

Roles of Select Cytochrome P450 Epoxygenases in Brown Adipogenesis

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ABSTRACT

Brown adipose tissue (BAT) has recently emerged as a novel target for obesity treatment and prevention. Cytochrome P450 (CYP) epoxygenases, primarily CYP2J and CYP2C isoforms, produce epoxy fatty acids (EpFAs), which have been suggested to play important roles in the regulation of white adipogenesis and obesity. However, adipocyte-specific expression of CYP epoxygenases has not been extensively analyzed, and the roles of CYP epoxygenases in brown adipogenesis remain unexplored. In this study, we examined mRNA levels of *Cyp2j* and *Cyp2c* isoforms during murine and human brown and white adipocyte differentiation and in various adipose tissue depots of high-fat diet (HFD)-fed and control mice. Moreover, we began to elucidate the roles of select CYP epoxygenases in brown adipogenesis through modulation of CYP expression. Stable transfection and lentiviral-mediated knockdown of murine *Cyp2j13* and stable transfection of human *CYP2J2* were performed in brown preadipocytes and their differentiation potentials were compared with cells that were transfected with empty vector or non-targeting knockdown controls. The effects on brown adipogenesis were evaluated by lipid accumulation and expression of brown adipocyte and general differentiation marker genes. We report that mRNA levels of CYP epoxygenases are increased in mature adipocytes compared to preadipocytes but decreased in the most strongly differentiated murine brown adipocytes. Furthermore, *Cyp2j* mRNA levels displayed an upward trend in the BAT of HFD-fed mice, implicating that *Cyp2j* expression may be associated with reduced BAT mass and activity. We show that *Cyp2j13* overexpression significantly suppressed murine brown adipocyte differentiation, while *Cyp2j13* knockdown enhanced differentiation, suggesting that *Cyp2j13* may be a negative regulator of brown adipogenesis. In contrast, *CYP2J2* overexpression did not remarkably affect human brown adipocyte differentiation. Together, our results suggest that CYP

epoxygenases may play important roles in brown adipogenesis. *Cyp2j13*, in particular, may be a novel target for brown adipogenesis, and consequently, for obesity treatment and prevention. Further studies are warranted to examine the roles of *CYP2J2* by gene knockdown and pharmacological antagonism, to determine whether *CYP2J2* overexpression may alleviate inflammation-induced suppression of brown adipocyte differentiation and activity, and to analyze roles of other CYP epoxygenase isoforms in brown adipogenesis for obesity treatment and prevention.

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CHAPTER I

INTRODUCTION

The worldwide prevalence of obesity has nearly tripled from 1975 to 2016. In 2016, nearly 1.9 billion adults were overweight, and 650 million of these adults were obese [1]. As global rates of obesity continue to rise, it is of increasing importance to develop novel therapeutic strategies to combat this pandemic along with its accompanying comorbidities such as type 2 diabetes and cardiovascular disease. Obesity is fundamentally characterized by a chronic energy imbalance, in which energy storage exceeds energy expenditure [2]. White adipose tissue (WAT) is the principal site of energy storage in the body, accumulating excess energy in the form of lipid droplets [3, 4]. In contrast, brown adipose tissue (BAT) is a site of energy expenditure due to its thermogenic potential [5, 6]. Metabolically active BAT was previously thought to only exist in human infants, as a method of adaptation to cooler extra-uterine temperatures following birth [5, 6]. More recently, significant amounts of functional BAT were found to be present in adult humans [7, 8]. This discovery has introduced a realm of research investigating strategies to manipulate the thermogenic capacity of BAT as a means of enhancing energy expenditure, thus aiding in reversal of the chronic energy imbalance that underlies obesity.

Polyunsaturated fatty acids (PUFAs) are fatty acids that are metabolized into a variety of signaling molecules known as eicosanoids, which are key mediators of inflammation [9]. The cytochrome P450 (CYP) epoxygenase pathway of PUFA metabolism produces epoxy fatty acids (EpFAs) such as arachidonic acid (ARA)-derived epoxyeicosatrienoic acids (EETs), which exert anti-inflammatory, anti-oxidative stress, vasodilatory, and angiogenic actions by activating receptor-mediated signaling pathways and ion channels [10]. EETs and other EpFAs have been

demonstrated to produce favorable effects on obesity and its related metabolic diseases such as attenuation of high-fat diet (HFD)-induced increases in body weight, adiposity, serum lipids, insulin resistance, and vascular dysfunction, in numerous studies [10-13]. These metabolic effects are partially mediated through adipocyte-specific actions, as increasing EET concentrations in white preadipocytes has been shown to decrease their ability to differentiate and accumulate lipids [14]. CYP epoxygenase overexpression is one method of increasing EpFA production to elicit these positive outcomes [15]. However, the specific CYP isoforms expressed in adipocytes that contribute to EpFA production are not well characterized. Additionally, the role of CYP epoxygenases and subsequent EpFA production remains unexplored in brown adipocytes, and it is unclear if the observed metabolic benefits can be partially attributed to an increase in BAT development and activation. In the current study, we sought to determine which CYP epoxygenase isoforms are expressed in brown and white adipocytes throughout differentiation and in diet-induced obesity. Furthermore, we began to elucidate the role of CYP epoxygenases in brown adipogenesis through modulation of CYP expression in brown adipocyte differentiation. The results presented contribute to our understanding of adipocyte CYP epoxygenase expression and suggest a role of these enzymes in brown adipocyte differentiation. Together, this provides promising areas for future research to uncover methods to manipulate the CYP pathway to ameliorate the debilitating consequences of obesity in our world.

CHAPTER II

LITERATURE REVIEW

2.1. Adipose Tissue and Metabolic Homeostasis

2.1.1. Types of Adipose Tissue

Three major types of adipose tissue are recognized to exist in mammals, each of which plays different roles in metabolic homeostasis and obesity development. White adipose tissue (WAT) is distributed throughout the body, with its primary function being the storage of energy as triacylglycerols (TAG) and the release of this energy through lipolysis to free fatty acids (FFAs) and glycerol. WAT is considered an endocrine organ and regulates a variety of physiological processes through the synthesis and secretion of biologically active compounds, such as hormones and cytokines, which are deemed as adipokines [3, 4]. In circumstances of energy surplus, WAT possesses the ability to expand and proliferate through hypertrophy and hyperplasia in order to accommodate excess lipids. Excessive lipid accumulation is characteristic of obesity and prompts a state of chronic inflammation and oxidative stress in WAT, leading to a dysregulated gene expression profile and altered adipokine production, and ultimately driving the metabolic diseases associated with obesity [3, 4].

Brown adipose tissue (BAT) exists in discrete depots throughout the body and is histologically and functionally distinct from WAT. While white adipocytes are large in size with a unilocular lipid compartment, brown adipocytes are smaller with numerous lipid droplets [6, 16, 17]. Most notably, brown adipocytes are far more concentrated in mitochondria, which gives the tissue its darker color and renders it more metabolically active. BAT possesses a unique protein on the inner membrane of its mitochondria known as uncoupling protein-1 (UCP1), which facilitates its ability to undergo non-shivering thermogenesis, a process of heat production.

In the electron transport chain, UCP1 uncouples the proton motive force from adenosine triphosphate (ATP) synthesis by allowing H^+ to leak into the mitochondrial matrix, thus dissipating this energy as heat and elevating overall energy expenditure. BAT is highly vascularized to allow for delivery of substrates and oxygen to facilitate this heat production and in order to effectively transmit generated heat to other tissues in the body [5, 6, 16, 17].

A third form of adipose tissue has also been described, in which adipocytes located within WAT can be recruited in response to various stimuli to express a BAT-like phenotype, with multilocular lipid droplets, increased mitochondria, and UCP1 expression, allowing these cells to facilitate thermogenesis much like classical brown adipocytes. These inducible brown adipocytes are referred to as beige adipocytes, and this process is known as the “browning” of WAT [18-20]. Despite similarities in thermogenic function, brown and beige adipocytes should be distinguished as different cell types due to numerous key discrepancies. Beige fat possesses a distinct gene expression pattern compared to classical BAT. In contrast with classical BAT, which elicits high basal UCP1 expression, expression of UCP1 in beige adipocytes is only present upon stimulation [18-20]. Additionally, a number of quantitative trait loci are associated with the induction of beige but not brown adipocyte development, signifying these cell types are likely differentially regulated [21]. The extent to which these beige fat cells comprise the functional BAT observed in adult humans is unknown [20], but nonetheless, both beige and brown adipocytes display exciting potential for therapeutic outcomes in obesity due to their energy expending capacity.

