT mice (Supplementary Figure 3F). We then analyzed GPR30 gene expression in fat tissue, liver, and primary hepatocytes of the

mice. We found that GPR30 mRNA was barely detectable in the liver and was completely absent in isolated mouse hepatocytes, but it was highly expressed in WAT of WT mice (Figure 3A). Interestingly, GPR30 mRNA abundance in WAT of female mice was greatly up-regulated by HFD feeding (Figure 3B). Collectively, these results provide strong evidence that WAT but not liver or intestine is the primary site for GPR30 to regulate adiposity in HFD-fed female mice.

GPR30 deficiency reduces fat depot mass and adipocyte size

To gain insight into alterations of body composition, we euthanized mice after 23 wks of HFD treatment and collected various fat pads and organs. The inguinal, gonadal, and perirenal fat pads from GPRKO mice weighted significantly less than those in WT mice (Figure 4A). There were no differences in brown fat mass and other organs except pancreas between WT and GPRKO mice. In addition, ectopic lipid accumulation in muscle, heart, aorta, and kidney was not observed in both genotypes. These results suggest that the reduced fat mass in GPRKO female mice fed HFD is not a result of decreased body growth. During the development of obesity, adipose tissue undergoes hyperplasia as well as hypertrophy for the increased demand for triglyceride storage (30,31). In order to determine if the differences in fat mass between KO and WT mice was due to differences in adipocyte size, we performed H&E staining of fat sections, which labels adipocyte plasma membranes, allowing for adipocyte size measurements in paraffin sectioned WAT to evaluate the contribution of adipocyte hypertrophy to WAT mass accumulation. We found that GPRKO female mice had smaller adipocytes as compared to WT female mice (Figure 4B, 4C). Further analyses of adipocyte size distribution revealed that GPR30 deficiency caused the shift toward smaller adipocytes across fat pads (Figure 4D-F).

Deletion of GPR30 has no effects on plasma E2 level and expression of ER and adipogenic factors in WAT

Because GPR30 is an ER (4,11), we wondered whether deletion of GPR30 in female mice affects circulating E2 levels and classical ER expression in WAT. We found that neither plasma E2 levels (Supplementary Figure 4A) nor ER α and ER β gene expression were altered by deletion of GPR30 (Supplementary Figure 4B), suggesting that GPR30 effect on adiposity was not due to the secondary action whereby its absence modulated E2 production or ER α / β expression. In addition, deletion of GPR30 had no significant effect on gene expression of several adipogenic transcription factors in WAT, including peroxisome proliferator-activated receptor- γ (Ppar γ), CCAAT/enhancer binding protein- α (Cebp α), Cebp β , C/ebp δ , and bone morphogenetic protein 2 (Bmp2) (Supplemental Figure 4C).

The effects of GPR30 on body temperature, energy expenditure, and fatty acid oxidation (FAO)

As the absence of GPR30 didn't alter the amount of food intake in mice, we examined if deletion of GPR30 increased energy expenditure, thereby resulting in reduced fat deposits. In this regard, we first measured body temperature at 12, 19 and 22 wks of treatment. Female GPRKO mice fed HFD had significantly higher rectal body temperature than that of HFD-fed WT female mice (38.7, 38.8, and 38.3 °C for KO mice vs 38.1, 38.1, and 37.6 °C for WT mice at 12, 19, and 22 wks, respectively) (Figure 5A). GPRKO female mice tended to have higher energy expenditure than the WT female mice (Figure 5B), but the difference was not statistically significant ($p=0.07$). In addition, female GPRKO mice fed HFD tended to be more active than WT female mice during

dark time ($p=0.0825$) (Figure 5C). Further, absence of GPR30 didn't alter FAO in WAT (Figure 5D) or muscle (Figure 5E).

GPR30 regulates adipogenesis of mouse adipose-derived stromal cells and 3T3-L1 preadipocytes

We further examined whether GPR30 directly regulates adipogenesis, which is crucial in driving the expansion of adipose tissue mass that leads to obesity. Consistent with the reduced fat mass in GPRKO mice, adipose-derived stromal cells from GPRKO displayed a significantly lower rate of differentiation as compared with WT cells (Figure 6A and B). We further confirmed that GPR30 gene was expressed in WT stromal cells (Figure 6C) and their differentiated fat cells (Figure 6D), but was completely absent in cells from GPRKO mice (Figure 6C and D). Consistently, activation of GPR30 by G1 increased adipogenesis of 3T3L1 cells (Figure 6E and F) without affecting cell proliferation (data not shown). We further show that GPR30 mRNA is abundantly present in both 3T3-L1 preadipocytes and fully differentiated adipocytes (Figure 6G). These data suggest that the reduced adiposity in GPRKO female mice may be due to decreased adipogenesis.

Discussion

In the present study, we found that deletion of GPR30 protected female mice from developing obesity, glucose intolerance, and insulin resistance when challenged with a HFD. Interestingly, all these effects are not observed in male mice. We also analyzed GPR30 mRNA levels in adipose tissues of male and female mice, and found that there was no significant gender difference in adipose expression of GPR30. These data demonstrate that GPR30 regulation of adipose tissue

energy metabolism in response to HFD exposure is female-specific and may be E2-dependent. While data from the present study show that GPRKO female mice fed the HFD displayed better insulin sensitivity and glucose homeostasis, these beneficial effects may be the secondary effects whereby deletion of GPR30 prevented obesity in mice fed a HFD, given that deletion of GPR30 had no effects on blood glucose, insulin, and insulin sensitivity in STD-fed mice. However, we can't exclude the possibility that GPR30 may directly modulate glucose metabolism in HFD-fed mice that could lead to the improved glucose tolerance in HFD-fed mice.

As GPR30 is not involved in regulating calorie intake in mice, we then addressed whether GPR30 affects energy expenditure or ambulatory activity, which can contribute to the reduced body fat mass. It is worth noting that in the present study energy expenditure was normalized to lean mass instead of BW, because fat tissue may contribute comparatively less to the total energy expenditure compared with lean mass due to its relatively low metabolic activity (32). While KO female mice fed with HFD tended to have higher energy expenditure and cage activity than those of WT female mice, the differences didn't reach statistical significance. Consistently, deletion of GPR30 had no significant effect on fatty acid oxidation in WAT and skeletal muscle, suggesting that the ability of mitochondria to oxidize fatty acids in these tissues was not altered by GPR30. However, there is possible that the small difference in increased daily energy expenditure and physical activity between WT and KO mice could lead to the large differences in the accumulation of fat mass over time (32,33). BAT plays a critical role in maintaining body temperature and balancing energy expenditure in mammals (34). We observed that GPRKO mice had higher body temperature, suggesting that GPR30 could regulate fatty acid (35,36) and/or glucose (37,38) metabolism in BAT, which dissipates energy from glucose and fatty acids into heat (39). This aspect needs to be further investigated.

Disruption of fat digestion and absorption attends HFD-induced obesity. However, this is not the case for GPRKO mice, as the fecal triglyceride and NEFA contents were similar between fed WT and KO mice. Increased secretion of VLDL accompanied with disturbed clearance of triglycerides contributes to the development of obesity. In the present study, neither fat tolerance nor NEFAs levels in the blood between KO and WT mice were different. In addition, we believe that GPR30 is not involved in regulating de novo lipogenesis and triglyceride secretion in the liver, as deletion of GPR30 did not alter hepatic triglyceride contents and gene expression of transcription factors CHREBP and SREBP-1c, which coordinate the expression of genes required for fatty acid synthesis. Indeed, GPR30 mRNA was barely detectable in the liver and was absent in isolated mouse hepatocytes, while it was highly expressed in WAT of HFD-fed WT female mice. Interestingly, GPRKO mice had significantly higher fasting plasma triglyceride levels than those in WT mice. The reason for this difference is unclear. It is possibility that WT mice may be able to accumulate more triglycerides in the fat tissues given that WT mice fed a HFD had larger adipocytes as compared with those in KO mice. In addition, deficiency of GPR30 could affect plasma lipoprotein lipase activity during fasting, thereby modulating triglyceride levels. Nevertheless, these results provide strong evidence that the reduced fat accumulation by deletion of GPR30 is not due to altered fat absorption, hepatic lipid metabolism, or lipoprotein lipase-mediated triglyceride clearance.

While how exactly GPR30 regulates adipose tissue fat metabolism is still unclear, our data demonstrate that the effect of GPR30 on fat mass in HFD-fed female mice was not due to a secondary action by which its deletion altered circulating E2 levels or expression of ER α , which is believed to play a major role in mediating estrogenic effects on energy homeostasis (40). Both human and rodent WAT expresses ER α , ER β , and GPR30, suggesting that E2 signaling could

occur through both ERs and GPR30. Interestingly, it was reported that GPR30 and ER α inhibit each other's actions in several types of cells (7,20,41). These data suggest that there may be a “ying-yang” relationship between GPR30 and ER α in regulating energy metabolism in adipose tissue in response to E2. In that regard, activation of ER α by E2 inhibits adiposity, whereas activation of GPR30 might promote obesity. It was demonstrated that plasma E2 levels were increased in ER α KO female mice (42), which could lead to increased E2/GPR30 signaling. If this is true, it is possible that HFD-induced obesity in ER α KO female mice could be at least partially due to enhanced E2/GPR30 signaling in the lack of ER α , an aspect that is currently under investigation in our lab.

Adipogenesis plays an important role in the expansion of WAT mass that leads to obesity. WAT-derived stromal cells is a rich source of preadipocytes and mesenchymal stem cells that can be induced to differentiate into adipocytes (43). We found that deletion of GPR30 reduced adipogenic differentiation of WAT-derived stromal cells. Consistently, activation of GPR30 increased adipogenesis of 3T3L-1 preadipocytes. These data, along with our in vivo finding that GPR30 expression was upregulated in mice fed HFD, suggest for the first time that GPR30 may play a role in promoting obesity in females by at least partially acting in WAT to regulate adipogenesis. It is presently unknown however how GPR30 regulates adipogenesis, given that deletion of GPR30 had no significant effects on the gene expression of several important adipogenic factors in WAT. Fatty acid synthase (FAS) is a key lipogenic enzyme that catalyzes the generation of palmitate from malonyl-CoA and acetyl-CoA (18). FAS is also expressed in WAT. Recently studies showed that adipose FAS plays an important role in adipogenesis and obesity development (44). It was recently shown that activation of GPR30 by E2 increases FAS gene expression in cancer cells (18). It is therefore possible that FAS may be the downstream target

of the GPR30 signaling pathway that mediates its adipogenic action, which is presently under investigation in our laboratory.

In summary, the present study provides evidence for the first time that GPR30 promotes adipogenesis and thereby obesity in mice exposed to excess fat energy. The HFD-induced increase in GPR30 expression in WAT may lead to increased E2/GPR30 signaling, which could counteract the role of ER α in regulating adipose tissue energy homeostasis, an aspect that need further investigation.

Methods

Animals

GPR30 heterozygous mice on 129 background were kindly provided by Dr. Deborah J. Clegg (UT Southwestern Medical Center, TX). Homozygous GPRKO and their littermate WT mice were generated by matting heterozygous mice and genotyped using quantitative RT-PCR. All mice were housed under constant temperature (23-24°C) with a 12-h light/dark cycle and ad libitum access to food and water. The BW of GPRKO and WT mice was similar at weaning (3–4 wks) and thereafter when exposed to a STD. At 12 wks old, female mice were divided into 4 groups with 7-8 mice per group and fed either a STD with 18% of calories from fat or a HFD (Research Diets Inc., NJ) with 58% of calories from fat for 23 wks. Food intake and BW were recorded weekly. The weights of major organ including fat pads were recorded after mice were euthanized. For comparing GPR30 gene expression in WAT between male and female mice, WT female mice and their male littermates fed a STD were euthanized at 18 wks old, and the gonadal fat tissues were then collected for this analysis. All animal studies were approved by the Institutional Animal Care

and Use Committee (IACUC) at Virginia Tech, and all experiments were strictly carried out in accordance with approved protocols and regulations by IACUC.

Body composition and energy expenditure measurements

Body composition of the mice was evaluated using a nuclear magnetic resonance-based instrument (Bruker Optics Inc, MA) at 0, 5, 10, 15, and 20 wks of the feeding experiment. Body temperature was measured using a thermometer probe placed at a 2.5 cm depth in the rectum. After 23 wks of treatment, mice were transferred to metabolic cages for assessing energy expenditure and voluntary cage activity using an indirect calorimetry system (TSE Systems, Inc, MO) (45). The rates (ml/kg/h) of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) for each mouse were recorded at 20-min intervals for 48 h. Total energy expenditure was calculated as $EE = VO_2 \times (3.815 + (1.232 \times RER))$ and normalized to body lean mass (kcal/kg/h). Home cage activity was recorded at 20-min intervals for 48h and expressed as total distance moved per hour.

Measurement of adipocyte area and size

The inguinal, gonadal, and perirenal adipose tissues were fixed in 10% of phosphate buffered formalin for 18h, dehydrated, and embedded in paraffin. Tissues were then sectioned and stained with hematoxylin and eosin (H/E, Fisher Scientific, PA) and photographed at 10× of magnification. Areas of adipocytes were measured using the ImageJ software (NIH, MD). At least 100 adipocytes were counted from each section and 3 sections from each mouse. The frequency distribution of adipocyte sizes in fat depots was quantified as described (46). Briefly, Data were loaded into a spreadsheet of Microsoft Excel program, and the number of adipocytes within the distribution from

0 to 5,000 μm^2 with 500 increment was then calculated using the frequency function. The frequency distribution of adipocyte sizes was expressed as a percentage of total adipocytes counted.

Measurements of fatty acid oxidation (FAO) in white adipose tissue (WAT) and skeletal muscle

Mice were fasted overnight and gonadal fat tissues and skeletal muscle from the gastrocnemius and quadriceps were collected after euthanization. Tissue homogenates were incubated in buffer containing [1- ^{14}C] palmitic acid. FAO was assessed by measuring and summing $^{14}\text{CO}_2$ production and ^{14}C -labeled acid-soluble metabolites from the oxidation of [1- ^{14}C] palmitic acid (American Radiolabeled Chemicals, MO) (47).

Fecal triglyceride analysis

Feces were collected for 3 consecutive days at 10 and 19 wks of feeding experiment. Fecal lipids were extracted as previously described (48). Briefly, feces were weighed and lipids were extracted with chloroform-methanol (2:1). After centrifugation, chloroform phase was collected and lipid extracts were dried under a stream of nitrogen gas and then resuspended in chloroform-1% Triton X-100. The samples were evaporated again and finally dissolved in ddH₂O. Total triglyceride concentrations were then measured using an assay kit (Teco Diagnostics, CA) and normalized to the dry weight of feces.

Fat tolerance test and triglyceride measurement in the liver

To perform the fat tolerance test, mice were fasted overnight before administration of 10 μ l/g BW of olive oil by gavage. Plasma triglyceride levels were measured at 0, 1, 2, 3, 4 and 8 h after oil administration. Liver triglyceride content was determined as reported (49). The liver triglyceride contents are expressed as mg of triglyceride per gram of the liver sample.

Blood chemistry

The fasting (FBG) and non-fasting (NFBG) blood glucose levels were measured at various time points throughout the experiment (50). Plasma total cholesterol, triglyceride and non-esterified fatty acid (NEFA) were measured by using enzymatic assay kits (Teco Diagnostics, CA; Wako Diagnostics, CA). Plasma insulin levels were measured by ELISA (Crystal Chem, IL). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following equation: $\text{HOMA-IR} = (\text{fasting plasma insulin (mU/l)} \times \text{fasting plasma glucose (mmol/l)})/22.5$. Plasma E2 and leptin concentrations were determined by ELISA (Caymen Chemical, MI) and RIA (SPIbio, Montigny le bretonneux, France), respectively.

Glucose and insulin tolerance tests

For intraperitoneal glucose tolerance test, mice were fasted for 12 h and then injected intraperitoneally with a single bolus of glucose (1 g/kg body weight). For insulin tolerance test, mice were fasted for 4 h and then intraperitoneally injected with human insulin (0.75 units/kg BW; Eli Lilly, IN). Glucose levels were measured post injection using a glucometer (50).

Isolation of hepatocytes

Mouse hepatocytes were isolated as previously described (51). Hepatocytes were cultured with DMEM medium overnight before used for RNA extraction.

Quantitative real-time RT-PCR

Total RNA was extracted from tissues with TRI reagent (Molecular Research Center, OH) and reverse-transcribed using GoScript™ Reverse transcriptase and random primers (Promega, WI). Amplification reactions were performed on an Applied Biosystems® 7500 Fast Real-Time PCR System as we previously described (52). Data were analyzed by the $2^{-\Delta\Delta C_t}$ method. The primers used are: GPR30 (5'- TCATTTCTGCCATGCACCCA-3' and 5'-GTGGACA-GGGTGTCTGATGT-3'), ER α (5'- CTGTCGGCTGCGCAAGTGTT-3' and 5'-CATCTCTCTGACGCTTGTGCT-3'), ER β (5'- GCCAACCTCCTGATGCTTCT-3' and 5'-TCGTACACCGGGACCACAT-3'), Chrebp (5'-CTGGGGACCTAACAGGAGC-3' and 5'-GAAGCCACCCTATAGCTCCC-3'), Srebp-1c (5'-GATCAAAGAGGAGCCAGTGC-3' and 5'-TAGATGGTGGCTGCTGAGTG-3'), Pparg (5'- ATTGAGTGCCGAGTCTGTGG-3' and 5'-GCAAGGCACTTCTGAAACCG-3'), Cebpa (5'- AGCAACGAGTACCGGGTACG -3' and 5'-TGTTTGGCTTTATCTCGGCTC-3'), Cebpb (5'- CGCAACCTGGAGACGCAGCA-3' and 5'-GGCTCGGGCAGCTGCTTGAA-3'), Bmp2 (5'- GACTGCGGTCTCCTAAAGGTCG -3' and 5'- CTGGGGAAGCAGCAACACTA -3'), 18S RNA (5'-ACCTGGTTGATCCTGCCAGTAG-3' and 5'-TTAATGAGCCATTCGCAGTTTC-3').