2.1.2. Therapeutic Potential of Brown Adipose Tissue

The ever-increasing global burden of obesity and scarcity of effective treatments has instigated a search for novel therapeutic targets. Due to its energy expending capacity, BAT

holds great promise in this realm. BAT is inversely associated with body mass index, adiposity, and type 2 diabetes incidence [22], suggesting that increasing BAT mass and activity can ameliorate these conditions. It is estimated that approximately 50 grams of active BAT in humans could account for a 20% increase in daily energy expenditure [23], which corroborates the clinical relevance of enhancing BAT mass in obese individuals. BAT activity also decreases with age, with an estimated 50% activity in individuals in their 20s compared to approximately 10% activity in those in their 50s and 60s [24]. This observation coincides with the known decrease in basal metabolic rate with advancing age, indicating potential for a more remarkable metabolic impact with enhanced activation in an older population [25].

In addition to associational evidence in humans, BAT-mediated thermogenesis has demonstrated to directly impact body weight homeostasis in mouse models, with UCP1 ablation inducing obesity and its associated complications in mice when housed at thermoneutral conditions [26, 27]. Conversely, these mice did not become obese when housed at room temperature [28]. BAT elicits additional functions that may act alongside body weight reduction as a means to improve overall metabolic health. Namely, its ability to rapidly uptake and dispose of circulating lipids and glucose may attenuate insulin resistance and hyperlipidemia, both of which are commonly present in obese individuals [29, 30]. Together, this provides compelling evidence that research examining factors to promote BAT development and activation is warranted for its therapeutic potential in obesity.

2.1.3. Brown Adipose Tissue Development and Activation

Brown adipocytes, in addition to skeletal muscle cells and certain populations of white adipocytes, are derived from Myf-5-expressing myogenic precursors [6, 31]. Once mesenchymal stem cells (MSCs) are determined as preadipocytes, differentiation occurs to allow for

development of the morphological and functional characteristics of mature adipocytes. This adipocyte development process, including determination of stem cell fate and differentiation into mature adipocytes, is referred to as adipogenesis [32]. Although there are numerous transcription factors and other regulatory factors involved in the complex process of brown adipogenesis, peroxisome proliferator-activated receptor gamma (PPAR γ), PR domain containing protein 16 (PRDM16), and PPAR gamma coactivator-1 alpha (PGC-1 α) represent the key players involved. PPAR γ is a ligand-activated nuclear receptor which dimerizes with retinoid X receptor (RXR) and binds to peroxisome proliferator response elements (PPREs) to regulate expression of target genes [33]. PPAR γ is primarily expressed in adipose tissue and is considered the master regulator of adipogenesis, but also plays an integral role in the regulation of lipid metabolism, insulin signaling, and adipokine production, among other functions [33]. PPAR α , another PPAR isoform, is more highly expressed in BAT rather than WAT and is predominantly involved in lipid metabolism. PPAR α can also induce adipogenesis, although not to the same extent as PPAR γ [34].

As the master regulator of adipogenesis, PPAR γ is essential for the development of both brown and white adipocytes [35, 36]; however, this transcription factor alone is not sufficient to commit MSCs to a brown adipocyte lineage. Together with early B-cell factor 2 (EBF2), PPAR γ stimulates expression of PRDM16 [21]. PRDM16 operates as a molecular bidirectional switch to determine whether MSCs become myocytes or brown adipocytes [37]. Knockdown of PRDM16 in brown adipocytes results in a loss of thermogenic potential and differentiation of these cells into myocytes, whereas ectopic expression of this protein induces a thermogenic phenotype in myoblasts [37, 38]. PRDM16 binds to and modulates the activity of a number of transcription factors in brown preadipocytes that are imperative for differentiation and

stimulation of thermogenic activity, including PPAR γ and PGC-1 α [6, 39]. PGC-1 α is recognized as the chief controller of mitochondrial biogenesis and respiration and activates thermogenesis in BAT through stimulation of UCP1 expression [39, 40]. Although PGC-1 α is essential for activation of thermogenic function in BAT, it is not believed to be involved in the commitment to the BAT lineage from Myf-5-expressing precursors, as BAT develops normally in PGC-1 α -null mice [41].

In addition to knowledge regarding molecular mechanisms of BAT development, comprehension of means to enhance BAT thermogenic activity is critical in order to unlock its therapeutic potential. BAT is highly innervated with sympathetic nervous fibers, and activation of the sympathetic nervous system (SNS) is recognized as a principal mechanism of BAT thermogenesis induction [5, 6]. Upon SNS activation, norepinephrine is released and binds to β 3-adrenergic receptors on the cell surface of brown adipocytes, which triggers an intracellular cascade resulting in the formation of cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A (PKA). PKA phosphorylates and activates hormone sensitive lipase (HSL), which initiates lipolysis, cleaving TAGs to FFAs and glycerol. These FFAs are shuttled to BAT mitochondria, where they can induce UCP1 activity and thermogenesis [17]. Aside from adrenergic stimulation, numerous other factors have been described to activate thermogenesis through interaction with various cell membrane and nuclear receptors in BAT, including thyroid hormones, irisin secreted from skeletal muscle, and natriuretic peptides released from the heart [21, 42].

Cold exposure results in norepinephrine release and serves as the most well-established trigger for BAT thermogenesis, as a means to maintain body temperature [5]. Cold exposure not only promotes activation of classical BAT but is also known to stimulate formation of beige fat

to aid in heat production [18]. Despite its profound effects on BAT stimulation, increasing exposure to cold temperatures is not a practical method to improve BAT activity for obesity treatment. Numerous studies have elucidated roles of various dietary and pharmacological factors that may mimic the effect of cold exposure on BAT. Capsaicin and its analog capsinoids, compounds found in red peppers, are among the most heavily studied dietary factors demonstrated to augment BAT-mediated thermogenesis, through activation of the calcium channel transient receptor potential vanilloid 1 (TRPV1), which subsequently results in β 3-adrenergic stimulation [43, 44]. Consumption of high doses of capsinoids has demonstrated to enhance thermogenesis in both rodents and humans, and ingestion of 9 mg per day of capsinoids for six weeks resulted in greater cold-induced thermogenesis and decreased fat mass in human subjects [45]. A meta-analysis examining the impacts of capsinoids and capsaicin on humans has confirmed their ability to enhance energy expenditure and fat oxidation; however, the magnitude of these effects suggests only a small benefit for long-term weight loss, and many successful studies were conducted in lean individuals [46]. Fish oil, curcumin, green tea catechins, and resveratrol are among other dietary compounds with substantial evidence proposing a role in BAT activation, although numerous others have also been described [47]. Ultimately, there is significant potential for dietary compounds to act as therapeutic agents through increased BAT mass and activity, but further research is warranted in order to obtain clinically relevant outcomes for obesity.

2.2. Polyunsaturated Fatty Acids and their Metabolites

2.2.1. Overview of Polyunsaturated Fatty Acids: Structure, Function, Dietary Sources

Fatty acids are generally categorized based upon the length of their carbon chain and the number of double bonds. Polyunsaturated fatty acids (PUFAs) are fatty acids that contain at least

two double bonds on their carbon chain. PUFAs can be further classified into n-3 and n-6 PUFAs, indicating the position of the first double bond is three or six carbons from the methyl end of the carbon chain, respectively. The 18-carbon n-3 and n-6 PUFAs, alpha-linolenic acid (ALA) and linoleic acid (LA), are considered essential, indicating they cannot be synthesized in the body and must instead be obtained through dietary sources. Vegetable oils such as soybean oil, canola oil, and sunflower oil are especially concentrated in LA but also contain small proportions of ALA, whereas the opposite is true for flaxseed oil. These essential fatty acids can also be found in significant quantities in chia seeds, hemp seeds, and walnuts [48, 49].

Longer chain (≥ 20 carbons) n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the n-6 PUFA arachidonic acid (ARA), are more biologically active and can be synthesized from ALA and LA through multiple steps of elongation and desaturation [48, 49]. However, this conversion rate is considered to be low in mammals, and many experts recommend obtaining these longer chain PUFAs directly from the diet to ensure biological processes are not compromised as a result of deficiency [50-52]. ARA is particularly abundant in most animal products, such as meat, cheese, and eggs. Insufficient intake of n-6 PUFAs is not a concern for the general population, as typical Western diets are highly concentrated in both LA and ARA. EPA and DHA, on the other hand, can only be found in cold-water oily fish, and as a result, there is a greater threat for deficiency in cultures where habitual seafood consumption is not typical [48, 49].