Adipogenesis analysis

Stromal vascular cells from female WAT of WT and GPRKO mice were isolated as previously described (53). The cells were grown to confluence in complete DMEM medium containing 10% FBS and then were cultured in adipocyte differentiation cocktail containing 5 μ M dexamethasone, 2.5 μ g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1nM T3 (54) . After 2 days, cells were cultured in DMEM medium supplemented with 1.5 μ g/ml insulin and 1nM T3 for 4 days. 3T3L1 preadipocytes were cultured and differentiated as we previously described (55). Briefly, post-confluent cells were incubated in complete DMEM containing 1 μ M dexamethasone, 0.5 mM IBMX, 1 μ g/ml insulin with or without 100 nM G1 for 2 days. The cells were then washed with PBS and cultured in complete DMEM supplemented with 1 μ g/ml insulin for additional 2 days. Afterwards, cells were maintained in DMEM medium for 8 days with medium changed every other day. The differentiated cells were visualized by Oil Red-O staining of intracellular lipids. Oil red-O stain in the cells was extracted with isopropanol and quantified using a microplate reader (55).

Statistical analysis

Data were analyzed with one-way ANOVA or the student's t-test using JMP software (SAS Inc., NC), where appropriate. Values are expressed as means \pm SEM. Treatment differences were subjected to t-test or Tukey's test. A $P < 0.05$ was considered significant. Real-time PCR data were analyzed using the $\Delta\Delta$ CT method, where 18S RNA served as the endogenous control and fat from control mice served as the calibrator sample. The Δ CT = CT target gene – CT 18S, and $\Delta\Delta$ CT = Δ CT target sample – Δ CT calibrator (56). Relative quantities, calculated as $2^{-\Delta\Delta$ CT}, were used for statistical analysis.

Figures

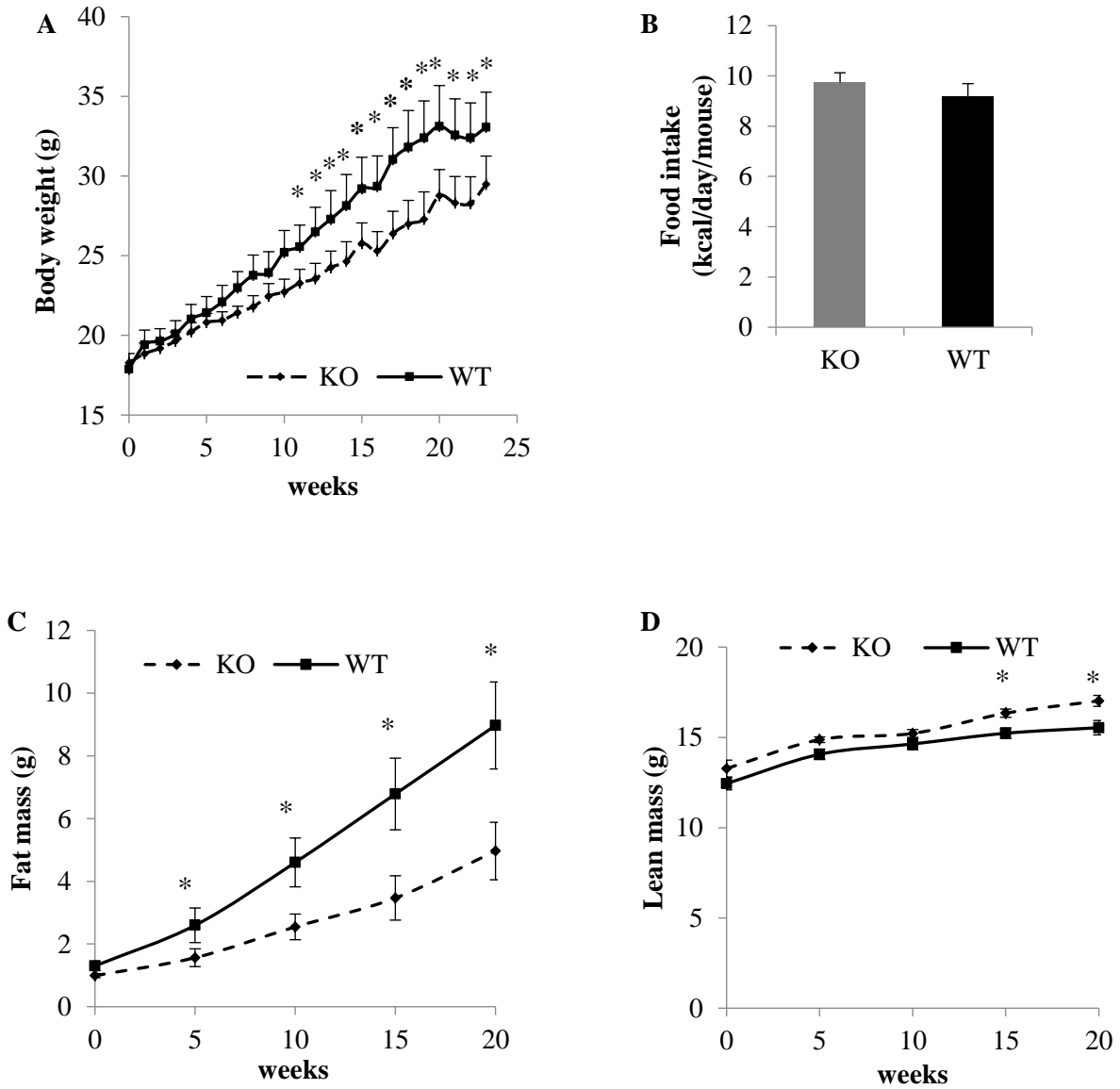


Figure 1. Deletion of GPR30 reduces adiposity in female mice fed a HFD.

WT and KO female mice (13 wks old) with identical initial body weight were fed a HFD for 23 wks.

Weekly body weight (**A**), food consumption as calorie intake (**B**), and fat (**C**) and lean (**D**) mass are shown.

Data are mean \pm SEM (n=8 mice/group). *, p<0.05.

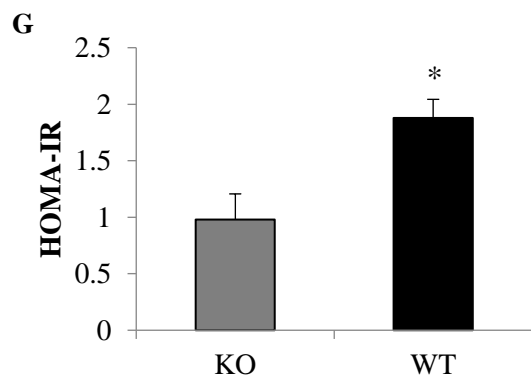
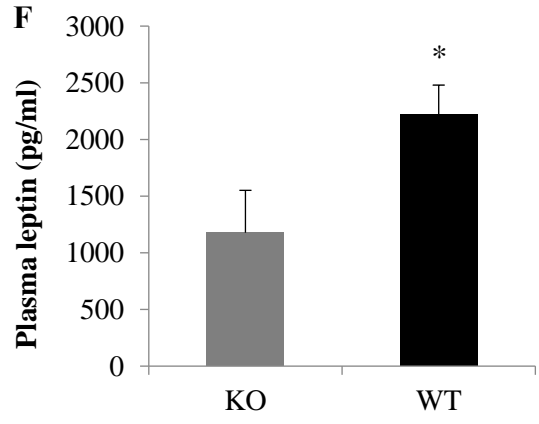
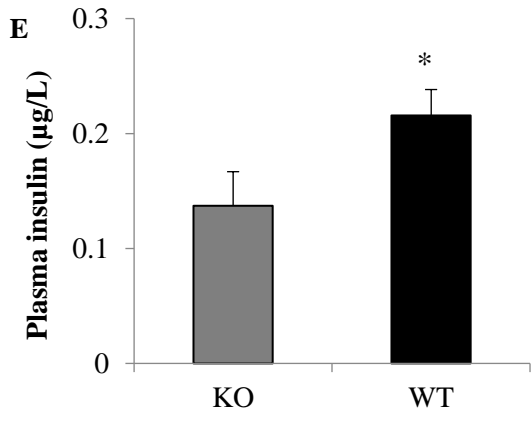
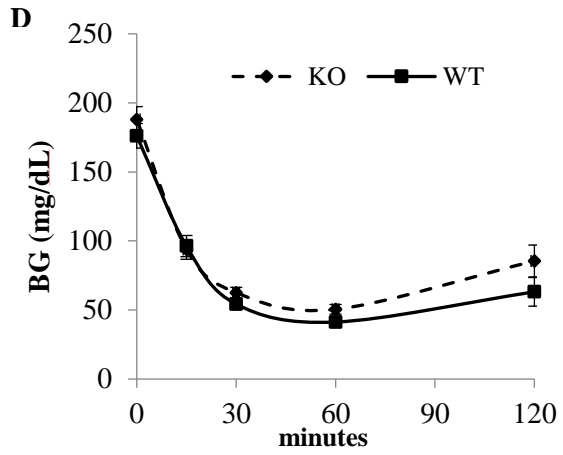
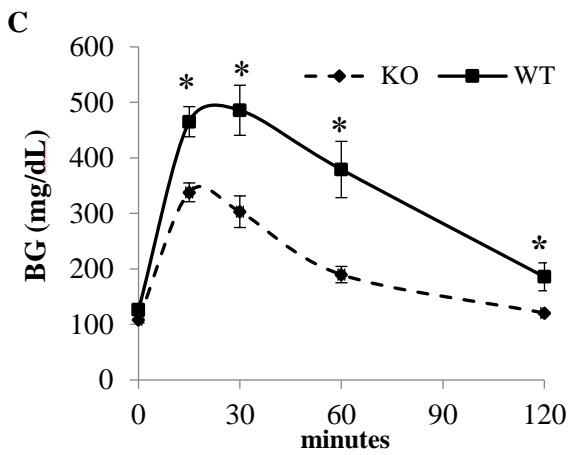
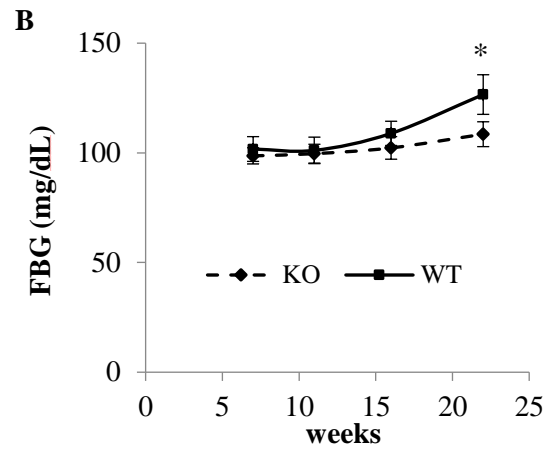
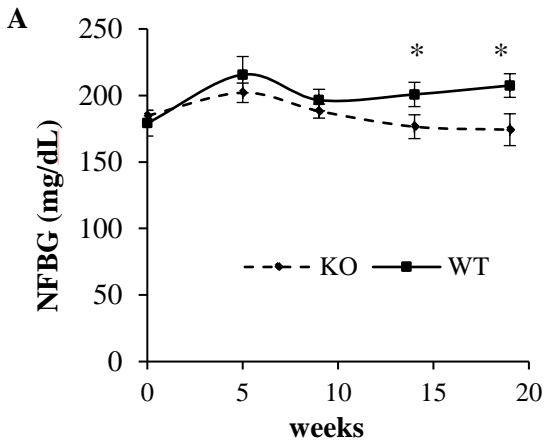


Figure 2. Deletion of GPR30 improves glucose homeostasis and insulin sensitivity in female mice fed a HFD.

(A) Non-fasting blood glucose (NFBG) and (B) Fasting blood glucose (FBG) levels were measured at indicated weeks of HFD feeding. Glucose (C) and insulin (D) tolerance tests were determined at 22 and 23 wks, respectively. Plasma insulin (E) and leptin (F) levels in overnight fasted mice after 23 wks on HFD were measured by enzyme immunoassay kits. HOMA-IR was calculated as stated in the “Methods” section (G). Data are mean \pm SEM (n=8 mice/group). *, P<0.05.

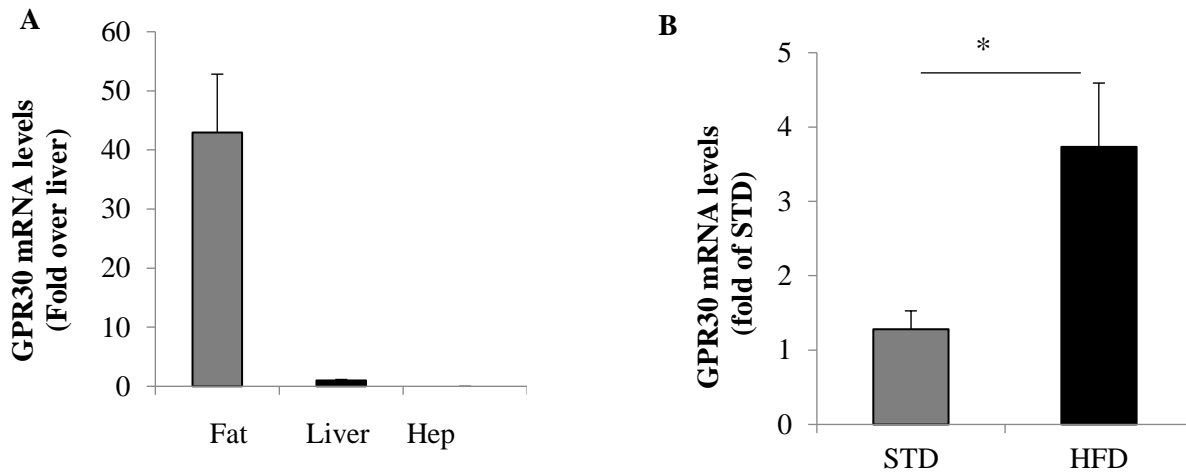


Figure 3. GPR30 mRNA expression was absent in liver hepatocytes but upregulated in WAT of female mice by HFD feeding.

A. GPR30 gene expression in fat tissue, liver, and primary hepatocytes (Hep) of the mice was measured by quantitative real-time RT-PCR. GPR30 mRNA expression in adipose tissue from female mice fed STD or HFD was also determined (**B**). Data are mean \pm SEM (n=8 mice/group). *, p<0.05.