PUFAs are involved in numerous functions throughout the body and have been extensively studied for their roles as inflammatory mediators. This ability to modulate levels of inflammation in the body is especially intriguing for therapeutic research, considering many of the health conditions that plague modern society are provoked and propagated through chronic

low-grade inflammation. The n-6 PUFAs produce metabolites primarily involved in pro-inflammatory actions, and conversely, n-3 PUFAs are widely recognized for their anti-inflammatory capabilities [53].

PUFAs occupy the sn-2 position of cell membrane phospholipids. Although cell membrane composition is regulated based on requirements for specific tissues, it is well established that increasing dietary consumption of certain PUFAs can subsequently elevate the concentration of that PUFA within cell membranes. This is particularly of interest with regard to n-3 PUFAs due to their anti-inflammatory roles and the concern for deficiency in typical Western diets. Insufficient intake of n-3 PUFAs can result in an imbalance that favors pro-inflammatory mediators [54]. Conversely, consumption of fish oil supplements has been shown to elevate EPA and DHA concentrations in cell membranes of cells and tissues involved in inflammation, such as immune cells and adipose tissue, in both a time- and dose-dependent manner [55-58]. This increase in EPA and DHA coincides with a decrease in ARA [57, 58]. Thus, it is of great interest to examine how these alterations in membrane PUFAs and their metabolites can elicit various downstream effects on health.

2.2.2. Eicosanoid Production

Upon cleavage by calcium-activated phospholipase A2, PUFAs are liberated from cell membranes and become subject to metabolism by different classes of enzymes in order to form bioactive compounds known as eicosanoids. Three major enzyme classes metabolize PUFAs, including cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP) monooxygenases. ARA is the most prevalent PUFA in most cell membranes and therefore is the dominant substrate for eicosanoid synthesis. Metabolism of ARA by COXs produces prostaglandins (PGs) and thromboxanes (TXs). Among the most biologically relevant is PGE₂, a

potent pro-inflammatory mediator that induces production of inflammatory cytokines by macrophages, such as tumor necrosis factor (TNF) α , interleukin (IL)-1 β , and IL-6. Other key players include TXA₂, an activator of platelet aggregation, and PGI₂, involved in vasodilation and inflammation resolution. LOXs give rise to various leukotrienes (LTs), which are also pertinent in facilitation of the inflammatory response [48, 59].

EPA is also capable of forming various PGs, TXs, and LTs upon metabolism by COXs and LOXs, although their biological potency is lesser than those derived from ARA and consequently, cytokine production and the overall inflammatory influence are attenuated [60]. Remarkably, both EPA and DHA can also produce a different class of eicosanoids when acted upon by COXs and LOXs that are anti-inflammatory and inflammation resolving, which are deemed as resolvins (from EPA and DHA), and protectins and maresins (from DHA) [48, 49, 60]. Altered eicosanoid production is a key mechanism through which these long-chain n-3 PUFAs confer benefits for chronic metabolic diseases. However, EPA and DHA exert additional contributory actions such as decreasing the expression of adhesion molecules involved in atherosclerosis development, limiting the production of pro-inflammatory transcription factors, cytokines, and angiogenic growth factors, and reducing lipogenesis via inhibition of sterol regulatory element binding protein 1c (SREBP1c) [48].

The third pathway of eicosanoid formation, the CYP monooxygenase pathway, is less characterized than the COX and LOX pathways and has only recently begun to receive appreciable attention in the literature. CYP hydroxylases can hydroxylate at or near the methyl end of PUFAs to produce hydroxy fatty acids. Terminal hydroxylation of ARA forms 20-hydroxyeicosatetraenoic acid (20-HETE), which has demonstrated pro-inflammatory capabilities. Elevated plasma and serum levels of this metabolite are associated with increased

incidence of metabolic disease [61]. CYP epoxygenases oxidize PUFAs across one of their double bonds, giving rise to various regioisomers of epoxy fatty acids (EpFAs). EpFAs produced from ARA are known as epoxyeicosatrienoic acids (EETs), whereas EPA and DHA are metabolized to epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs), respectively. 11,12-EET and 14,15-EET are the most abundantly generated regioisomers from ARA in most mammalian tissues. In the n-3 varieties, CYP epoxygenases preferentially oxidize the n-3 bond, resulting in 17,18-EEQ and 19,20-EDP being the predominant n-3 EpFAs formed [10]. EpFAs have sparked intrigue among researchers due to their observed anti-inflammatory actions and proposed benefits for numerous diseases [10-13]. The CYP epoxygenase pathway and its potential impacts for metabolic health will be discussed in greater detail in the following sections.

2.2.3. CYP Epoxygenase Pathway: CYP Isoforms and Degradation by sEH

CYP enzymes comprise a complex superfamily of genes, with 57 isoforms reported to be functional in humans [62]. These enzymes function to catalyze the metabolism of various xenobiotics and endogenous compounds. CYP enzymes are most highly expressed in the liver but are also expressed in various extrahepatic tissues such as the kidney, heart, skeletal muscle, adipose tissue, pancreas, and vasculature [63]. Isoforms belonging to the CYP2C and CYP2J subfamilies are considered the primary epoxygenases responsible for EpFA formation in mammals. Humans possess four *CYP2C* isoforms, which are *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19* [10, 62]. Murine distribution of isoforms is increasingly complex with 15 described functional isoforms: *Cyp2c29*, *Cyp2c37*, *Cyp2c38*, *Cyp2c39*, *Cyp2c40*, *Cyp2c44*, *Cyp2c50*, *Cyp2c54*, *Cyp2c55*, *Cyp2c65*, *Cyp2c66*, *Cyp2c67*, *Cyp2c68*, *Cyp2c69*, and *Cyp2c70* [10, 62, 64]. Humans only possess one *CYP2J* isoform, which is *CYP2J2*. In mice, once again, significant

allelic expansion has resulted in seven functional isoforms: *Cyp2j5*, *Cyp2j6*, *Cyp2j8*, *Cyp2j9*, *Cyp2j11*, *Cyp2j12*, and *Cyp2j13* [10, 62, 65]. The wide variety of epoxigenase isoforms and inconsistencies between mice and human varieties creates additional layers of complexity in the effort to elucidate the key players driving formation of EETs and other EpFAs.

Soluble epoxide hydrolase (sEH) is a cytosolic enzyme whose C-terminal domain rapidly hydrolyzes EpFAs into their corresponding diols following their formation [66]. These diol counterparts, known as dihydroxyeicosatrienoic acids (DHETs) in the case of EETs, are much less biologically active and do not exert the same beneficial metabolic effects as EpFAs [10, 67]. In fact, certain diol products of sEH have even illustrated pro-inflammatory potential [61, 68]. EpFAs can be metabolized through other pathways [69]; however, hydrolysis by sEH represents the dominant pathway of metabolism [67]. Due to these observations, various pharmacological inhibitors of sEH have been generated in order to stabilize and enhance endogenous EpFA levels for therapeutic purposes with remarkable levels of efficacy demonstrated in a variety of conditions, including cardiac arrhythmias, hypertension, atherosclerosis, chronic obstructive pulmonary disease, renal failure, and insulin resistance, among others [70].

2.2.4. Epoxy Fatty Acids: Functions and Mechanisms of Action

EETs are by far the most heavily studied of the CYP-derived EpFAs, and therefore much of the evidence summarized here regarding EpFAs will be specific to EETs. EETs stimulate vasodilation, thrombolysis, angiogenesis, and cellular proliferation, and attenuate overall inflammation and nociception, acting through both autocrine and paracrine processes [63, 67, 71]. EEQs and EDPs are also anti-inflammatory, anti-nociceptive, and vasodilatory, but these n-3 derived epoxides differ from EETs in that they elicit anti-proliferative, proapoptotic, and anti-angiogenic effects on target cells [63, 67, 71].

There are several mechanisms through which these EpFAs exert their biological actions. EETs are thought to bind to cell-surface receptors in order to activate downstream signal transduction pathways. For example, EET-dependent vasodilation occurs through activation of the cAMP/protein kinase A (PKA) pathway. Additional EET actions are mediated through activation of phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein (MAP) kinase, AMP-activated protein kinase (AMPK), and Src kinase signaling pathways, among others [67, 72]. It has been suggested that a specific EET cell membrane receptor exists; however, there is currently little evidence to substantiate this claim and the supposed EET receptor has yet to be identified or cloned [67, 71].