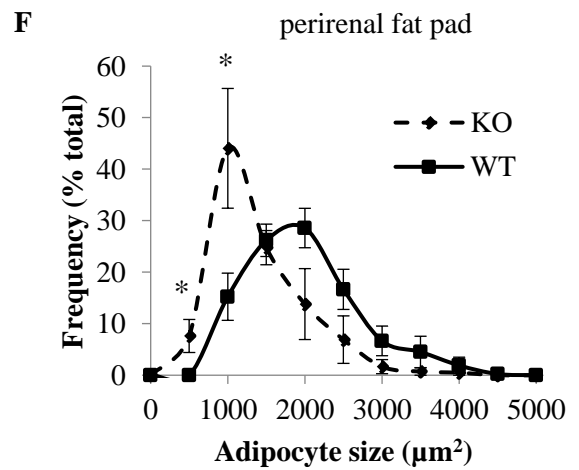
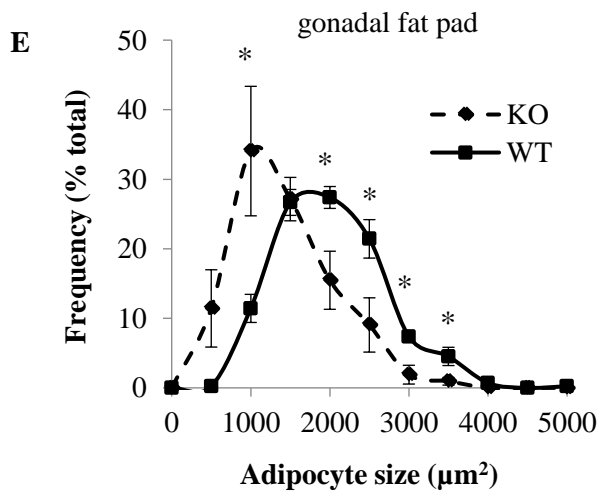
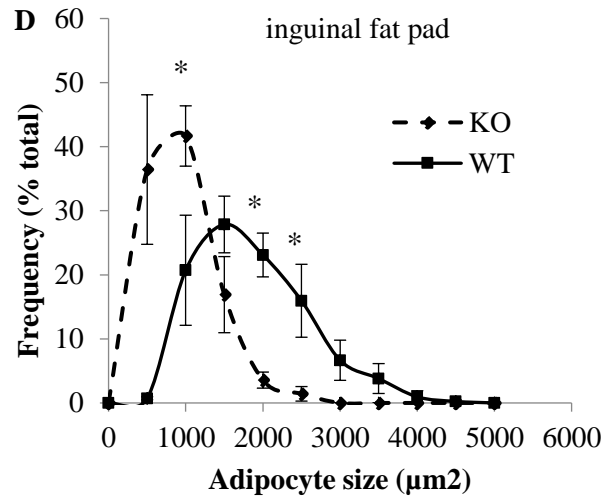
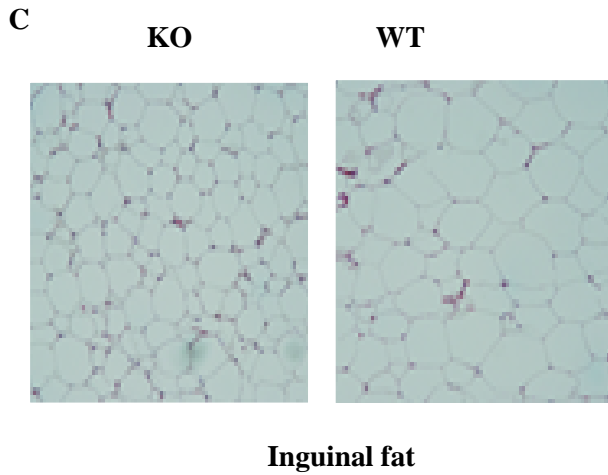
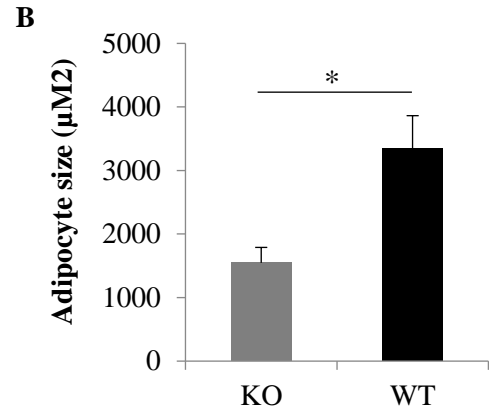
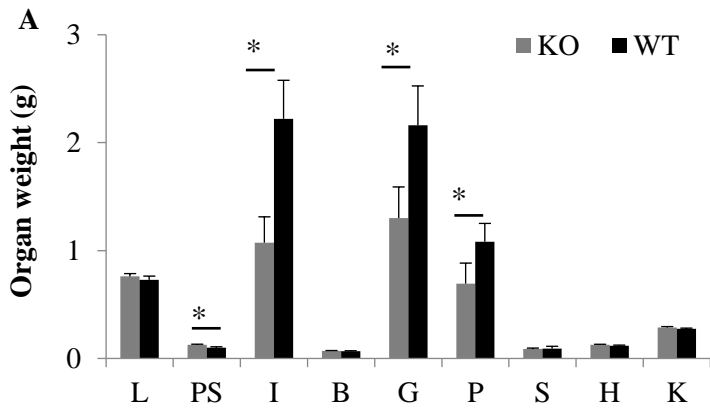


Figure 4. GPR30 KO reduces fat mass and adipocyte size in female mice fed a HFD.

A. Fat pad and organ weights of female mice fed a HFD for 23 wks. **B.** Average adipocyte size of inguinal fat. **C.** Representative images from inguinal fat tissue. The distribution of different sizes of adipocytes in inguinal (**D**), gonadal (**E**) and perirenal (**F**) fat pads. Data are mean \pm SEM (n=8 mouse/group). *, p<0.05.

Note: L=liver, PS=pancreas, I=inguinal fat, B=brown fat, G= gonadal fat, P=perirenal fat, S=spleen, H=heart, K=kidney.

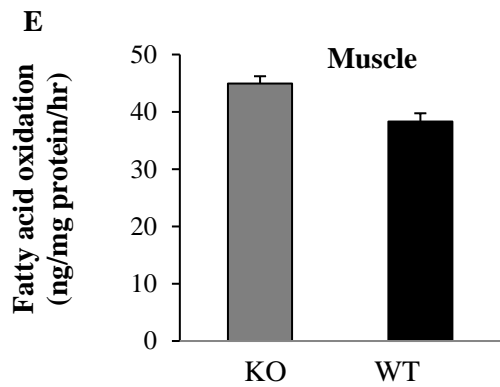
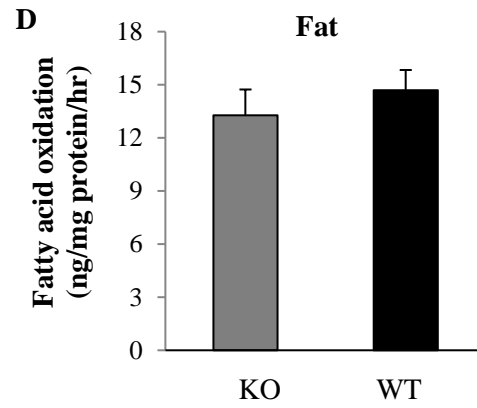
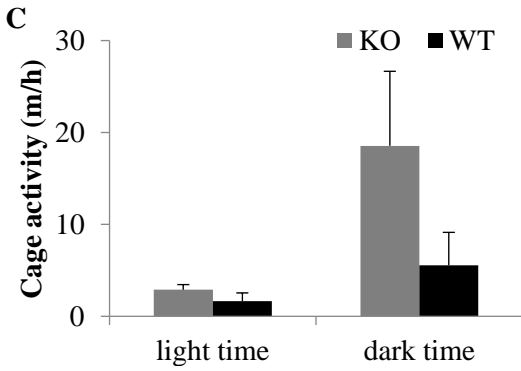
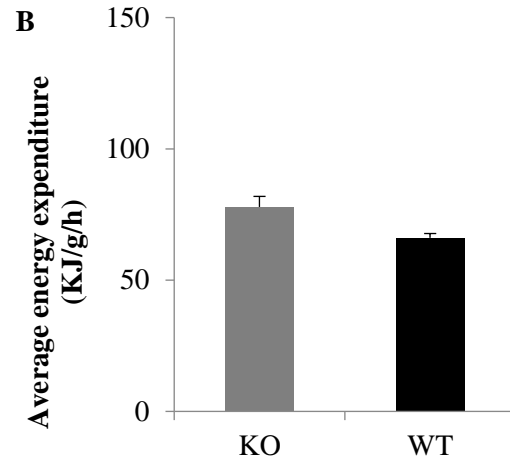
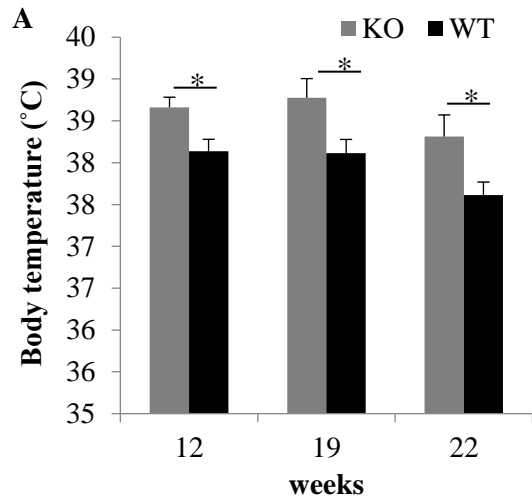


Figure 5. GPR30 deficiency increased body temperature but had no significant effect on energy expenditure and fatty acid oxidation.

Female WT or KO mice were fed a STD or HFD for 23 wks. Rectal body temperatures were measured at 12, 19 and 22nd wk (**A**). The average energy expenditure (**B**) and cage activity during light time and dark time (**C**) were measured after 23 wks of treatment. Fatty acid oxidation in white adipose tissues (**D**) and skeletal muscle (**E**) from WT and KO female mice were also determined. Data are mean \pm SEM (n=8 mice/group). *, p<0.05.

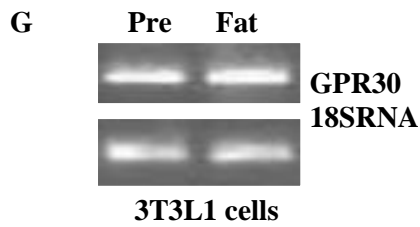
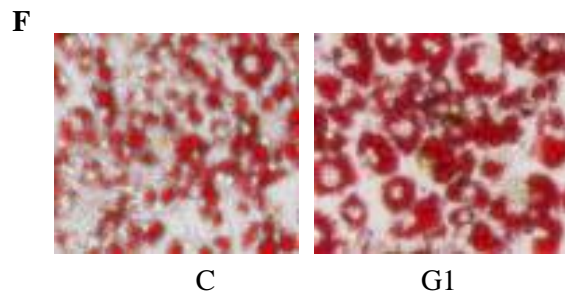
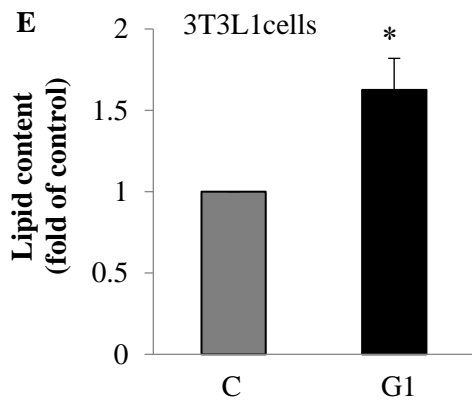
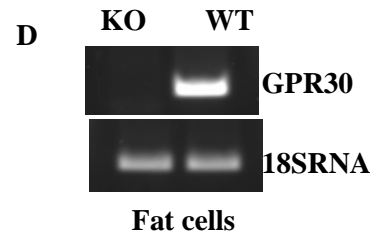
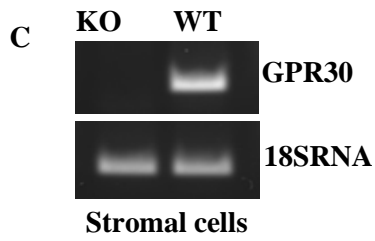
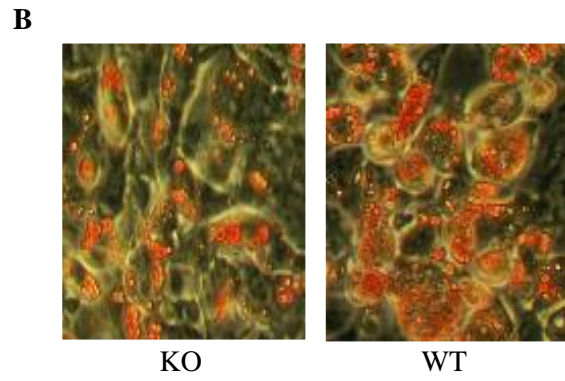
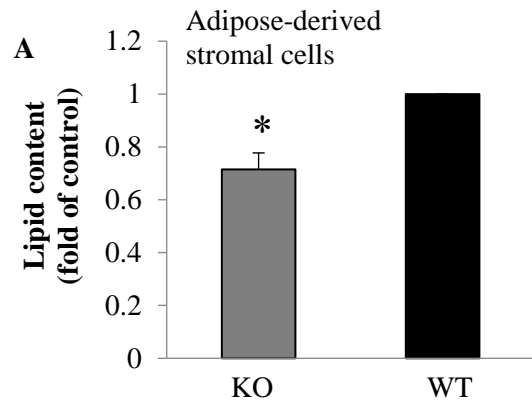


Figure 6. GPR30 regulates adipogenesis.

Adipose-derived stromal cells from GPRKO or WT female mice were cultured in differentiation medium for 6 days. **A.** The differentiated cells were visualized by staining with Oil Red O and stained lipids were extracted and quantified. **B.** Shown are representative images from four experiments. GPR30 gene expression was measured in WT and KO adipose-derived stromal cells (**C**) and differentiated fat cells from stromal cells (**D**) by RT-PCR (a representative full-length gel image is included in the Supplementary Information). Post-confluent 3T3L1 cells were incubated in differentiation medium with or without G1 (100 nM) as described in “Materials and Methods”. Lipid accumulation in the differentiated cells were measured (**E**), and representative images of Oil Red O staining of intracellular lipids from three experiments in triplicated determinations each are shown (**F**). **G.** GPR30 gene expression in 3T3L-1 preadipocytes (Pre) and fully differentiated fat cells (fat) as analyzed by RT-PCR. Data are mean \pm SEM. *, $p < 0.05$ vs. control.

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Chapter Four

Does estrogen act through GPR30 to affect adiposity in female mice?

Abstract

Estrogen plays an important role in maintaining the well-being of postmenopausal women as well as men, in whom the enzyme aromatase (Ar) is the primary regulator to convert C19 steroids to 17 β -estradiol (E2). Though GPR30 was officially named as G protein-coupled ER (GPER) and found to express in numerous tissues, the convincing evidence that E2 acts through GPR30 to elicit significant physiological events is still lacking. To investigate whether E2 is necessary for GPR30 action in fat metabolism, we generated three transgenic mouse models to examine the metabolic role of GPR30, Ar knockout (ArKO), GPR30 knockout (GPRKO), and Ar and GPR30 double knockout (DKO). We discovered that GPR30 deficiency had limited effects on energy metabolism in mice fed a standard chow diet (STD). However, deletion of GPR30 promoted metabolic flexibility in both genders fed a HFD regardless of the presence of estrogen, suggesting GPR30 may not solely act as an ER. Further investigation clarifying the detailed signaling regulated by GPR30 in adipose tissue would largely contribute to our understanding of the sex differences in the development of obesity and provide novel therapeutic option for obesity treatment.

Keywords: GPR30, estrogen, aromatase, metabolic flexibility

Introduction

Based on estimates by the Centers for Disease Control and Prevention in 2017, approximately 40% of adults are obese in the US (1). It has been recognized that estrogen plays an important role in maintaining the well-being of the postmenopausal women as well as men (2,3). Premenopausal women tend to have an increased lower body fat or pear-shaped body, whereas men and postmenopausal women tend to have an accumulation of central body fat (visceral fat) or apple-shaped body (4), which is associated with a higher risk of breast cancer, insulin resistance, and cardiovascular disease (5-8). The shift of body fat from the gluteal to the abdominal region coincides with a reduction of circulating estrogen in menopausal women, thereby linking estrogen deficiency to central obesity (9). In addition, postmenopausal women receiving estrogen replacement therapy do not share the abdominal adiposity accumulation pattern, which often thought to be the characteristic of menopause (10,11), again emphasizing the strong correlation between estrogen and adiposity.

The significant role of estrogen in lipid metabolism is primarily mediated via the classic nuclear estrogen receptor α (ER α) (12), whereas the effect of estrogen receptor β (ER β) in maintaining fat metabolism is inconclusive (12-14). GPR30, a seven transmembrane-domain G-protein coupled receptor (GPCR), was first discovered and cloned in 1997 (15) and recently has been acknowledged as a novel membrane-associated estrogen receptor, officially renamed as GPER (G-protein coupled estrogen receptor) (16). All three aforementioned ERs are expressed in many different tissues in humans and rodents, including the brain (17-20), adipose tissue (21-23), blood vessels (24,25), intestines (26,27), and adrenal glands(28). Therefore, tissues where expressing one or more ERs are the major targets of estrogenic regulations.

In men and postmenopausal women, the conversion of C19 steroids to estrogen in adipose tissues is the primary mechanism to produce estrogen (29-31). Aromatase (Ar) is the key enzyme that catalyzes the biosynthesis of C18 estrogens from C19 androgens (32,33). In human, aromatase is encoded by the *cyp19* gene and expressed in several tissues including the tests, bone, adipose tissue, and various sites in the brain (34,35). Unlike the ovaries where estrogen is essentially generated in premenopausal non-pregnant women, these extragonadal sites synthesize a small amount of estrogen but exert significant biological functions locally (36). Notably, these extragonadal sites do not possess the capacity to synthesize C19 precursor steroids to aromatize estrogen. Hence, extragonadal estrogen synthesis depends on the circulating C19 precursor steroids androgen and the bioavailability of local aromatase (37,38).

We previously discovered that GPR30 knockout (GPRKO) female mice had about 50% less body fat mass compared with wild type (WT) female mice when fed a high-fat diet (HFD) (39), which is in clear contrast to the established phenotype of mice lacking ER α or estrogen. We speculated that there might be a “Yin-Yang” relationship between GPR30 and ER α regarding their roles in the development of obesity, meaning they counteract each other’s action in regulating fat metabolism. In fact, it has been shown that GPR30 activation opposes the effects of ER α in several different types of cells (40-42). Furthermore, GPR30 gene expression levels in fat tissues of HFD-induced obese female mice were significantly increased while neither the circulating estrogen nor ER α / β gene expressions were altered due to the deletion of GPR30, suggesting the excess energy intake induced fat accumulation could be, or at least partially, caused by the enhanced estrogen-GPR30 signaling in adipose tissue. To test our working hypothesis, we generated three animal models, GPR30 and aromatase double knockout (DKO), GPRKO, and aromatase knockout

(ArKO), to investigate whether the obesity-promoting action by GPR30 requires extragonadal estrogen.

Results

Deletion of GPR30 and/or Ar had limited effects on body weight and fat mass regulation in mice fed a STD.

In the course of current study, we observed that estrogen-insufficient ArKO animals had a progressive body weight gain as compared with the WT littermates in both genders (Fig. 1A&B). Adult male ArKO mice were heavier than WT littermates from 12 weeks of age and onward, while female ArKO mice displayed slightly increased body mass at 20 weeks of age compared to WT, indicating a transition stage in menopause when the ovaries senescence gradually lead to a low level of circulating estrogen (43,44). GPRKO animals, not only female but also male mice, had slightly lower body weight as compared to WT, which is consistent with our previous results (39). Interestingly, DKO female mice still had slightly lower body weights as compared to ArKO mice at 20 weeks of age, suggesting the absence of GPR30 still exert a moderate anti-obese effect when the endogenous estrogen is limited. In agreement with the body weight result, whole body composition analyses showed that GPRKO and DKO female mice had slightly lower body fat mass as compared to WT and ArKO females, while ArKO male mice had higher percentage of fat mass as compared with the WT, GPRKO, and DKO male mice (Fig. 1 C and D).

Deletion of GPR30 and/or Ar had no effect on glucose homeostasis in mice fed a STD.

To determine if the deletion of GPR30 and/or Ar affects glucose homeostasis, non-fasting blood glucose (NFBG) were measured at 11, 14, 16, and 19 weeks of age. The NFBG amongst the four groups of female mice (Fig. 2A) and male mice (Fig. 2B) were similar along the observation, suggesting the beneficial effect of GPR30 deletion on glucose homeostasis we previously discovered [41] were blunted when the mice were not challenged with excessive energy intake.

Deletion of GPR30 suppressed body weight gain in HFD fed mice and this effect was blunted when endogenous estrogen is limited.

In order to investigate whether estrogen is the endogenous ligand of GPR30 and acts through GPR30 to promote adiposity, we examined GPRKO, ArKO, DKO, and WT mice challenged with the high-fat diet (HFD) for 16 weeks. The BW were similar before HFD feeding, however, a notable difference of body weight change due to the ablation of Ar appeared around 30 weeks of age. As seen in Fig. 3A, ArKO as well as DKO female mice were significantly heavier than WT and GPRKO groups. Starting from 5 weeks of HFD feeding, the two groups without Ar were heavier onward, indicating the decreased level of circulating estrogen due to the hormone fluctuation around 8 months, which is the established menopause stage in lab rodents (44). Intriguingly, the disparity of body weight between WT and GPRKO became slightly wider at the same time, while the food intake of all groups were same (data not shown). GPRKO female mice had the lowest body weight during the whole HFD feeding period, which is consistent with our previous findings (39), suggesting a potential protective effect of GPR30 ablation in obesity development. Notably, we observed similar phenotype in the old male mice as well. At 24 weeks of age, all four groups of male mice had similar body weights. However, after 5 weeks of HFD

feeding, WT and GPRKO mice were lighter as compared to those lacking Ar groups, ArKO and DKO (Fig. 3B). Interestingly, when comparing GPRKO and WT, GPR30 deficiency had a potential anti-obese property whereas this effect was blunted when comparing ArKO and DKO groups. The deletion of GPR30 had no effect on body weight regulation when the androgen failed to be aromatized to estrogen, suggesting that estrogen is, or at least partially, the endogenous ligand of GPR30.

Deletion of GPR30 reduces adiposity and enhances body lean mass in both genders fed a HFD.

Results from NMR-based whole body composition analysis showed that fat mass in GPRKO female mice were lower after 10 weeks of HFD feeding, while the lean mass was higher (Fig. 4 A and B). Interestingly, the fat mass of DKO female were slightly lower as compared to ArKO, suggesting the deletion of GPR30 has a subtle yet notable effect when endogenous estrogen level was limited. However, as shown in Fig. 4C, the fat mass of ArKO and DKO male mice were similar through the entire study. Considering that Ar is the key enzyme for estrogen production in males, it indicates that the pro-obese effect of GPR30 activation failed to increase body fat accumulation in the absence of estrogen in males, suggesting that the effect of GPR30 as an “obesity gene” requires the activation by estrogen.

Deletion of GPR30 improves glucose homeostasis in both female and male mice fed a HFD.

At the time when mice were exposed to the HFD, there was no difference in NFBG level among four groups of female or male mice (data not shown). However, HFD-fed female GPRKO mice

had significantly lower fasting blood glucose (FBG) levels compared with the other genotypes littermates (Fig. 5A) while DKO male mice had higher FBG as compared to the rest (Fig. 5B). As insulin signaling plays a pivotal role in maintaining glucose homeostasis, we performed insulin tolerance test (ITT) and glucose tolerance test (GTT) to investigate whether the capability of GPR30 potentially mediating the metabolic changes depends on ligation of estrogen. ITT results showed no statistically significant group differences in both genders (data not shown). GTT results, however, showed that deletion of GPR30 significantly improved glucose tolerance in female mice (Fig. 5C&D). Fig. 5E shows the results of GTT in male mice performed after 10 weeks of HFD feeding. Consistently, GPRKO male mice displayed improved glucose tolerance as demonstrated by a significantly lower AUC following ip glucose injection (Fig. 5F).

Deletion of GPR30 might regulate thermogenesis via promoting heat production in mice fed a HFD.

While the absence of GPR30 didn't alter the amount of food intake in mice, we observed GPRKO female mice fed the HFD had higher body temperature and energy expenditure than those of WT mice (39), which likely contributes to the reduced fat deposits. We then measured the body temperature at 5, 10, and 15 weeks of HFD feeding. GPRKO mice, female and male, consistently exhibited a higher body temperature throughout the recording. In females, GPRKO mice were 0.3°C higher as compared to that of WT mice, while DKO mice were 0.33°C higher than that of ArKO at week 15, suggesting the potential effect of GPR30 deficiency on regulating body temperature even with a limited or low level of endogenous estrogen in female mice. A not quite similar pattern was observed in male mice. A higher body temperature of GPRKO mice was seen throughout the study, however, in contrast to females, the ArKO and DKO male mice showed

similar body temperature, suggesting that deletion of GPR30 would not affect the regulation of body temperature in the absence of estrogen in males. Taken together, GPR30 has a counter effect on regulating body temperature thereby suppressing the energy metabolism leading to excess fat accumulation.

Discussion

GPR30 has been classified as a novel ER, but it remains to be determined whether GPR30 mediates endogenous E2 effects *in vivo*. Here, our results reinforce the concept that estrogen plays a critical role in maintaining energy homeostasis. In the first animal study when mice were fed a STD, ArKO mice displayed higher body weight while GPRKO mice maintained the lowest body weight as compared to their littermates. Though the results were not significant, it shed a light on the role of GPR30 and the interaction between GPR30 and endogenous estrogen in energy metabolism since the mice were fed a STD, representing a physiologically relevant situation. The rodents, rats, and mice share the similar endocrine changes and steroid fluctuation found in human menopause, with menopause starting approximately by 8 months of age (44). By 20 weeks of age when female mice have normal ovarian function releasing estrogen (45), DKO female mice had lower body weights as compared to ArKO female mice, sharing a similar pattern when comparing GPRKO and WT mice, indicating that GPR30 deficiency might have an effect on energy metabolism independent of endogenous estrogen. In the present study, we found that GPRKO female mice had less body fat mass as compared to WT littermates. Similarly, the DKO female mice also had lower percentage of body fat mass than that of ArKO female mice, further suggesting the pro-adiposity effect of GPR30 may be not mediated by estrogen.

The ArKO male mouse model is a great estrogen responsive model since aromatase is the key enzyme for estrogen formation in male (38). However, in both young healthy male mice study and old obese male mice study, no difference of body weight gain between ArKO and DKO groups were observed throughout the study, while GPRKO male mice displayed a slightly decreased body weight gain, which is consistent with our finding that GPR30 deficiency had no effect on adiposity in male mice (39). It is presently unclear the reason that GPR30 is inactive in male mice. We speculate that this is due to lower levels of circulating 17 β -estradiol concentrations in males, which should be responsible for the female-specific effect of GPR30. Plasma 17 β -estradiol levels in male mice are only about 40 pM (46). However, the estimated binding affinity of E2 to GPR30 is in the range of 2.7-6 nM (47-51), which is much lower as compared with its binding affinity for classical ERs that are in the range of 0.1-1.0 nM (52). Therefore, endogenous E2 levels in male mice might be not high enough to partially or fully activate GPR30.

Measures of whole body composition showed that GPRKO female mice had significantly less body fat and higher proportion of lean body than that of ArKO mice, which raised an interesting question regarding the metabolic interaction between GPR30 and estrogen, and the complex signaling involved. In light of our recent speculation that deletion of GPR30 may enhance thermogenesis, the increased lean body mass in GPRKO female after 10 weeks of HFD feeding could be due to the browning of WAT and enhanced thermogenesis, thereby leading to a decreased fat mass. Indeed, our findings also showed that GPRKO had higher body temperature as compared to the other littermates after 15 weeks of HFD feeding in both female and male. It is interesting to note that DKO female mice had comparable body temperature as GPRKO, suggesting that activation of GPR30 may suppress thermogenesis. In males, DKO and ArKO had similar body

temperature which were lower than that of GPRKO male mice, indicating the effect of GPR30 is diminished in the absence of estrogen.

It was shown that men with disrupted mutations in the Ar genes displayed impaired glucose tolerance and hyperglycemia, and estrogen treatment improves the metabolic status in men with Ar deficiency (53,54). Interestingly, DKO male mice showed slightly better glucose tolerance as compared to ArKO while GPRKO had improved glucose tolerance in HFD-fed mice. Since deletion of Ar in males blocks the conversion from androgen to estrogen, it is assumed that estrogen is absent in ArKO and DKO male mice. Still, GPR30 deficiency tend to ameliorate glucose intolerance in diet-induced obese male mice in the absence of estrogen, suggesting estrogen may not be the only ligand that activates GPR30 signaling. The cell-based experiments proposed a potential collaboration between the classical ER α and GPR30 (reviewed in Ref. (55)), suggesting the signaling of estrogen requires the assembly of a large protein complex that communicates with GPR30. Therefore, the loss of any one of the links lead to a disrupted signaling cascade. Currently, few details are known regarding the interaction between the classical ER α and GPR30 upon the action of estrogen.

The drawback of the present studies is the lack of estrogen measurements. Without the determinant parameter, it is difficult to interpret the fluctuations in metabolic changes observed in female mice. As discussed above, competition binding assays revealed a binding affinity for estrogen to GPR30 ranged 3-6 nM (49,51), which is 3-4 folds higher than the circulating estrogen in female mice (56). Regardless of the circulating level or extragonadal production of estrogen, deletion of GPR30 has an influence on energy metabolism and may attenuates or reverses the impaired metabolic flexibility caused by insufficient estrogen.

In summary, preliminary study of animal models we generated aimed at exploring the metabolic role of GPR30 provides unique insight regarding the GPR30 signaling and energy metabolism in response to estrogen. The present study provides valuable findings that deletion of GPR30 has certain capability to improve the metabolic flexibility, including glucose homeostasis and thermogenesis, regardless of the amount of estrogen. Further investigation clarifying whether estrogen acts as an endogenous ligand to activate GPR30 signaling would largely contribute to our understanding of the sex differences in obesity development and provides a novel therapeutic target to fight obesity.

Research Design and Methods

Animals

Homozygous GPRKO, ArKO, and DKO and their littermate WT mice were generated by mating heterozygous mice and genotyped using quantitative RT-PCR. All mice were housed under constant temperature (23-24°C) with a 12-h light/dark cycle and *ad libitum* access to food and water. As for the first observation study, the young mice were fed a standard chow diet and started recording body weight, food intake, body composition, and blood glucose at 10 weeks of age. All mice were then changed to HFD with 58% of calories from lard (Research Diets Inc., New Brunswick, NJ, USA) for the second study. Ethics approval was obtained from the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech and all experiments were strictly carried out in accordance with approved protocols and regulations by IACUC.

Body weight and food intake measurements.

To investigate the effect of GPR30 and estrogen deficiency on energy metabolism, body weight and food intake were determined weekly at the same time in the morning for 10 weeks for the first study and 16 weeks for the second study.

Blood glucose measurements.

NFBG and FBG were measured at 11, 14, 16, and 19 weeks of age in the first study. Two measurements were conducted two days apart in the same week allowing animal to rest. The duration of fasting for FBG measurement is 15 hours.

Body composition measurements.

Body composition of the mice was evaluated using a nuclear magnetic resonance-based instrument (Bruker Optics Inc, MA) at 20 weeks of age before changing diet. At age of 6 month old, all animals were maintained on HFD and the body composition were determined at 5, 10, and 15 weeks of HFD feeding.

GTT and ITT (second study only).

For intraperitoneal glucose tolerance test, mice were fasted overnight and then injected intraperitoneally with a single bolus of glucose (1 g/kg body weight). For insulin tolerance test, mice were fasted for 4 h and then intraperitoneally injected with human insulin (0.75 units/kg BW; Eli Lilly, IN). Blood glucose levels were measured post injection using a glucometer at 15, 30, 60, and 120 minutes.

Body temperature measurements (second study only).

Body temperature was measured using a thermometer probe placed at a 2 cm depth in the rectum at 5, 10, and 15 weeks at the same time in the morning.

Figures

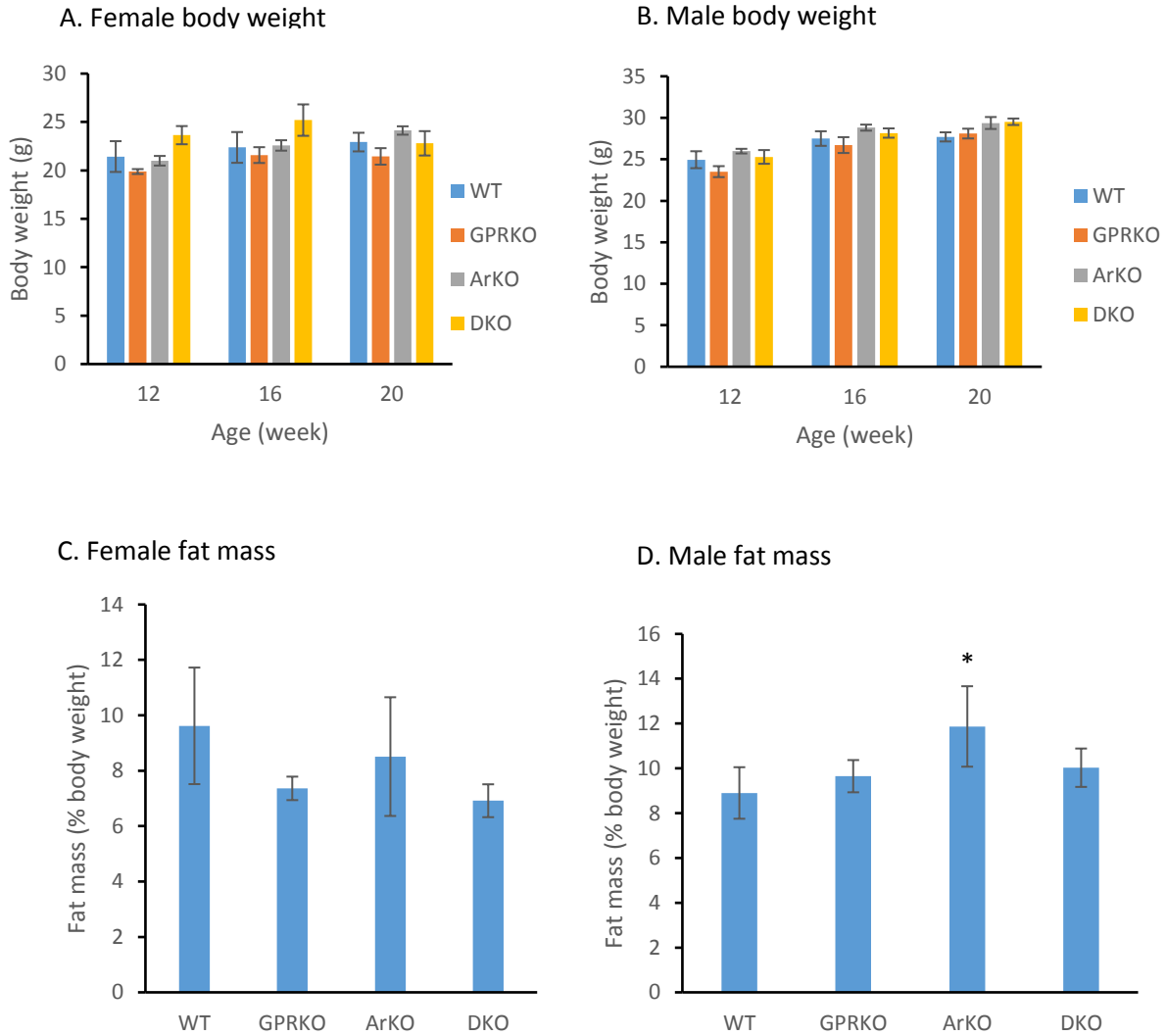
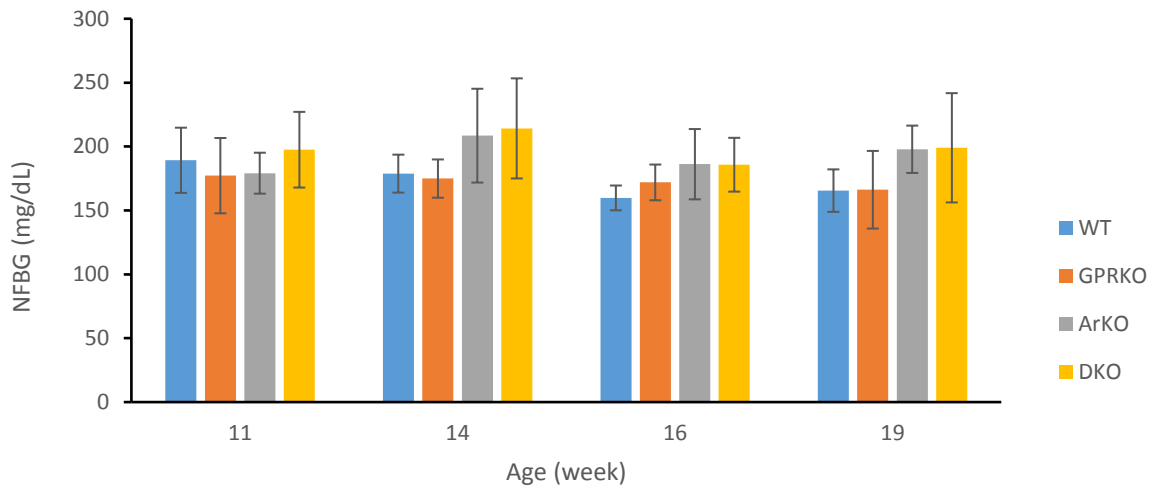


Figure 1. Deletion of GPR30 and/or Ar had limited effects on body weight and fat mass regulation in mice fed a STD.