EETs and DHETs can also be taken up directly by cells and incorporated into phospholipids through a coenzyme-A-dependent mechanism [67, 73, 74]. The physiological relevance of this incorporation into cell membranes remains to be determined, and it is thought this may provide an intracellular storage pool of EETs for later usage [71]. Cytosolic fatty acid binding proteins (FABPs) have been shown to bind EETs and may help to facilitate their uptake into cells [67, 75]. EETs attached to FABP are also protected from sEH-mediated hydrolysis, indicating this interaction may serve to prolong EET actions within the cell [76]. In addition to cell-surface interactions, EET actions are also mediated through interaction with various intracellular effectors such as ion channels and transcription factors [67]. Most notably, EETs have illustrated an ability to bind and activate the PPARs [77-80], which likely accounts for many of their observed metabolic benefits related to adipose tissue and inflammation. A detailed summary of the evidence surrounding EpFA generation in relation to adipose tissue and obesity is provided in the next section.

2.3. CYP Epoxygenase Pathway, White Adipose Tissue, and Obesity

The potent anti-inflammatory capabilities of EpFAs have acquired significant attention for a potential therapeutic role in the progression of obesity and its associated metabolic diseases. The effects of EpFAs on body weight and cardio-metabolic health appear to be mediated through a variety of cell types and tissues in the body, including endothelial cells, hepatocytes, skeletal muscle, adipose tissue, and immune cells [10-13]. This section will focus particularly on the evidence pertaining to adipocyte-specific effects of the CYP pathway and the implications for obesity development.

2.3.1. CYP and sEH Expression in White Adipose Tissue

There are several observations that indicate obesity is associated with dysregulation of the CYP epoxygenase pathway in white adipose tissue. sEH expression is upregulated upon differentiation of 3T3-L1 preadipocytes into mature adipocytes while CYP epoxygenase expression is decreased [14, 81]. As expected, this corresponds with a stark decline in EET levels as adipocytes develop [14]. This observation is replicated at the human stem cell level, with *CYP2J2* expression and EET levels being shown to decrease as human MSCs become mature adipocytes [82]. Elevated sEH expression is also present in the adipose tissue of HFD-fed mice and overweight humans when compared to lean controls, and total adipose sEH activity is higher in obese mice, even after normalization for body weight [81, 83]. Furthermore, *CYP2J2* expression decreased by 71% in the subcutaneous adipose tissue of obese women compared to lean women [84]. A comprehensive analysis of lipid mediators produced from the COX, LOX, and CYP pathways in adipose tissue of HFD-fed mice revealed that CYP-derived EpFAs are the most severely altered in obesity, with almost all types of EpFAs being reduced by 70-90% in the adipose tissue of obese mice [85]. Together, these observations provide compelling evidence that

adipocyte-specific expression of CYPs and sEH contribute to decreased EETs in obesity and implicate a therapeutic role for enhancing EpFA production in adipose tissue. There are three main approaches used to augment EpFA concentration in order to assess therapeutic outcomes: overexpression of CYP epoxygenase enzymes, administration of stable EET analogs, and inactivation of sEH through pharmacological inhibition or targeted gene deletion. The following sections will outline findings obtained in relation to white adipose tissue and obesity utilizing these tactics.

2.3.2. CYP Epoxygenase Pathway: Adipogenesis and Obesity

Sustained white adipogenesis is a hallmark of obesity, driving both hyperplasia and hypertrophy of white adipocytes in order to accommodate excess lipids. Key transcriptional mediators of this process include PPAR γ , CCAAT/enhancer-binding protein (C/EBP) α , and SREBP1c [14, 32]. Accumulating evidence suggests that EETs and other EpFAs elicit regulatory functions in relation to white adipogenesis. Administration of stable EET analogs dose-dependently suppressed differentiation of 3T3-L1 preadipocytes and MSCs into mature adipocytes with reduced lipid accumulation and decreased gene expression of adipogenic markers, including PPAR γ , C/EBP α , SREBP1c, and fatty acid synthase (FAS) [14, 82]. Furthermore, EET analogs given to mice in the drinking water attenuated HFD-induced increases in body weight, fat mass, and adipocyte size, accompanied by an improvement in glucose tolerance [14]. Administration of sEH inhibitors and targeted gene deletion of *Ephx2*, the gene encoding for the sEH protein, has been shown to produce similar anti-adipogenic effects through an increase in EpFA bioavailability [14, 82, 86].

The suppressive effect of CYP-derived EpFAs on white adipogenesis is further corroborated by the study of *Cyp2j4* deletion in rats and MSCs [87]. The epoxygenase *Cyp2j4* is

expressed in the major rat organs and shares 79% homology with human *CYP2J2* [88]. Remarkably, *Cyp2j4*^{-/-} MSCs underwent spontaneous adipogenesis, in that these cells differentiated into mature adipocytes without the addition of adipogenic differentiation media, displaying significant lipid accumulation and expression of *Fabp4*. Moreover, WAT from *Cyp2j4*^{-/-} rats exhibited elevated expression of PPAR γ and C/EBP α compared to wild-type (WT) control rats [87].

CYP2J2 gene therapy in mice, which resulted in significant CYP2J2 protein expression in adipose tissue, was confirmed to elevate circulating EETs. Overexpression of this key human epoxygenase mitigated the burden of a HFD, with significantly reduced total body weight, adiposity, serum triglycerides and cholesterol in the transgenic mice [89]. These observed effects on cholesterol metabolism appear to be at least partially due to adipocyte-derived EpFAs, as incubation of 3T3-L1 differentiated adipocytes with an sEH inhibitor enhanced CD36-mediated recognition and degradation of oxidized low-density lipoproteins, and improved cholesterol efflux to form high-density lipoproteins through upregulation of ATP-binding cassette transporter (ABCA1) [90]. Moreover, this enhancement of reverse cholesterol transport through sEH inhibition was also demonstrated in adipocytes *in vivo* [91].

However, effects on white adipose tissue are not solely due to adipocyte-specific EpFA production, as other studies have demonstrated similar outcomes mediated through epoxygenase overexpression in other organs. For example, a similar study examining *CYP2J2* gene therapy in diabetic mice reported that CYP2J2 protein expression was primarily present in the liver, with no detectable levels in adipose tissue. Despite this, these transgenic mice still experienced a marked decrease in body weight, serum triglycerides, and cholesterol levels [92]. Moreover, endothelial-specific overexpression of *CYP2J2* in mice significantly attenuated HFD-induced increases in

body weight and adiposity when compared to WT controls, while also improving vascular dysfunction [93]. The anti-adipogenic effects of epoxygenase overexpression may be partially attributed to enhanced lipid oxidation, as endothelial *CYP2J2* overexpression has also been shown to activate the AMPK pathway with increased phosphorylation of acetyl-CoA carboxylase (ACC) and increased expression of carnitine palmitoyltransferase I (CPT1) and PPAR α [94]. The studies of endothelial-specific *CYP2J2* overexpression implicate EpFAs from the endothelium may evoke paracrine effects on adipocytes. Given that endothelial cells comprise a significant portion of the adipose stromal vascular fraction [95], it is likely that EpFAs can arise from these neighboring cells in order to exert their functional effects in adipocytes.

It is important to note that physical outcomes of altering EpFA bioavailability *in vivo* on adiposity are not apparent until subjects are placed under significant metabolic stress from energy-dense diets or advancing age. While endothelial *CYP2J2* overexpression in mice attenuated HFD-induced increases in body weight and adiposity compared to WT controls, no differences between groups were detected when mice were fed regular chow [94]. Similarly, these transgenic mice were also protected from aging-induced increases in fat mass and adipocyte size, but no differences in these parameters were present in the young mice [96]. *Cyp2j4*^{-/-} rats fed a high-fat, high-sugar cafeteria diet (CAF) displayed increased body weight and fat mass gain with greater adipocyte size compared to WT rats fed the same diet, and these effects were also mirrored under the pressure of aging [87]. Together, these outcomes demonstrate the beneficial impacts of CYP epoxygenase enzymes on body weight and adiposity under circumstances of metabolic stress.

EETs have been demonstrated to bind and activate PPAR γ , so it is rather surprising that the anti-adipogenic effect of increased EpFAs seems to be at least partially facilitated through downregulation of PPAR γ in WAT under metabolic stress, as summarized above. It has been speculated that EET interaction with ERK, AMPK, and cAMP/PKA signaling pathways may play a role to evoke this downregulation of adipogenic transcription factors, particularly in the early phases of adipogenesis [14]. However, the inhibitory effect of EETs and other EpFAs on white adipogenesis is not straightforward and some findings are even contradictory. One study demonstrated that EET analogs in the presence of an sEH inhibitor activated PPAR γ and increased *Fabp4* mRNA expression in 3T3-L1 preadipocytes, a general marker of adipocyte differentiation [77]. Furthermore, *CYP2J2* overexpression in the livers of diabetic mice increased PPAR γ mRNA and protein levels by 4.5-fold, potentially as a means to attenuate dyslipidemia present in diabetes [92]. Therefore, the effects of EpFAs on PPAR γ expression are likely contingent upon interaction of EpFAs with other signaling pathways and may be influenced by the tissue microenvironment, and further studies are needed to fully elucidate these complex interactions.

2.3.3. CYP Epoxygenase Pathway: Inflammation in White Adipose Tissue

Under circumstances of chronic energy surplus, the expansion of WAT exceeds the rate of neovascularization, resulting in a state of localized hypoxia and fibrosis. Reduced tissue plasticity and increased mechanical stress ultimately triggers a persistent inflammatory response, with altered adipokine secretion and infiltration of macrophages into adipose tissue. Further caloric overload eventually exceeds the storage capacity in WAT, leading to ectopic lipid accumulation in tissues such as the liver, skeletal muscle, and heart, a phenomenon commonly referred to as lipotoxicity. These alterations in adipocyte function and structure provoke the

downstream cardio-metabolic abnormalities characteristic of many chronic diseases, such as insulin resistance and endothelial dysfunction [97-100].

In the inflammatory state, altered adipokine secretion is characterized by a stark increase in TNF α accompanied by a decline in adiponectin levels [97]. TNF α is known to stimulate the activation of nuclear factor kappa B (NF- κ B), a central mediator of the inflammatory response that prompts expression of various pro-inflammatory genes [101]. Inhibition of NF- κ B is a key mechanism through which EpFAs exert their anti-inflammatory actions [70]. EpFAs can block nuclear translocation of NF- κ B through inhibition of I κ B kinase and can also indirectly reduce its transcriptional activity through activation of PPAR γ [77, 80, 102]. *CYP2J2* overexpression, sEH inhibition, and EET infusion via osmotic mini pumps have each demonstrated to mitigate HFD-induced activation of NF- κ B and MAPK signaling pathways in WAT [89]. This function of EpFAs demonstrates a manner through which these lipid mediators can alleviate the inflammatory consequences of obesity within adipose tissue.

Additionally, EpFAs have been shown to reverse the decline in adiponectin associated with inflammatory WAT. Adiponectin reduces inflammatory cytokines and oxidative stress and helps to regulate glucose and lipid metabolism [103]. Incubation of differentiated 3T3-L1 cells with an EET analog increased adiponectin levels in the culture medium and decreased the pro-inflammatory cytokines IL-1 α and IL-6 [104]. Similarly, treatment with an EET analog reversed the HFD-induced decrease of serum adiponectin in mice, with a reduction in serum TNF α and IL-6 [105]. Incubation of lipopolysaccharide (LPS)-induced 3T3-L1 mature adipocytes with an sEH inhibitor dose-dependently enhanced adiponectin levels, and this effect was blocked by the addition of the PPAR γ antagonist GW9662, indicating that upregulation of adiponectin occurs in a PPAR γ -dependent manner [90]. Furthermore, *Cyp2j3* gene delivery in rats and 3T3-L1 cells

stressed by a HFD or thapsigargin resulted in increased adiponectin levels, which was mediated through alleviation of endoplasmic reticulum (ER) stress [106]. sEH inhibition or deficiency has also been reported to attenuate HFD-induced ER stress in the adipose tissue of mice [83].

CYP2J2 overexpression and sEH inhibition have also been shown to reduce macrophage infiltration and fibrosis within the WAT of HFD-fed mice, with downstream effects on insulin sensitivity [89, 93, 107]. Furthermore, an increase in EpFA bioavailability through these means hindered the switch of macrophages to the classically active macrophage (M1) phenotype associated with WAT inflammation and preserved the alternatively activated macrophage (M2) phenotype. Conversely, *Cyp2j4*^{-/-} rats fed CAF displayed increased crown-like structures and fibrotic collagen within WAT compared to WT mice on the same diet. These rats also experienced ectopic lipid accumulation particularly in the liver, indicative of severely overloaded and inflamed WAT [87]. Collectively, these findings indicate the anti-inflammatory activity of EETs and other EpFAs can ultimately aid in reversal of the altered adipokine production and macrophage infiltration characteristic of dysfunctional WAT, demonstrating ample significance for prevention of obesity-associated metabolic diseases.

2.3.4. CYP Epoxygenase Pathway: Browning of White Adipose Tissue

In addition to the anti-adipogenic and anti-inflammatory functions of EpFAs in WAT, these eicosanoids have also been reported to stimulate mitochondrial biogenesis and other markers of WAT browning. EETs and other EpFAs are thought to induce PGC-1 α activation, resulting in downstream effects on mitochondrial function, thermogenesis, and overall energy expenditure. Stable EET analogs have been demonstrated to increase white adipocyte expression of PGC-1 α , PRDM16, UCP1, uncoupling protein-2 (UCP2), and cytochrome c oxidase subunit 1 (COX-1), both *in vitro* and *in vivo* [104, 108, 109, 110]. Additionally, EET analogs normalized

VO₂ levels and the respiratory quotient in HFD-fed mice, with an elevation of mitochondrial proteins [108]. Outcomes on thermogenic and mitochondrial markers are PGC-1 α -dependent, as these effects are diminished in PGC-1 α -deficient cells and animals [104, 108, 109, 110]. *CYP2J2* overexpression and sEH inhibition also have been shown to elicit similar elevations in PGC-1 α , UCP1, and UCP2 in WAT of HFD-fed mice [89, 93, 111]. Ultimately, the promotion of WAT browning is likely a manner through which EpFAs reduce adiposity under metabolic stress, working together with the other described anti-inflammatory and anti-adipogenic functions to combat obesity and its related diseases.

2.4. CYP Epoxygenase Pathway and Brown Adipose Tissue

2.4.1. Evidence for a Role in Brown Adipose Tissue

Despite the described probable functions of EpFAs in browning and energy expenditure, studies examining the CYP pathway specifically in classical BAT are extremely limited. BAT is highly vascularized to facilitate substrate delivery and heat transmission, with endothelial cells representing the most abundant cell type within BAT [6]. Given that elevated EpFAs from the endothelium seemingly evoke paracrine effects on WAT, it can be presumed these endothelial-derived EpFAs play additional roles in BAT.

Brown adipocytes also produce EpFAs, which may contribute to favorable metabolic outcomes. Our recent data illustrated that sEH expression is upregulated in *in vitro* brown adipogenesis and in the BAT of diet-induced obese mice, mimicking the pattern observed in white adipocytes [112]. Furthermore, we found that sEH inhibition by *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy-benzoic acid (*t*-TUCB) dose-dependently promoted brown adipogenesis *in vitro* and increased protein expression of genes imperative for lipid metabolism in the BAT of diet-induced obese mice [112]. A different study examining sEH

inhibition in diet-induced obesity reported a 50% increase in UCP1 protein expression in the interscapular BAT (iBAT) of mice, accompanied by enhanced metabolic rate and weight loss [113]. Usage of a different sEH inhibitor in another study increased the iBAT mass of *fat-1* mice, which are mice that express an n-3 desaturase that elevates endogenous cell membrane n-3 PUFAs [107]. Additionally, EET analogs have been shown to upregulate UCP1 mRNA expression in the BAT of HFD-fed mice [14]. Collectively, these data indicate the beneficial metabolic effects of EpFAs may be partially mediated through enhanced BAT development and activation.

It is worth noting that in the study which reported increased iBAT mass with an sEH inhibitor, the effect on BAT was only apparent in the *fat-1* mice and not the WT mice [107]. These *fat-1* mice presented with significantly elevated n-3-derived EpFAs due to increased cell membrane n-3 PUFAs, suggesting that n-3-derived EpFAs such as 17,18-EEQ and 19,20-EDP may be more important for the promotion of BAT development and activity rather than ARA-derived EETs. This hypothesis is further substantiated when considering that fish oil is believed to enhance brown and beige fat development and thermogenesis based on numerous studies [114-118], and this may occur in an adipocyte-autonomous manner, given that EPA stimulates brown adipogenesis *in vitro* [119-121]. Primary mechanisms cited for these effects of fish oil include EPA- and DHA-mediated activation of TRPV1 and G protein-coupled receptor 120 (GPR120) [122]. Perhaps, fish oil partially exerts these effects through increased formation of n-3-derived EpFAs, potentially inducing more potent activation of TRPV1 and GPR120 with subsequent thermogenic stimulation. Indeed, it has been postulated that many of the cardioprotective properties of fish oil may be attributed to their epoxide metabolites [123], suggesting potential for a similar role in BAT. Elevation of cell membrane n-3 PUFAs from fish

oil supplementation has been shown to increase serum n-3 EpFAs more than any other lipid mediator [124], further validating the relevance of these eicosanoids in facilitating the effects of fish oil. Overall, it appears likely that EpFAs play a significant role in brown adipocytes, and further studies examining the CYP pathway in BAT are warranted to elucidate these functions and their relation to obesity pathogenesis.