All mice (10 weeks old) with identical initial body weight were fed a STD for 10 weeks. Measurements of body weight at weeks 12, 16, and 20 of female (**A**) and male (**B**) are shown. Fat mass of female (**C**) and male (**D**) are shown as the percentage of whole body weight. Data are mean \pm SEM (n=6-8 mice/group).

A. Female non-fasting blood glucose (NFBG)



B. Male non-fasting blood glucose (NFBG)

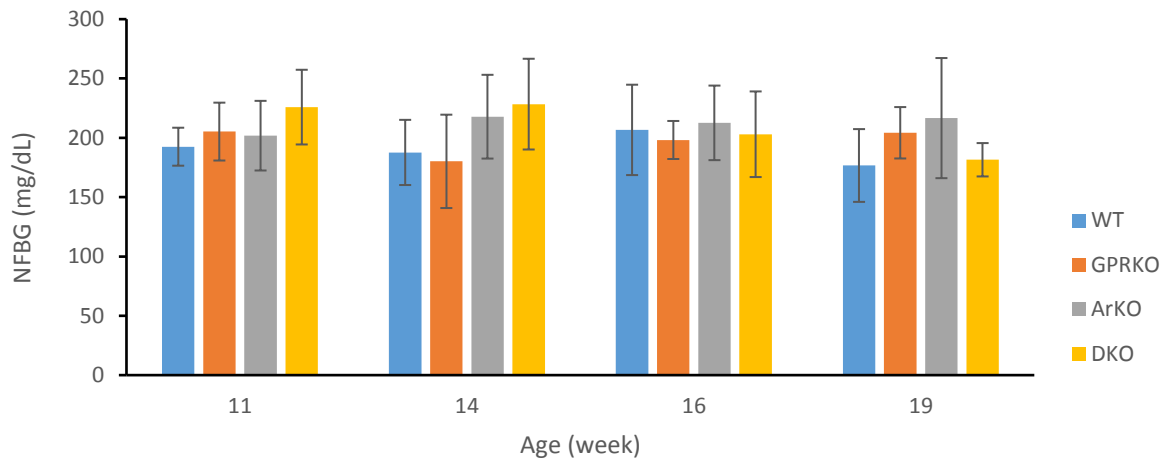
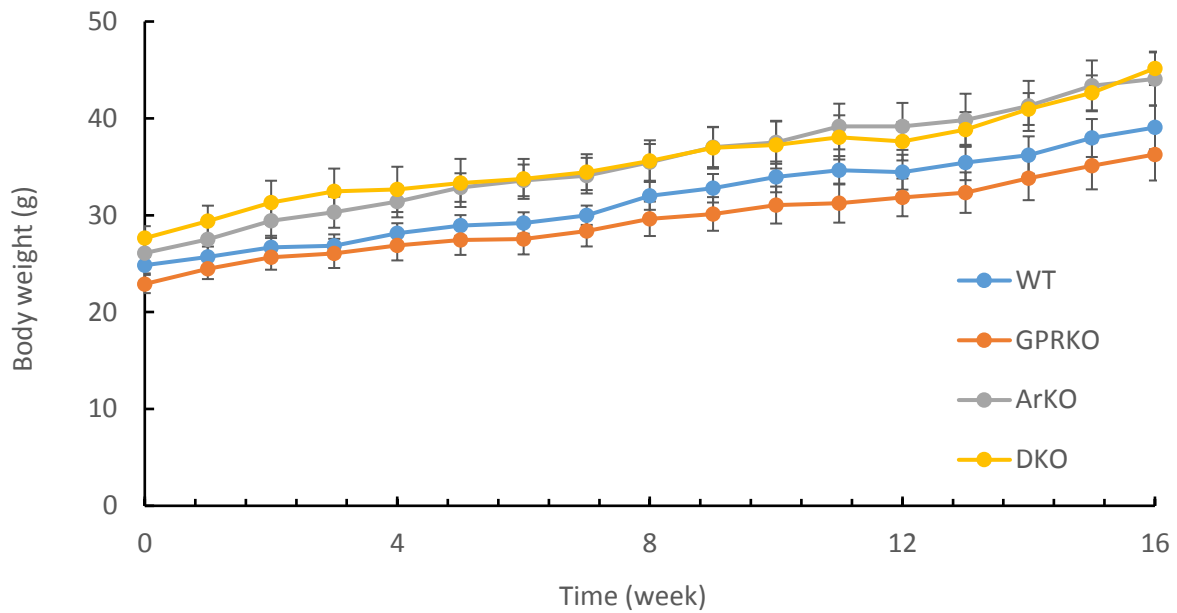


Figure 2. Deletion of GPR30 and/or Ar had no effect on glucose homeostasis in mice fed a STD.

Non-fasting blood glucose (NFBG) levels were measured at age of 11, 14, 16, and 19 weeks, both female (**A**) and male (**B**). Data are mean \pm SEM (n=6-8 mice/group).

A. Female body weight



B. Male body weight

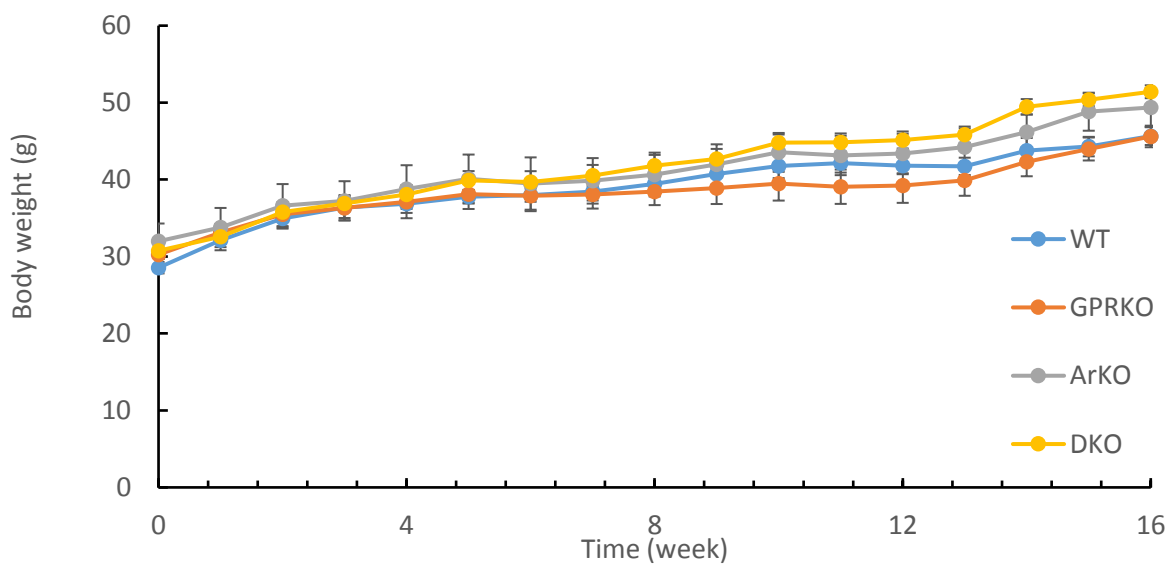


Figure 3. Deletion of GPR30 suppressed body weight gain in HFD fed mice and this effect was blunted in DKO mice.

WT, GPRKO, ArKO, and DKO mice (6-month old) were fed a HFD for 16 weeks. Body weight of female (A) and male (B) were measured weekly. Data are shown as mean \pm SEM (n=6-12 mice/group).

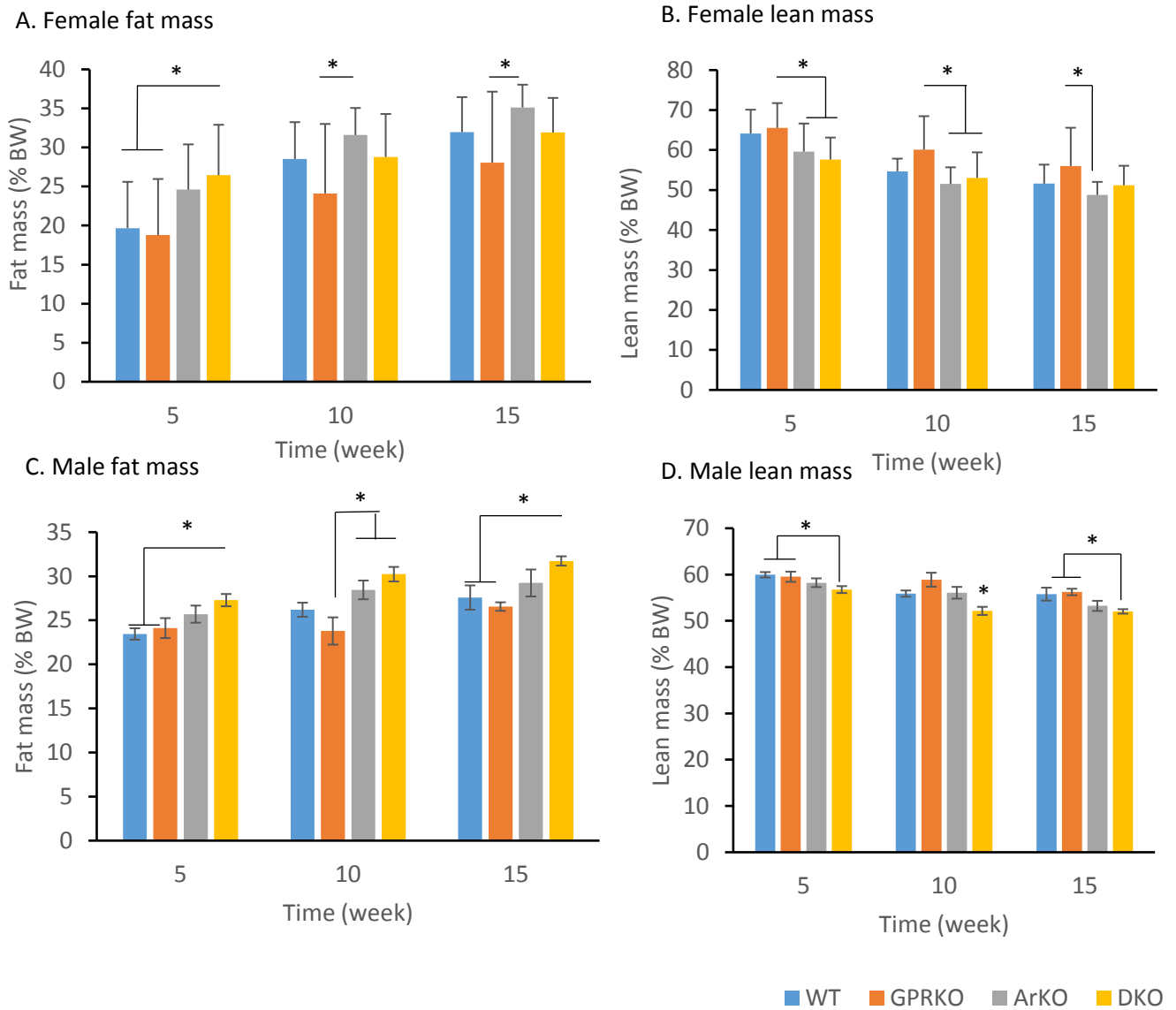
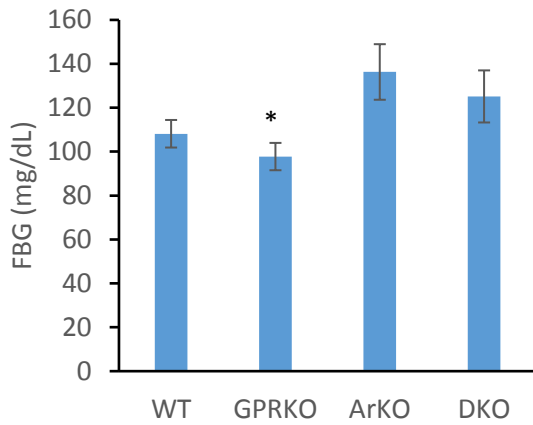


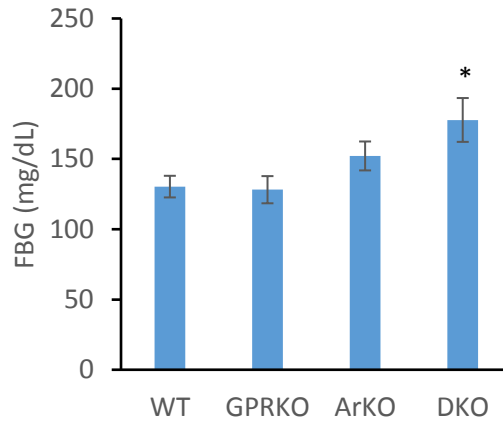
Figure 4. Deletion of GPR30 reduces adiposity and enhances body lean mass in both genders fed a HFD.

NMR-based body composition were measured at weeks 5, 10, and 15. Female fat mass (**A**), lean mass (**B**) and male fat mass (**C**) and lean mass (**D**) are shown. Data are mean \pm SEM (n=6-12 mice/group). *, p<0.05.

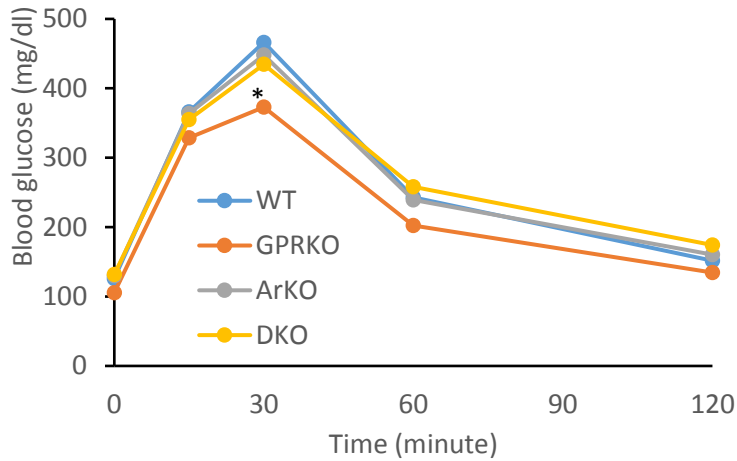
A. Female fasting blood glucose (FBG)



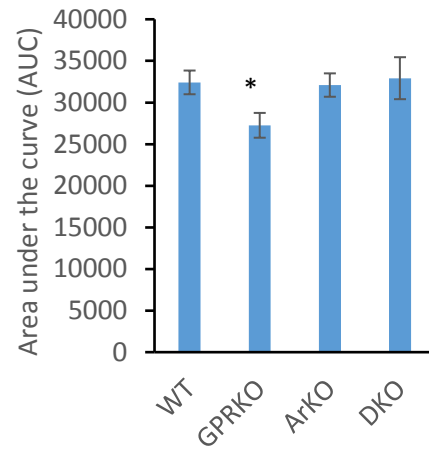
B. Male FBG



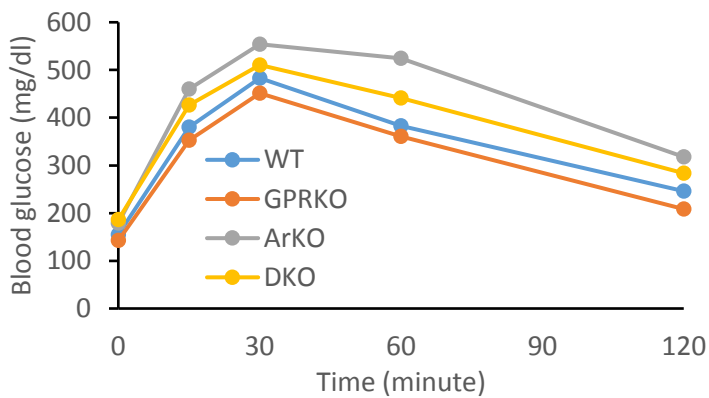
C. Female glucose tolerance test (GTT)