2.4.2. The Research Gap

In summary, it has been well established that the CYP epoxygenase pathway holds therapeutic promise for obesity and its related metabolic diseases, and that these effects are administered at least partially in an adipocyte-autonomous manner. CYP overexpression is capable of producing these favorable outcomes through an increase in EpFA bioavailability. However, the vast array of CYP isoforms makes identification of the primary epoxygenases driving these metabolic effects to be much more complex and challenging. There are currently very limited data examining CYP expression in white adipocytes, and a comprehensive analysis of all known epoxygenase isoforms has not been conducted. Additionally, to our knowledge, no studies have determined which CYP isoforms are expressed specifically in BAT, and the role of CYP epoxygenase expression in brown adipogenesis has not yet been explored.

In this thesis, we sought to characterize adipocyte CYP epoxygenase expression throughout adipogenesis and in diet-induced obesity. This was accomplished through a comprehensive analysis of *Cyp2j* and *Cyp2c* mRNA expression in brown and white adipocytes of murine and human origin throughout differentiation, and in the BAT and WAT of mice fed HFD and regular chow. Furthermore, we began to elucidate the role of the CYP pathway in brown adipocytes through modulation of CYP epoxygenase expression in murine and human brown adipogenesis. Collectively, these data contribute to our understanding of the CYP

epoxygenase pathway in adipocytes and suggest a role of these enzymes in brown adipogenesis, further contributing to our comprehension of how EpFAs can help regulate overall metabolic homeostasis.

CHAPTER III

MATERIALS AND METHODS

3.1. Materials and Reagents

Anti-UCP1 antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-PGC-1 α antibody was purchased from Millipore (Temecula, CA). Anti-CYP2J2 antibody was purchased from Kerafast (Boston, MA). Anti-PPAR γ , anti-Myc-Tag, anti-ERK1/2, horseradish peroxidase-conjugated goat anti-rabbit, and horseradish peroxidase-conjugated goat anti-mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA).

The Myc-DDK-tagged *Cyp2j13* cDNA plasmid and empty vector control (pCMV6) were purchased from OriGene (Rockville, MD). The lentiviral particles against mouse *Cyp2j13* and a non-targeting scrambled control were obtained from MISSION Sigma-Aldrich (St. Louis, MO). The *CYP2J2* cDNA plasmid was a gift from Dr. Ahmed Bettaieb at the University of Tennessee Knoxville, and the empty vector pSBtet-Pur was a gift from Eric Kowarz [125] (Addgene plasmid # 60507; <http://n2t.net/addgene:60507>; RRID:Addgene 60507). The pSBtet-Pur-*CYP2J2* construct was generated by PCR amplifying *CYP2J2* full cDNA fragment flanked with two different SfiI sites followed by ligation into the SfiI pre-cut pSBtet-Pur vector. The complete *CYP2J2* cDNA sequences in pSBtet-Pur were confirmed by sequencing.

3.2. Cell Culture and Differentiation

The murine brown preadipocyte cell line was a gift from Dr. Johannes Klein (University of Lubeck, Lubeck, Germany), and was generated from the interscapular brown adipose tissue of newborn C57BL/6J mice [126]. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) in a humidified 37°C and 5% CO₂ incubator until they reached confluence

(designated as day 0 (D0)). The cells were then induced to differentiate in differentiation media containing DMEM supplemented with 20% FBS, 1 nM T3, and 20 nM insulin every 2 days for 6 days. Select cells received induction media containing differentiation media supplemented with 0.125 mM indomethacin, 2 µg/ml dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (MIX) for 24 hours prior to being changed into differentiation media to induce a stronger degree of differentiation.

The murine 3T3-L1 cell line was obtained from ATCC (Manassas, VA). The cells were maintained in DMEM supplemented with 10% calf serum (Hyclone, Logan, UT) until they reached confluence. Preadipocytes were then induced to differentiate with differentiation media containing DMEM supplemented with 10% FBS, 1 µM dexamethasone, 10 µg/ml insulin, and 0.5 mM MIX for 3 days (D0-D3). Media were subsequently changed to DMEM with 10% FBS and 10 µg/ml insulin for an additional 2 days (D4-D5). Cells were then maintained in DMEM with 10% FBS until fully differentiated on D7.

The human brown cell line was a gift from Dr. Yu-hua Tseng at Joslin Diabetes Center. Cells were cultured and differentiated, as previously described [127]. Briefly, the preadipocytes were cultured in DMEM supplemented with 10% FBS until confluency was achieved. Cells were then induced to differentiate in DMEM supplemented with 10% FBS, 0.5 µM insulin, 33 µM biotin, 17 µM pantothenate, 0.1 µM dexamethasone, 2 nM T3, 500 µM MIX, 30 µM indomethacin, and 5 µM rosiglitazone. Media were replaced every 3 days until cells were fully differentiated at 3 or 4 weeks. For the *CYP2J2* overexpression experiments, human brown cells received either 1 µg/ml doxycycline or the vehicle control (water) in the media at 48 hours prior to confluency, and then with every medium change thereafter in order to induce stronger expression of human *CYP2J2*.

293T (human embryonic kidney cells) were obtained from Sam Lee at Beth Israel Hospital (Boston, MA) [128] and were used to generate positive controls for the CYP2J13 and CYP2J2 protein expression. The cells were maintained in DMEM with 10% FBS.

3.3. Animals

All mice studies were approved by the Institutional Animal Care and Use Committee at the University of Tennessee. C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). The diet-induced obese mice and their chow-fed controls have been described elsewhere [129, 130]. Briefly, six-week old male mice were fed either a HFD (60% kcal from fat, D12492) (Research Diets Inc., New Brunswick, NJ) or regular chow (RC) for 20 weeks before sacrifice at 26 weeks of age. Upon sacrifice, iBAT, epididymal and subcutaneous inguinal WAT (eWAT and iWAT) were excised and immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

3.4. Lipofectamine Transfection

Murine and human brown preadipocytes were seeded in 12- or 6-well plates at ~50% confluency. Cells were then transfected using Lipofectamine 3000 and Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and were split into selection media 48 hours later. *Cyp2j13*-stably transfected murine brown preadipocytes were selected for two weeks with 600 µg/ml neomycin. Individual stable clones were used for analysis. *CYP2J2*-stably transfected human brown preadipocytes and 293T cells were selected for two weeks with 2 µg/ml puromycin. Stably transfected pooled cells were used for analysis. 293T cells transfected with *Cyp2j13* did not undergo selection and were immediately harvested at 48 hours after transfection.

3.5. Lentiviral shRNA Particle Infection

Murine brown preadipocytes were seeded at ~50% confluency in 6-well plates. Cells were then transduced with lentiviral particles against mouse *Cyp2j13* or a scrambled non-targeting control (MISSION Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, and were split into selection media after 48 hours. Stably infected cells were selected for two weeks with 600 µg/ml neomycin. Individual stable clones were used for analysis.

3.6. RNA Preparation and Quantitative Real-Time PCR Analysis

Total RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA yield was quantified with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse Transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions. mRNA expression of target genes and the housekeeping gene 36B4 (a ribosomal protein that is a component of the 60S subunit) were measured quantitatively using the PowerUp SYBR master mix (Applied Biosystems, Austin, TX) and gene-specific primers (primer sequences available upon request). PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 2 min, 40 cycles of 95°C for 15 s, then 60°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method, after normalization to the housekeeping gene 36B4.

3.7. Western Blot Analysis

Total cell lysates were prepared using 1xRIPA lysis buffer (Cell Signaling, Danvers, MA). Protein concentrations were quantified using the BCA assay kit (Thermo Scientific,

Waltham, MA). Proteins were separated on a 10% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked in TBST buffer (20 mM Tris Base, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4)) containing 5% nonfat milk. Membranes were then immunoblotted with the primary antibodies at 4°C overnight, followed by 1-hour incubation with secondary antibodies conjugated with horseradish peroxidase at room temperature. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA). Band densitometry was quantified using the ChemiDocXRS+ system with Image Lab software (Bio-Rad, Hercules, CA).

3.8. Statistical Analysis

Statistical analysis was performed using SigmaPlot 14.0 (Systat Software, San Jose, CA). One-way ANOVA followed by the Holm-Sidak method was performed in the time course studies to determine differences in group means compared to cells collected on day 0. Murine brown cells receiving induction media and those receiving only differentiation media were compared separately to day 0. One-way ANOVA followed by the Student-Newman-Keuls method for multiple groupwise comparisons was conducted to determine differences in group means compared to the control groups in the animal studies and *Cyp2j13* overexpression and knockdown experiments. In the studies of *CYP2J2* overexpression in human brown cells, two-way ANOVA followed by the Student-Newman-Keuls method was performed to determine the differences between *CYP2J2* overexpression and the empty vector controls and between treatment with doxycycline and the vehicle control. The level of significance was set at $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1. Results

4.1.1. CYP Epoxygenase Expression in Murine Brown Adipocyte Differentiation

We first examined CYP epoxygenase expression in murine brown adipocyte differentiation. Brown adipocyte marker genes *Ucp1*, *Pgc-1 α* , *Ppar γ* , *Prdm16*, and *cell death-inducing DFFA like effector A (Cidea)* were significantly increased in a time-dependent manner during the course of differentiation, and their expression were significantly higher in cells that received induction media (Figure 1A). mRNA expression of a total of 19 murine *Cyp2j* and *Cyp2c* isoforms (*Cyp2j5*, *Cyp2j6*, *Cyp2j7*, *Cyp2j8*, *Cyp2j9*, *Cyp2j11*, *Cyp2j12*, *Cyp2j13*, *Cyp2c29*, *Cyp2c37*, *Cyp2c38*, *Cyp2c39*, *Cyp2c40*, *Cyp2c44*, *Cyp2c50*, *Cyp2c54*, *Cyp2c55*, *Cyp2c65*, and *Cyp2c66*) were examined in the murine brown cells. Of these, *Cyp2j5*, *Cyp2j7*, *Cyp2j11*, *Cyp2j12*, *Cyp2c29*, *Cyp2c38*, and *Cyp2c40* were not detectable in all or a majority of the time points examined. mRNA expression levels of the remaining *Cyp2j* isoforms and select *Cyp2c* isoforms during differentiation are shown (Figure 1B, C). Interestingly, expression of all the *Cyp2j* isoforms were either elevated or not changed in the cells receiving only differentiation media but suppressed in the more strongly differentiated cells that received induction media (Figure 1B). The cells receiving only differentiation media had statistically significant elevations in mRNA levels of *Cyp2j6*, *Cyp2j9*, and *Cyp2j13* compared to day 0, with an upward trend also observed with *Cyp2j8*. Conversely, in the cells receiving induction media, mRNA expression of *Cyp2j6*, *Cyp2j9*, and *Cyp2j13* significantly decreased compared to day 0, with a downward trend in *Cyp2j8* mRNA levels. (Figure 1B).

Similar outcomes were observed in the *Cyp2c* isoforms, with slightly elevated or unaffected mRNA levels in cells receiving only differentiation media, and lower expression in cells receiving the induction media (Figure 1C). However, expression patterns were not as consistent between samples as with the *Cyp2j* isoforms. Of the tested isoforms, *Cyp2c44* displayed a unique expression pattern, which was significantly elevated both in cells receiving only differentiation media and receiving induction media (Figure 1C). mRNA levels of *Cyp2c37*, *Cyp2c50*, *Cyp2c55*, *Cyp2c65*, and *Cyp2c66* were increased in cells receiving only differentiation media, reaching statistical significance for *Cyp2c55* ($p < 0.05$). Similarly, mRNA levels of these *Cyp2c* isoforms showed a downward trend in cells receiving the induction media. Overall, these data demonstrate that mRNA levels of tested murine *Cyp2j* and *2c* isoforms are inversely correlated with the degree of brown adipocyte differentiation, suggesting roles of these enzymes in brown adipogenesis.

Next, we sought to compare the relative abundance of mRNA levels of the examined CYP isoforms both in the brown pre- and differentiated adipocytes. Of the *Cyp2j* isoforms, *Cyp2j6* mRNA levels were the most abundant, followed by *Cyp2j9*, then *Cyp2j13*, and *Cyp2j8*. *Cyp2c65* mRNA levels were the most abundant of the *Cyp2c* isoforms, followed by *Cyp2c66*, and *Cyp2c55* expression was the least pronounced. Between both *Cyp2j* and *Cyp2c* subfamilies, *Cyp2c65* was the most highly expressed (Figure 1D).

4.1.2. CYP Epoxygenase Expression in 3T3-L1 Adipocyte Differentiation

After thoroughly studying CYP epoxygenase expression patterns in brown adipocyte differentiation, we then examined the expression of these genes in 3T3-L1 white adipocyte differentiation. Strong differentiation was indicated by time-dependent increases in mRNA levels of adipogenic markers *Fabp4*, *Fasn*, *Ppar γ* , *C/ebp α* , and *stearoyl-Coenzyme A desaturase 1*

(*Scd1*) during the course of differentiation (Figure 2A). Consistently, *Cyp2j* mRNA levels, except for *Cyp2j8*, appeared to be decreased at day 3 and 5 compared to that of day 0, but were increased by day 7, with statistically significant elevations observed in *Cyp2j8* and *Cyp2j13* ($p < 0.01$) (Figure 2B). Interestingly, while majority of *Cyp2j* isoforms decreased at day 3, this regression appeared to be compensated for by an enhancement of *Cyp2c* mRNA expression. mRNA levels of *Cyp2c37*, *Cyp2c50*, *Cyp2c55*, *Cyp2c65*, and *Cyp2c66* each showed pronounced and statistically significant elevations at day 3 (Figure 2C). The only tested isoform showing a different pattern was *Cyp2c44*, which increased throughout differentiation (Figure 2C). Similar to the *Cyp2j* isoforms but to a greater degree, expression of all the *Cyp2c* isoforms were elevated in the fully mature white adipocytes at day 7 compared to the preadipocytes (day 0). Overall, these data demonstrate that mRNA levels of tested murine *Cyp2j* and *2c* isoforms are positively correlated with the degree of white adipocyte differentiation. Notably, the relative mRNA abundance of the tested CYP genes in the white adipocytes followed the same pattern as in the brown adipocytes, suggesting that similar CYP isoforms are the most prevalent EpFA producers in both brown and white adipocytes (Figure 2D). However, unlike the brown adipocytes, the relative *Cyp2j6* mRNA level was overall the most abundant among both the *Cyp2j* and *Cyp2c* isoforms in the 3T3-L1 cells (Figure 2D).

4.1.3. CYP Epoxygenase Expression in Human Brown Adipocyte Differentiation

After surveying CYP expression patterns in murine brown and white adipocytes, we next sought to examine the mRNA expression of human CYP isoforms in human brown adipocyte differentiation. The human brown preadipocytes achieved a high level of differentiation by week 4, displaying significant increases in mRNA levels of brown adipocyte marker genes *UCP1*, *PGC-1 α* , and *PPAR γ* (Figure 3A). Interestingly, mRNA levels of *CYP2C* isoforms were more

pronounced in the more differentiated state, with statistically significant elevations observed at week 4 in each of the four isoforms (Figure 3B). mRNA levels of *CYP2C9* and *CYP2C18* showed approximately 50-fold increases of mRNA levels at week 4 compared to day 0 ($p < 0.001$). This pattern contrasts slightly with the observed decline in *Cyp2c* mRNA levels in the murine brown cells receiving the induction media but aligns with the slight increase observed in the cells receiving only differentiation media. Considering these human brown cells did not fully differentiate to the extent of the murine brown cells receiving induction media, this observation suggests a bell-shaped pattern between CYP mRNA expression and brown adipocyte differentiation, in that enhanced mRNA levels are associated with a smaller degree of differentiation, but these levels are decreased as stronger degrees of differentiation are achieved. Interestingly, while *CYP2C* mRNA levels increased during human brown differentiation, *CYP2J2* displayed an opposing pattern. *CYP2J2* was found to be minimally expressed in our human brown cell line, and these low levels completely diminished with no detectable levels observed by week 4 (Figure 3B). Of the human CYP isoforms, *CYP2C19* was the most abundant, followed by *CYP2C18*, then *CYP2C9*, *CYP2C8*, and finally *CYP2J2* (Figure 3C).