D. Female GTT area under the curve (AUC)



E. Male GTT



F. Male GTT AUC

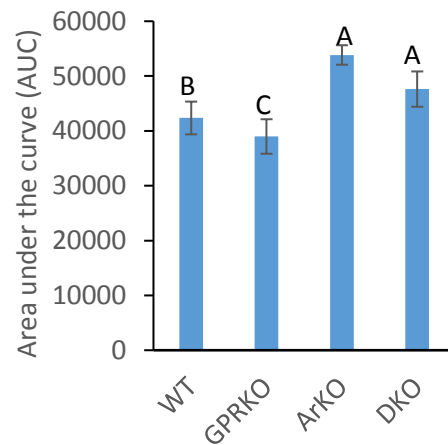
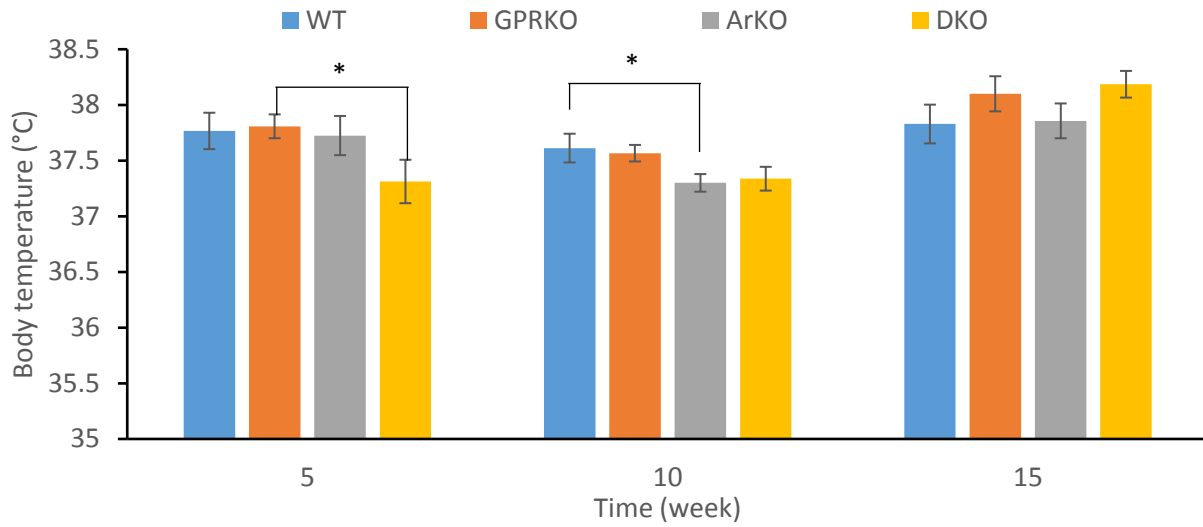


Figure 5. Deletion of GPR30 improves glucose homeostasis in both female and male mice fed a HFD.

Fasting blood glucose (FBG) levels of female (**A**) and male (**B**) were measured at week 12. Glucose tolerance tests (GTT) of female (**C**) and male (**E**) were performed as described in the Materials and Methods at week 11. The area under the curve (AUC) of female (**D**) and male (**F**) were calculated using the trapezoidal rule. Data are mean \pm SEM (n=6-12 mice/group). *, p<0.05. Lettering ABCD indicates significance at p<0.05 with comparisons for each pair using Student's t test.

A. Female body temperature



B. Male body temperature

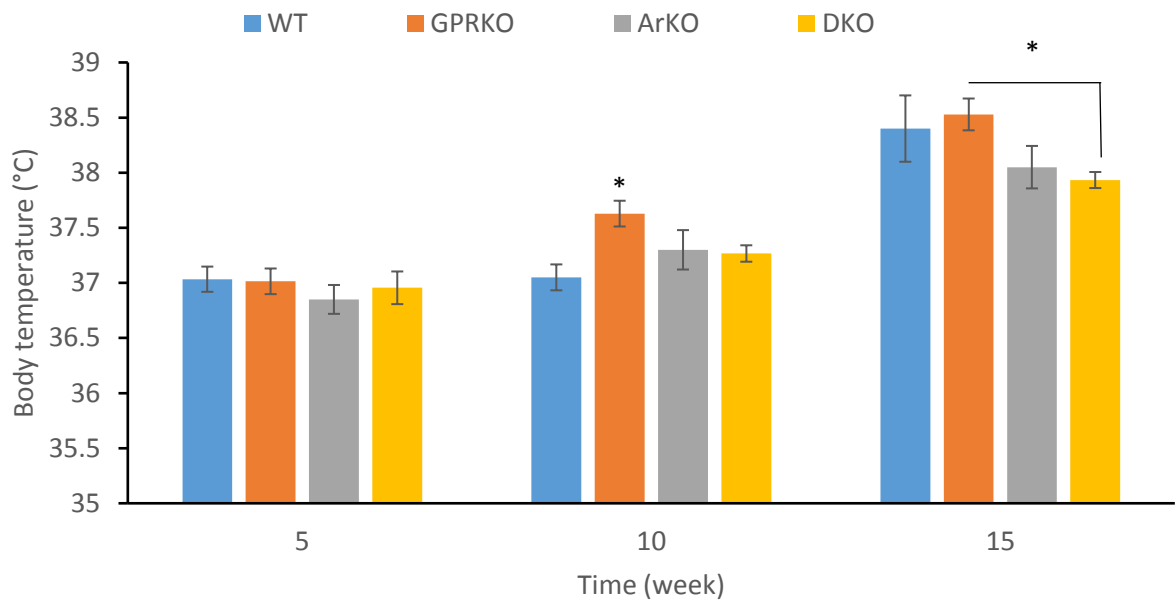


Figure 6. Deletion of GPR30 might regulate thermogenesis via promoting heat production in mice fed a HFD.

6-month old female and male mice were fed a HFD for 16 weeks. Core body temperature of female (A) and male (B) were measured using a rectal probe at weeks 5, 10, and 15. Data are mean \pm SEM (n=6-12 mice/group). *, p<0.05.

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Chapter Five

Deletion of GPR30 promotes beiging of white adipose tissue and thermogenesis in mice

Abstract

We previously discovered that deletion of GPR30 (GPRKO) protected female mice from developing obesity, glucose intolerance, and insulin resistance in female mice challenged with a high-fat diet (HFD), which were associated with higher body temperature and energy expenditure as compared with those of wild-type (WT) mice. In this study, I further explored whether GPR30 is involved in regulating browning of adipose tissue and thermogenesis in mice. Briefly, female mice were fed a HFD for 23 weeks and the expression of UCP-1, the key regulator of thermogenic browning, were higher in the adipose tissue of GPRKO female mice as compared with that of WT mice, both in brown adipose tissue (BAT) and white adipose tissue (WAT). Consistently, deletion of GPR30 enhanced the mitochondrial respiration in BAT, suggesting that GPR30 deficiency at least partially suppressed the fat accumulation by promoting thermogenesis and dissipating energy. *Ex vivo*, the expression of thermogenic genes and UCP-1 protein level are upregulated in beige adipocytes differentiated from GPR30-deficient stromal vascular fraction (SVF) cells. These findings provide novel aspects to understand the role of GPR30 in energy metabolism and may be instrumental in the development of new therapeutic strategies for obesity.

Keywords: GPR30, adipose tissue, beigeing/browning, thermogenesis

Introduction

The escalation in the prevalence of obesity worldwide has led to an increasing number of related health conditions, including type 2 diabetes, cardiovascular disease, and cancer (1-4). Obesity can be caused by excessive energy intake, reduced energy expenditure, or a combination of both (5,6). Therefore, impacting energy homeostasis in humans without altering energy intake or physical activity would pose a challenging yet promising intervene option for preventing/treating obesity.

The excess energy due to the excess calorie intake and/or reduced physical activity primarily stored as fat in white adipose tissue (WAT) (6,7). In contrast to WAT, brown adipose tissue (BAT) dissipates excess energy to generate heat through the action of mitochondrial membranous protein uncoupling protein-1 (UCP-1), thereby protecting against hypothermia and obesity (8). Although it has only been a decade that functional BAT has been recognized in adult humans (9-13), it is well acknowledged that BAT plays an important role in regulating energy homeostasis (12,14-16). Importantly, there is a third type of adipocytes in WAT discovered over the past decade (17), termed as beige or brite (brown-in-white) adipocyte, which possess a similar energetic characteristic as brown adipocyte in response to some environmental or hormonal stimuli, such as cold or high fat-diet (HFD) exposure (18-20).

BAT obtains the ability to uncouple electron gradients via the activation of UCP-1 and promote energy expenditure (21,22). Beige adipocytes have low basal levels of UCP-1, which however, are highly inducible in response to β adrenergic receptor stimuli, including cold and overfeeding (23-26). Since these novel fat cells contain a relatively high density of mitochondria and UCP-1 (27), it brings a hope that promoting the amount and/or the activity of the inducible beige cells in WAT could be a novel therapeutic option to control obesity. We previously discovered that deletion of GPR30 protected female mice from high-fat diet (HFD)-induced

obesity (28). While the absence of GPR30 didn't alter the amount of food intake in mice, GPRKO female mice fed HFD had higher body temperature and energy expenditure as compared with HFD-fed WT female mice, suggesting that the difference in energy expenditure likely contributes to the difference in the accumulation of fat mass over time between GPRKO mice and WT mice fed the HFD. Thus, we speculate that deletion of GPR30 may directly enhances thermogenesis and the browning program of WAT, thereby exerting the protective effects against diet-induced obesity.

In the present study, we demonstrated that deletion of GPR30 promotes thermogenesis by up-regulating UCP-1 to protect female mice from HFD-induced obesity. We further showed that GPR30 deficiency enhances beige adipocyte differentiation and GPRKO mice have higher mitochondrial respiration as compared to WT littermates. These data indicate a novel role of GPR30 as an “obesity gene” that might act to prevent beige fat development and thermogenesis in female mice exposed to excess amount of calories.

Results

UCP-1 in adipose tissues is upregulated in HFD-fed GPRKO female mice.

As previously observed (28), GPRKO female mice had higher body temperature than that of WT littermates when challenged a HFD. In mice, body temperature is a fundamental parameter in the evaluation of animal wellbeing (29-31) and in the assessment of thermogenesis (16,32). Given that BAT is the major site for adaptive nonshivering thermogenesis, a physiological process during which heat production increases in response to overfeeding through the action of UCP-1 (19,24,33,34), interscapular BAT of WT and GPRKO female mice was isolated to assess the effect of GPR30 on thermogenesis. As shown in Fig. 1 A&B, the relative UCP-1 protein levels in BAT were significantly higher in GPRKO animals as compared to WT female mice, suggesting that

deletion of GPR30 promotes thermogenesis in BAT by up-regulating UCP-1. While BAT plays an essential role in thermogenesis and balancing energy expenditure, the program of browning or stimulated UCP-1 activity within subcutaneous WAT is shown to improve metabolic phenotypes (35-37). Similarly, protein lysates from subcutaneous WAT of WT and GPRKO female mice were probed against UCP-1 and it was only detectable in one of the WT samples while half of the GPRKO samples displayed relative high levels of UCP-1 (Fig. 1 C&D). Taken together, our results indicate for the first time, as to our knowledge, that GPR30 deficiency exerts the thermogenic effect by up-regulating UCP-1 in HFD-induced obese female mice.

Deletion of GPR30 promotes the mitochondrial respiration and function in BAT.

Several experiments have utilized the XF24 Seahorse bioanalyzer to assess the mitochondrial respiration and function in adipose tissues (38-40). To further test whether GPR30 deficiency has a metabolic promoting effect in fat tissues, we measured the mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in white and brown adipose tissues from same area of each animal using the Agilent Seahorse (Santa Clara, CA) XF24 islet microplate. As shown in Fig. 2A, GPRKO animals had significantly higher basal OCR as compared to WT littermates. Interestingly, the maximal OCR stimulated by FCCP were similar between WT and GPRKO (data not shown) but the average OCR of the total 20 measurements showed that deletion of GPR30 promoted the overall mitochondrial OCR in BAT regardless of the addition of the mitochondrial complexes inhibitors (Fig. 2B). To understand the difference of mitochondrial metabolic phenotype of WT and GPRKO, the bioenergetics mapping was plotted using the overall OCR and ECAR (41,42). As seen in Fig. 2C, all measurements of WT animals fall into the quiescent quadrant whereas the majority of the GPRKO measurements fall under the energetic

quadrant with higher OCR and ECAR, suggesting that deletion of GPR30 promotes the mitochondrial energetic phenotype.

Deletion of GPR30 dynamically affects cAMP production in SVF cells

As reported before, the well-known second messenger cAMP activates protein kinase A (PKA), which breaks triglycerides to glycerol and free fatty acids, latter of which is the acute substrates for mitochondria thermogenesis (18,43). To examine whether GPR30 deficiency modulates the cAMP/PKA/CREB pathway that results in increased mitochondrial respiration, SVF cells from WAT were preincubated with KRB for 20 min and then treated with 0.2 mM IBMX in the absence or presence of 5 μ M forskolin for 15 min. As shown in Fig. 3A, when mice were kept on STD, basal cAMP production in both WT and GPRKO cells were same but upon the activation by forskolin, the cAMP level was higher in WT stromal cells as compared to GPRKO cells. Interestingly, this phenomenon reversed when the stromal cells were isolated from HFD-fed mice. The production of intracellular cAMP increased by 2.34 fold in GPRKO cells than that of WT when treated with forskolin (Fig. 3B). Consistently, the cAMP-regulated transcription as determined by measuring cAMP-response elements (CRE)-driven luciferase activity was significantly higher in GPRKO stromal cells as compared to WT cells (Fig. 3C&D).

Deletion of GPR30 promotes beige adipocyte differentiation and activates the thermogenic browning of subcutaneous WAT.

Since the discovery of inducible beige adipocytes in WAT, it has been shown that activation of PPAR γ or β_3 -adrenergic receptor (β_3 AR) induces the BAT-like gene program in WAT (44-47). To

characterize the potential role of GPR30 in browning of WAT, we isolated SVF from subcutaneous WAT and treated with the induction cocktail containing rosiglitazone, indomethacin, dexamethasone, and IBMX as instructed (47). As seen in Fig. 4 A&B, Oil Red O staining on day 8 showed that cells from GPRKO animals were differentiated to beige adipocytes with a higher efficiency as compared to the WT control, suggesting that GPR30 deficiency promotes the beige adipocytes differentiation.

To further address the potential role of GPR30 in the thermogenic browning of WAT, we examined the expression of the brown and beige fat selective genes (*Ucp-1*, *Prdm16*, and *Cidea*) (48-51) in the differentiated beige adipocytes from SVF. The expression levels of *Prdm16*, *Ucp-1*, and *Cidea* were 2-4 fold higher ($P < 0.05$) in GPRKO beige adipocytes than that of WT (Fig. 4C). We also examined the protein levels of UCP-1 in the SVF differentiated beige adipocytes on day 8. As shown in Fig. 4 D&E, the expression of UCP-1 in GPRKO beige adipocytes was 53% higher than that of WT group ($P < 0.05$). Collectively, these results confirm that deletion of GPR30 efficiently differentiated beige adipocytes and promoted the thermogenic browning of WAT.

Discussion

Our previous work, using both *in vivo* and *in vitro* approaches, demonstrated that deletion of GPR30 protected female mice from HFD-induced obesity, which was associated with higher body temperature and energy expenditure (52). We then speculated that GPR30 acts as an “obesity gene” and suppresses the thermogenic browning program of WAT thereby leading to the development of obesity. The present study provides evidence that GPR30 is a negative regulator of thermogenesis, which at least partially contributes to the reduced adiposity in the GPRKO female mice. First, UCP-1, the key regulator of brown and beige fat thermogenesis (8), is upregulated in

the absence of GPR30. Second, the mitochondria in BAT are highly energetic in GPRKO animals while the WT mitochondria remain at a relative quiescent stage. Last, deletion of GPR30 promotes the differentiation of beige adipocytes and induces the BAT-specific genes transcription in WAT. Together, our findings provide insight into the mechanism by which GPR30 regulates fat metabolism and adiposity in female mice exposed to excess calorie.

One confusing issue is how the newly discovered beige adipocytes differ functionally from the classic white and brown adipocytes. It has been presumed that white and brown adipocytes are developmentally related (53), as they express common enzymes and, importantly, both require PPAR γ for their differentiation (54,55). While beige cells may have similar morphology and function as those of brown fat cells, it has been demonstrated that beige cells have a unique gene expression pattern different from that of either white or brown fat cells. Recently, the widely used adipocytes cell line 3T3-L1 cells have been reported to express the BAT-selective gene *Ucp-1* (56), suggesting that there is no common boundary between white and brown/beige adipocytes as previously thought. The 3T3-L1 cells can be induced to beige-like adipocytes with the treatment of rosiglitazone, T3, and IBMX, which are the known inducers for beige differentiation (47). Like the white adipocytes, brown adipocytes store triglycerides in their multilocular droplets as well as beige adipocytes. Therefore, in addition to the differences in morphological structures and functions between WAT and BAT, there are distinct transcriptional factors involved in BAT development and thermogenesis (18,57,58), including *Sirt1*, *Pgc-1 α* , *Prdm16*, etc. (49,59,60). *Pgc-1 α* has been shown to induce numerous genes transcription that comprise the thermogenic browning program, including *Ucp-1* and *Dio2* (61). However, we discovered that the mRNA level of *Pgc-1 α* was similar between WT and GPRKO (data not shown). It is notable that isolated brown

adipocytes still express BAT-selective genes in the absence of *Pgc-1 α* (62). Thus, the upregulation of UCP-1 in GPRKO adipose tissue may be *Pgc-1 α* -independent.

We demonstrated that deletion of GPR30 highly boost the agonism of adenylyl cyclase releasing cAMP in energy-enriched stromal cells. As reported before, mitochondrial cAMP signaling is an indispensable mechanism regulating mitochondrial energetics and homeostasis (63) and the major downstream target, protein kinase A (PKA), then activates the transcription factor cAMP-responsive element-binding protein (CREB), initiating the transcriptional cascades involved in mitochondrial metabolism and biogenesis (64-66). Surprisingly, however, the ablation of GPR30 fails to alter the phosphorylation of CREB in stromal cells from WAT (data not shown). Besides CREB, cAMP/PKA signaling is involved in many other regulatory pathways (67), including protein kinase C (PKC) (68), phosphoinositide 3-kinase (PI3K) (69), phospholipase C (PLC) (70), Epac/inositol trisphosphate (IP3) (71). Hence, the increased production of cAMP in GPRKO stromal cells could stimulates multiple signaling pathways, which are not reflected by the phosphorylation of CREB. Nevertheless, the result of CRE-luciferase activity clearly indicates that GPR30 deficiency indeed enhanced transcriptional cascades, which require further investigation.

The primary function of mitochondria is to produce ATP as energy by using the nutritional substrates (72). However, not all of the external energy supply is fully coupled to the ATP synthesis. The extra energy is, thereby, dissipated as heat via the process of proton leak, which is regulated by the uncoupling proteins located on the innermembrane of mitochondria (73,74). FCCP, a widely used mitochondria uncoupler (75), stimulated the oxygen consumption in WT adipose tissue, but it failed to manipulate the mitochondrial respiration of GPRKO BAT (data not shown). Given that GPR30 is a membrane-associated receptor and has been found in mitochondria in C2C12 cells (76) and breast cancer cells (77), it is possible that GPR30 blocks the activity of UCPs in mitochondria

of adipocytes. Therefore, deletion of GPR30 promotes thermogenesis, resulting in enhanced energy metabolism and reduced fat accumulation (Fig. 5). To address the role of GPR30 in mitochondria, the use of isolated mitochondrial measurements can be of clear advantage, together with the whole living tissue measurements, which represent the physiologic condition.

In summary, we recently revealed for the first time that the beneficial effects of GPR30 deficiency on energy metabolism and fat mass control in female mice exposed to a HFD. We further demonstrate that GPR30 ablation promotes beige fat development and the key thermogenic gene expression in WAT, thereby providing the insight into the underlying mechanism by which GPR30 regulates fat metabolism and adiposity in female mice. Further work will be required to delineate the detailed mechanism of GPR30-regulated WAT browning, weight loss, as well as the possible interaction between GPR30 and ER α -mediated metabolic actions, which could potentially lead to a novel therapeutic strategy to more efficiently prevent the development of obesity, and obesity related metabolic diseases in females.

Materials and Methods

Materials

Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, triiodothyronine (T3), indomethacin, rosiglitazone, forskolin, DMEM-high glucose, bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) were from HyClone (Logan, UT, USA). Antibiotic Antimycotic Solution (Ab/Am) was purchased from Corning (Manassas, VA, USA), and Collagenase was obtained from Worthington (Lakewood, NJ, USA).

Animals

GPRKO female mice were generated as previously described (28). All mice were housed under constant temperature (23-24°C) with a 12-h light/dark cycle and *ad libitum* access to food and water. As for the first study, mice were fed a HFD with 58% of calories from lard (Research Diets Inc., New Brunswick, NJ, USA) for 23 weeks. Protein samples of adipose tissues from previous study (28) were analyzed after 23 weeks of HFD feeding. For the second study, mice were housed separately with 58% HFD *ad libitum* for one month. Ethics approval was obtained from the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech and all experiments were strictly carried out in accordance with approved protocols and regulations by IACUC.

Adipose stromal vascular fraction (SVF) cells isolation

SVF from WAT of WT and GPRKO female mice were isolated as described (78). Briefly, white fat pads were washed in Krebs-Ringer bicarbonate buffer (KRB) supplemented with 1% BSA then quickly transferred to the collagenase solution for approximately 50 min at 37°C. After the collagenase digestion, stromal cells were separated by centrifugation twice at 300 rcf for 5 min and washed twice with prewarmed PBS supplemented with 1% BSA afterwards. SVF cells were then seeded for subsequent experiments.

Intracellular cyclic AMP (cAMP) production measurements

Stromal cells isolated from HFD- or STD-fed mice were seeded in 6-well plates at 1×10^6 cells/well. On the next day after seeding, culture medium was changed to KRB for 20 min and the SVF were then treated with vehicle or forskolin (5 μ M) for 15 min. The intracellular cAMP

production were measured by an EIA kit as previously described (79). Data were normalized to the cellular protein concentration in the same samples.

CRE-luc activity measurements

The reporter plasmids were co-transfected using 1 µg of DNA or 2 µl of Lipofectamine 2000 (Invitrogen)/µg of DNA into the isolated stromal cells at 80% confluence in combination with pCRE (Clontech). The pGL4.73 (1 ng, Promega) that carries Renilla luciferase was also co-transfected as an internal control to monitor the transfection efficiency. After overnight transfection as instructed, cells were starved with serum-free medium for 4 hours before forskolin treatment. After 16 hours treatment, cells were washed with PBS once and harvested using passive lysis buffer. The luciferase activities were analyzed using the Dual-Luciferase assay kit (Promega) as recommended by the manufacturer.

Beige adipocyte differentiation

Beige adipocyte differentiation was induced by treating confluent but not packed SVF cells with DMEM medium containing 10% FBS, 1% Ab/Am, 125 µM indomethacin, 5 µM dexamethasone, 0.5 mM IBMX, 0.5 µM rosiglitazone. Two days after induction, cells were maintained in medium containing 10% FBS, 1% antibiotics, 5 µg/ml insulin, and 1 nM T3 for additional two days, and then cultured in complete medium for 3-4 days until the multilocular droplets formed and filled with oil (47). The triglycerides stored in the droplets of beige adipocytes was quantified by Oil Red O staining assay as previously described (28).

Western Blot

Adipose tissue lysates with equal amounts of protein from WT and GPRKO mice were subjected to immunoblot analysis as previously described (80,81). The immune-reactive proteins were detected by chemiluminescence and quantified using ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). The relative protein levels were normalized to those of housekeeping protein and compared to the WT group.

Real-time quantitative PCR

Total RNA were extracted from adipose tissues and beige adipocytes using TRI reagent (Molecular Research Center, OH) and reverse-transcribed using GoScript™ Reverse transcriptase and random primers (Promega, WI). Amplification reactions were performed on an Applied Biosystems® 7500 Fast Real-Time PCR System as we previously described (82). Data were analyzed by the $RQ=2^{-\Delta\Delta C_t}$ method. The primers used are: Ucp-1 (5'-AGCCGGCTTAATGACTGGAG-3' and 5'-TCTGTAGGCTGCCCAATGAAC-3'), Prdm16 (5'-CAGCACGGTGAAGCCATTC-3' and 5'-GCGTGCATCCGCTTGTG-3'), mCidea (5'-TGCTCTTCTGTATCGCCCAGT-3' and 5'-GCCGTGTTAAGGAATCTGCTG-3'), Pparg (5'-ATTGAGTGCCGAGTCTGTGG-3' and 5'-GCAAGGCACTTCTGAAACCG-3'), and 18S RNA (5'-ACCTGGTTGATCCTGCCAGTAG-3' and 5'-TTAATGAGCCATTCGCAGTTTC-3').

Mitochondrial respiration measurements

Mitochondrial respiration of the whole adipose tissue were assessed as instructed (38,83) with adjustments. Briefly, approximately 10 mg of white and brown adipose tissues were isolated from

same area of each animal and the fat pads were cut into small pieces and placed into the Agilent Seahorse (Santa Clara, CA) XF24 tissue culture plate. After wash with the assay buffer (Seahorse XF base medium) three times, mitochondrial respiration via oxygen consumption rate (OCR) was measured in the XF24 plate reader following the established protocol (38) to determine the mitochondrial response: 8 $\mu\text{g/mL}$ oligomycin, 8 μM FCCP (mitochondrial oxidative phosphorylation uncoupler), 12 μM antimycin A (AA), and 3 μM rotenone (Rote). Basal OCR was determined by five consecutive measurements after tissue equilibration and the overall OCR was calculated by averaging the total 20 measurements. Data were normalized to the wet weight of fat pads, respectively.

Statistical analyses

Data were analyzed using the software JMP (SAS Institute, Cary, NC, USA). Group differences were compared using a Student's t test to compare means that were significantly different ($p < 0.05$).

Data are presented as mean \pm SEM.

Figures

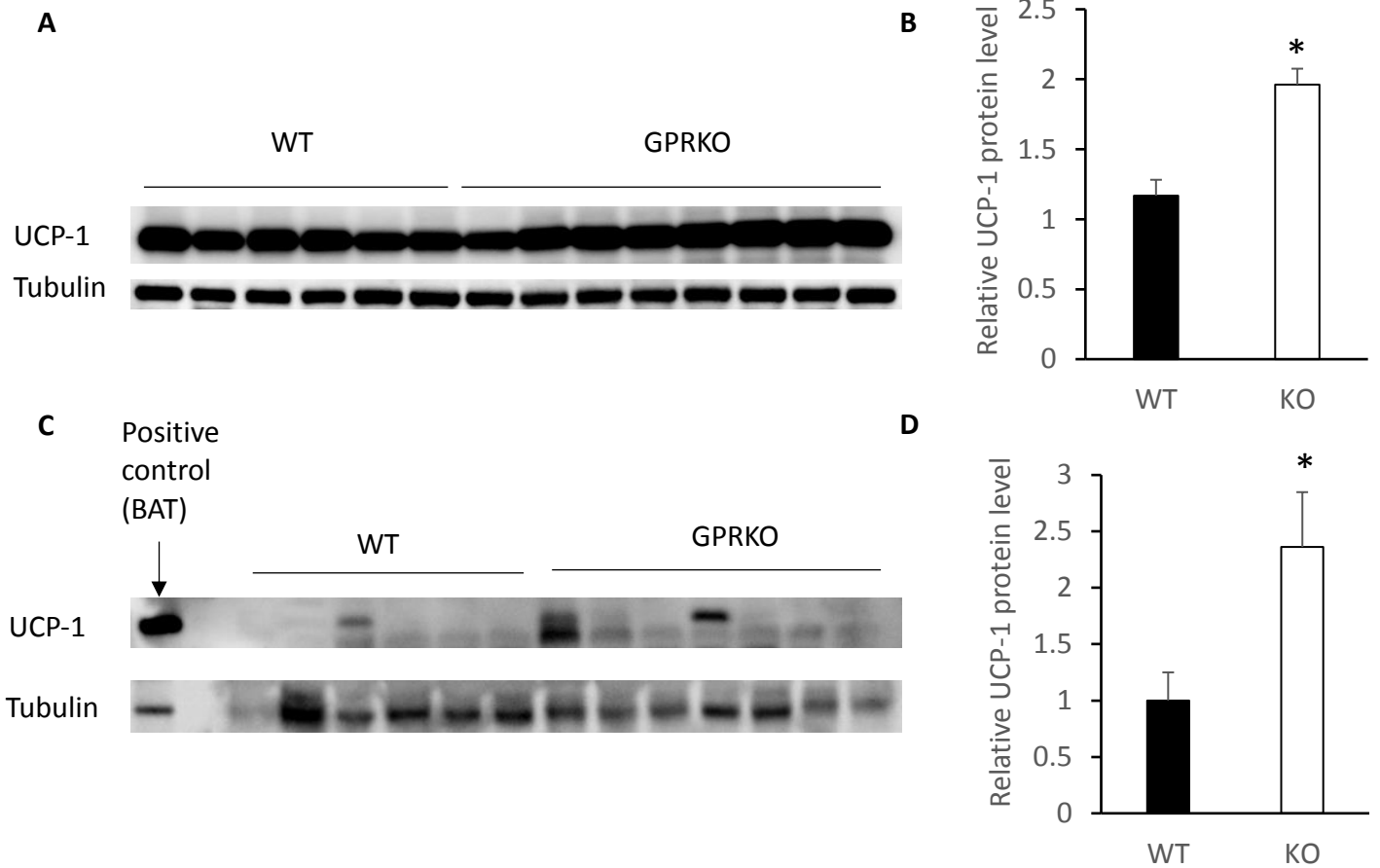


Figure 1. UCP-1 in adipose tissues is up-regulated in HFD-fed GPRKO female mice.

Western blot analysis of UCP-1 and tubulin loading control from BAT (A) and subcutaneous WAT (C).

Densitometric analysis of UCP-1 protein levels normalized to tubulin from BAT (B) and subcutaneous

WAT (D). All data are presented as means \pm SEM (n=6-8). *, p<0.05.

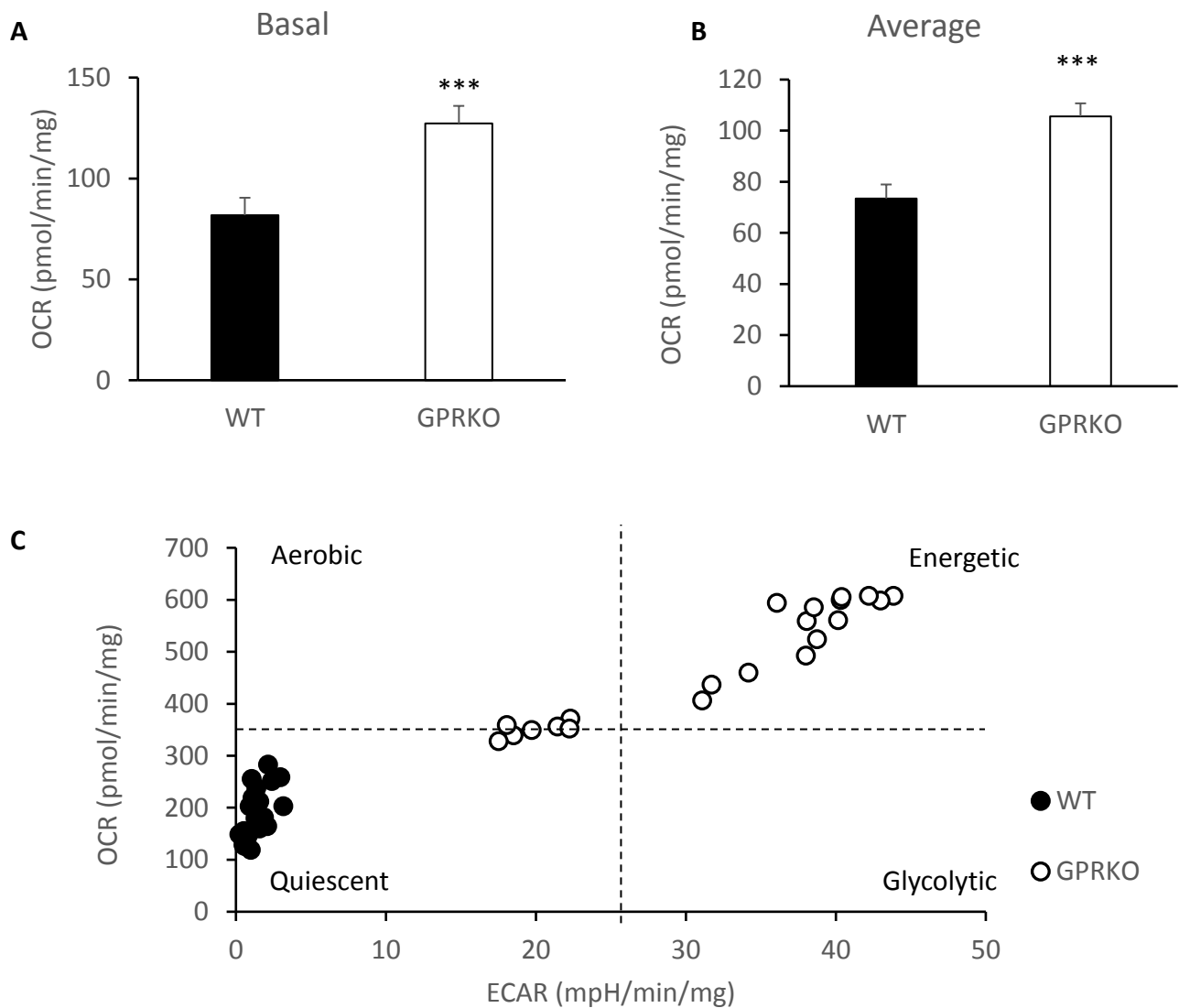


Figure 2. Deletion of GPR30 promotes the mitochondrial respiration and function in BAT.

BAT from three WT and GPRKO female mice, respectively, were analyzed using the XF24 Islet Capture Microplate as described in the Materials and Methods. **(A)** Basal OCR were calculated after the equilibration of tissues and before oligomycin injection. **(B)** Average OCR were determined using the total measurements regardless of the sequential chemicals injection. **(C)** The overall OCR and ECAR were plotted to represent the mitochondrial metabolic states. Data are normalized to wet weight of fat pads, respectively, and represented as means \pm SEM (n=3). ***, p<0.001.

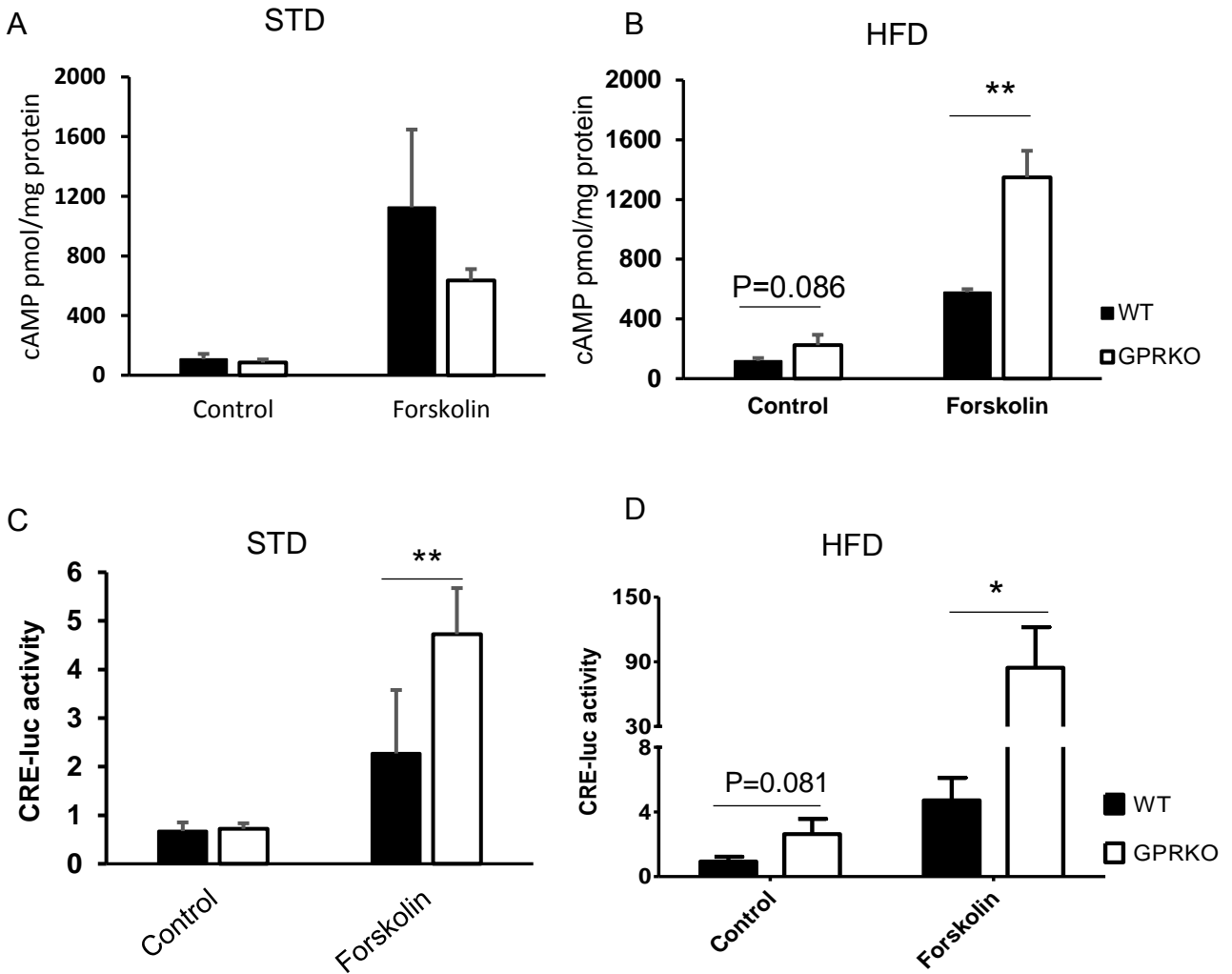


Figure 3. Deletion of GPR30 affects cAMP signaling pathway in WAT-derived primary stromal cells.

Primary SVF from subcutaneous WAT of WT and GPRKO were isolated from both STD- and HFD-fed mice. The intracellular cAMP production was measured as described in Materials and Methods. Forskolin stimulated cAMP level was 2.34 fold higher in HFD-fed GPRKO cells than that of WT (B), whereas this effect was blunted in SVF isolated from STD-fed lean mice (A). CRE-luciferase activity was alleviated in GPRKO cells in both mouse models when treated with adenylyl cyclase agonist forskolin (C&D). Data are represented as means \pm SEM (n=3-4). *, $p < 0.05$; **, $p < 0.01$.

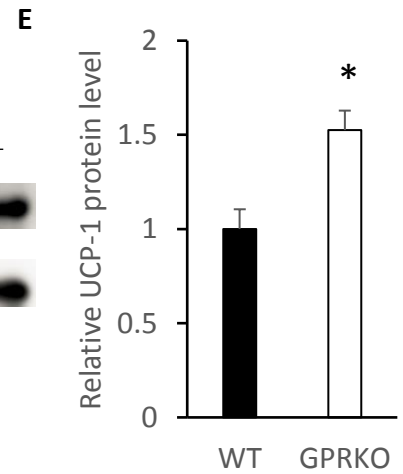
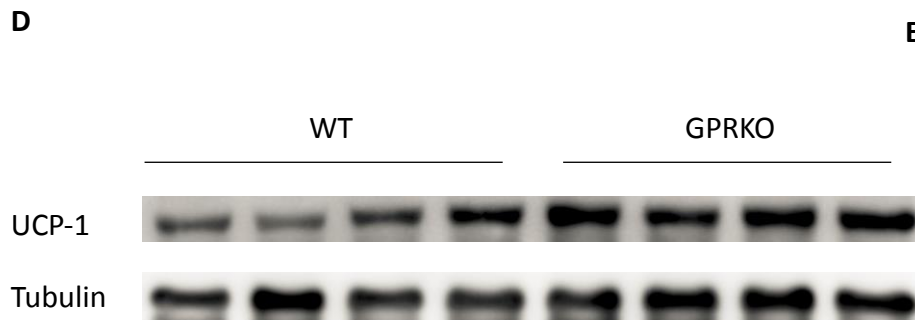
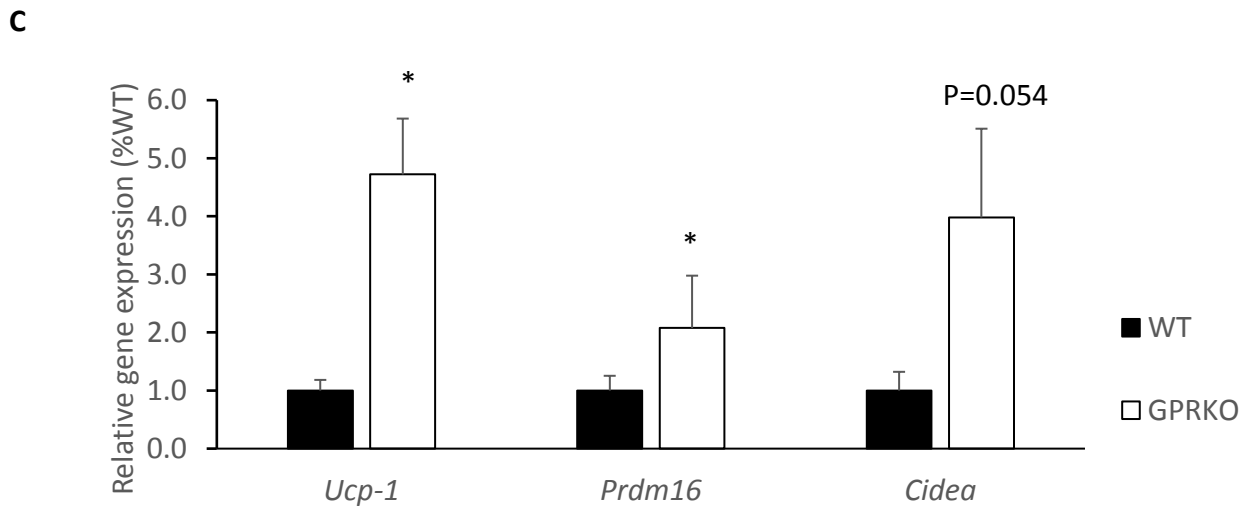
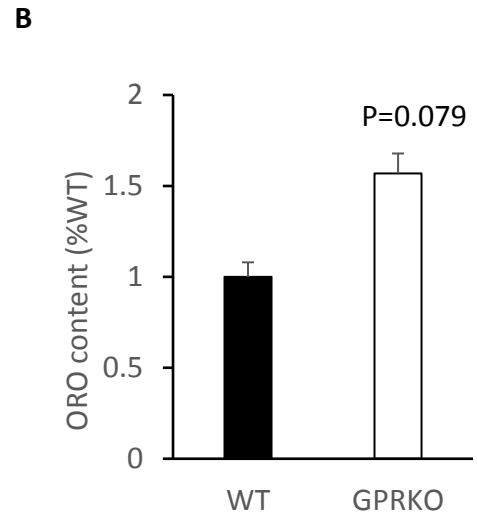
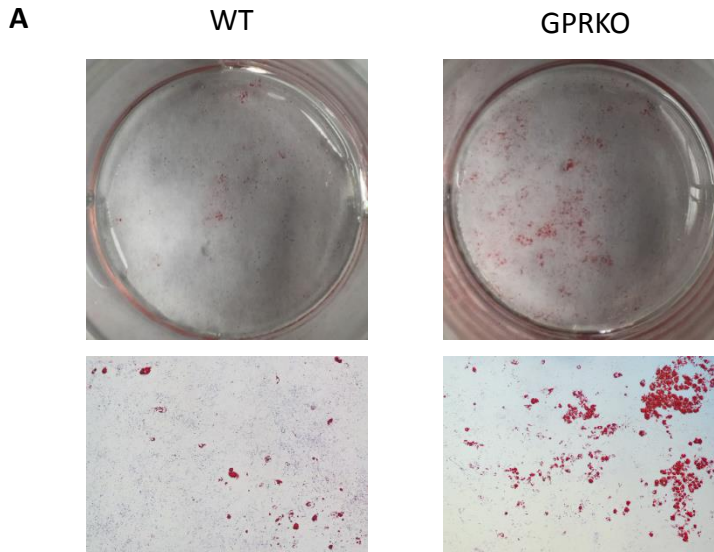


Figure 4. Deletion of GPR30 promotes beige adipocytes differentiation and activates the thermogenic browning program in subcutaneous WAT.

Primary SVF from subcutaneous WAT of WT and GPRKO differentiated into beige adipocytes as described in Materials and Methods. The triglycerides stored in the multilocular lipid droplets were visualized using Oil Red O staining as indicated respectively **(A)**. The Oil Red O stain content were extracted and normalized to WT **(B)**. Data are represented as means \pm SEM (n=3). **(C)** mRNA levels for brown fat-selective genes *Ucp-1*, *Cidea*, and *Prdm16* were analyzed by RT-qPCR. Data are normalized to WT and represented as means \pm SEM (n=7). *, p<0.05. **(D)** Western blotting against UCP-1 in mature beige adipocytes. Tubulin served as loading control. Data are normalized to WT **(E)** and represented as means \pm SEM (n=4). *, p<0.05.

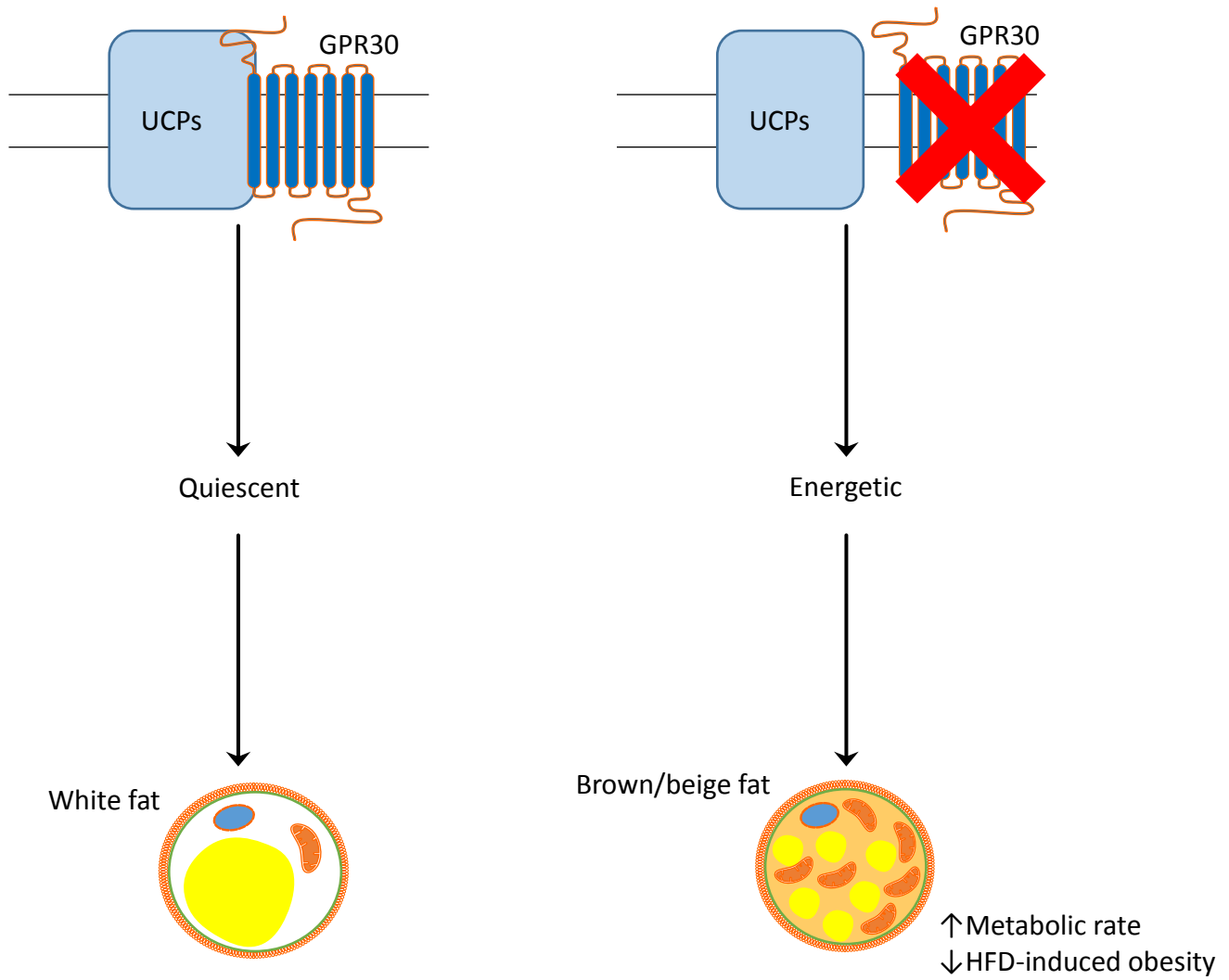


Figure 5. Schematic model of the proposed role of GPR30 in regulating thermogenic browning of WAT.

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Chapter Six

Conclusions

Significance

Although GPR30 has been officially acknowledged as a novel ER (1), whether it is an endogenous GPER that directly binds to estrogen and mediates estrogen effects remains controversial. My research project is aimed at investigating the metabolic effects of GPR30. We first demonstrated that deletion of GPR30 protects female mice from excess energy-induced adiposity and ameliorates glucose intolerance. Consistently, activation of GPR30 by selective agonist G-1 promotes adipocyte differentiation in 3T3-L1 cells. Interestingly, ablation of GPR30 is still capable to improve the metabolic flexibility, including glucose homeostasis and thermogenesis, in estrogen-insufficient mice. Thus, we speculate that GPR30 may not solely acts as an ER and further studies clarifying GPR30 signaling would largely facilitate our understanding of the sex differences in obesity development. In addition, we discovered that GPR30 deficiency promotes thermogenic browning program in white adipose tissue, which results in a reduction of fat accumulation in mice fed a HFD. Together, the results from this research project provide invaluable findings that GPR30 plays an important metabolic role in mice. Further investigations are needed to elicit the detailed signaling of GPR30, as well as the metabolic role of GPR30 in humans.

Future research directions

Although we discovered the promising anti-obese effect by deletion of GPR30, several puzzling issues remain. Our speculation that GPR30 deficiency suppress fat accumulation by promotes beigeing of WAT could be an indirect effect of trans-differentiation. The adipose tissue-derived

stem cells from SVF, like other stem cells, have the remarkable potential to develop into many different cell types (2). We have induced the differentiation of stromal cells into white and beige adipocytes and, as discussed in Chapter Five, there is no common boundary between these two types of adipocytes. Therefore, it is possible that deletion of GPR30 triggers the trans-differentiation from white to beige adipocytes, which indirectly suppressed the excess white fat accumulation. Alternatively, GPR30 may influence the fate commitment of the stem cells from beginning. Until recently, the mechanism of reprogramming of adipose-derived stromal cells remains elusive.

Another interesting finding is that after cold exposure, the GPR30 mRNA level increased in the stromal cells isolated from WT WAT, which could support our hypothesis that GPR30 impede the activity of mitochondrial uncoupling proteins. Therefore, deletion of GPR30 could fully liberate the mitochondrial UCP-1 and dissipate excess energy, resulting in reduced adiposity. Further investigation on deciphering the metabolic role of GPR30 is needed.

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