4.1.4. CYP Epoxygenase Expression in Diet-induced Obesity

In order to characterize CYP expression patterns in adipose tissue under circumstances of metabolic dysfunction, we next examined relative mRNA levels of these enzymes in adipose tissue from HFD-induced obese mice compared to RC-fed controls. Interestingly, mRNA levels of *Cyp2j* isoforms in the iBAT trended upwards in HFD-fed mice compared to controls, but statistical significance was only achieved for *Cyp2j9* ($p < 0.05$) (Figure 4A). *Cyp2c* mRNA expression in the iBAT displayed high levels of variation among individual mice and among isoforms (Figure 4B). There were no clear and consistent patterns across isoforms observed in

the eWAT, but *Cyp2j6* mRNA expression was significantly elevated in the HFD-fed mice, whereas *Cyp2c65* and *Cyp2c66* mRNA levels were significantly suppressed ($p < 0.05$). *Cyp2j13* mRNA level also trended downwards, but this did not reach statistical significance (Figure 4C, D). In the iWAT, mRNA expression of *Cyp2j* isoforms again appeared to trend upwards in the HFD-fed mice, with *Cyp2j8* reaching statistical significance ($p < 0.05$) (Figure 4E). No significant differences in iWAT *Cyp2c* mRNA levels were detected between groups (Figure 4F). *Cyp2c55* expression was also examined in the tissue samples, but mRNA levels were primarily undetectable and therefore the data is not shown. Overall, these data suggest that obesity may be associated with dysregulation of CYP expression in different adipose tissue depots.

4.1.5. *Cyp2j13* Overexpression Suppresses Murine Brown Adipocyte Differentiation

Considering that *Cyp2j* isoforms displayed more consistent but differential mRNA expression patterns in brown vs. white adipocyte differentiation in the cell culture studies, we chose to specifically focus on *Cyp2j* isoforms in brown adipocyte differentiation for the next portion of our study. *Cyp2j13* was selected for further investigation due to its unique mRNA expression pattern showing an upward trend in the iBAT and a downward trend in the eWAT of HFD-fed mice. We first examined the impact of *Cyp2j13* overexpression on murine brown adipogenesis. CYP2J13 protein expression was increased by ~6-fold in the murine brown preadipocytes stably transfected with the *Cyp2j13* expression plasmid (pCMV6-*Cyp2j13*) compared to preadipocytes stably transfected with the empty vector (pCMV6) (Figure 5A). At day 6 of differentiation, *Cyp2j13* mRNA expression was increased by ~16-fold in the overexpressing murine brown adipocytes (Figure 5C). Remarkably, overexpression of this murine epoxygenase induced a strong suppression of brown adipocyte differentiation. At day 6 of differentiation, *Cyp2j13* overexpressed brown cells displayed significantly reduced lipid

accumulation (Figure 5B left panel) with an approximate 50% decrease in oil red O (ORO) absorbance ($p < 0.001$) (Figure 5B right panel). Furthermore, mRNA expression of key brown marker genes *Ucp1* and *Cidea* and general adipocyte differentiation markers *Pparγ* and *Fabp4* were significantly suppressed with *Cyp2j13* overexpression ($p < 0.01$, $p < 0.001$, and $p < 0.05$, respectively) (Figure 5C). Protein levels of UCP1 and PPAR γ were also consistently suppressed in the *Cyp2j13* overexpressing cells ($p < 0.001$). In contrast, mRNA expression of the thermogenic marker *Pgc-1α* was significantly elevated in the *Cyp2j13* overexpressing cells (Figure 5C), but protein expression was significantly reduced ($p < 0.05$) (Figure 5D), suggesting differential regulation of *Pgc-1α* expression at the mRNA and protein levels by *Cyp2j13* overexpression in brown adipocytes. *Pparaα* mRNA levels also trended downwards in the *Cyp2j13* overexpressing cells, but this did not reach statistical significance (Figure 5C). Together, these findings suggest that increasing *Cyp2j13* expression may repress brown adipocyte differentiation.

4.1.6. *Cyp2j13* Knockdown Enhances Murine Brown Adipocyte Differentiation

We next utilized lentiviral-mediated knockdown of *Cyp2j13* to assess if the opposite outcome could be produced. Due to the lack of an available antibody specific for CYP2J13, knockdown efficacy was validated solely on the mRNA level. mRNA expression of *Cyp2j13* in the *Cyp2j13* knockdown (KD) brown preadipocytes was reduced by ~70% compared to cells infected with the scrambled non-targeting control ($p < 0.01$) (Figure 6A). mRNA expression of the brown marker gene *Cidea* and general differentiation markers *Fabp4* and *Fasn* were significantly enhanced in the *Cyp2j13* KD cells on day 6 of differentiation ($p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively) (Figure 6B). *Pgc-1α* mRNA expression also trended upwards, and PGC-1 α protein levels displayed a significant elevation ($p < 0.001$) (Figure 6B, C). There were

no observed differences in *Ucp1* mRNA expression between the two cell populations, however, a significant increase in UCP1 protein expression was observed ($p < 0.01$) (Figure 6C). In contrast, mRNA levels of both *Ppar γ* and *Ppara* were significantly reduced in the *Cyp2j13* KD cells ($p < 0.01$), but protein levels of PPAR γ were unaffected. Overall, these data appear to corroborate the suppressive effect of *Cyp2j13* overexpression on brown adipogenesis and suggest that *Cyp2j13* may be a negative regulator of murine brown adipogenesis.

4.1.7. *CYP2J2* Overexpression Does Not Affect Human Brown Adipocyte Differentiation

In attempt to gain insight into the role of CYP epoxygenases in human brown adipogenesis, we next analyzed the impact of *CYP2J2* overexpression on human brown adipocyte differentiation. Human brown preadipocytes were stably transfected with the *CYP2J2* expression plasmid (pSBtet-*CYP2J2*) or the empty vector (pSBtet). Robust *CYP2J2* expression was induced by treatment with 1 μ g/ml doxycycline and compared with cells receiving the vehicle control (water). After 48 hours of treatment with doxycycline or water, only the human brown preadipocytes transfected with *CYP2J2* and treated with doxycycline demonstrated significant *CYP2J2* protein expression (Figure 7A). However, mRNA analysis in the human brown adipocytes after differentiation for three weeks demonstrated that cells transfected with the *CYP2J2* plasmid still displayed a significant induction of *CYP2J2* mRNA expression without doxycycline, although not to the extent of the cells which received doxycycline. Of the *CYP2J2* transfected cells, those receiving water experienced a ~2600-fold increase in *CYP2J2* mRNA expression compared to the empty vector controls treated with water, while cells treated with doxycycline displayed nearly a 195,000-fold increase ($p < 0.001$) (Figure 7B). These strong elevations of *CYP2J2* expression in the differentiated human adipocytes were also confirmed at the protein level (Figure 7C). Surprisingly, despite the highly successful induction of *CYP2J2*

overexpression in the human brown adipocytes, no visible differences in differentiation potential were observed between the four groups after differentiation for three weeks. *CYP2J2* overexpression did not change *UCP1*, *PPAR γ* , *FABP4*, *FASN*, or *PPAR α* mRNA expression levels. However, *CYP2J2* overexpression did elicit a significant reduction in *PGC-1 α* mRNA levels compared to the empty vector controls ($p < 0.05$). Protein expression of *PPAR γ* was significantly increased with *CYP2J2* overexpression ($p < 0.01$), but there were no changes in *PGC-1 α* or *UCP1* protein levels compared to the empty vector controls. There were no significant alterations observed between the *CYP2J2* overexpressing cells receiving doxycycline or water, suggesting that increasing *CYP2J2* expression beyond the extent observed in the cells treated with water may not be sufficient to confer further changes in gene and protein expression, at least in the markers that were tested. These data suggest that overexpression of *CYP2J2* may not be sufficient to affect human brown adipocyte differentiation.

4.2. Discussion

BAT has recently emerged as a novel target for treatment of obesity and its related metabolic disorders. Accumulating evidence suggests that CYP epoxygenase overexpression may confer benefits for metabolic disease, presumably due to increased EpFA bioavailability, but it is unknown if CYP epoxygenase overexpression directly affects brown adipogenesis and whether the beneficial effects of CYP epoxygenase overexpression in metabolic diseases are partially mediated through enhanced BAT development and activation. Increasing EpFA concentrations in white adipocytes has been shown to inhibit white adipocyte differentiation [14, 82], suggesting these epoxygenase enzymes may play a regulatory role in adipogenesis. However, to our knowledge, adipocyte-specific expression of CYP epoxygenases has not been

extensively analyzed, and no studies on CYP epoxygenase expression and brown adipocyte differentiation have been reported.

This study is the first to report CYP epoxygenase expression patterns throughout the course of adipocyte differentiation. We show that similar CYP isoforms (*Cyp2j6*, *Cyp2j9*, *Cyp2j13*, *Cyp2c44*, *Cyp2c65*, *Cyp2c66*) are the most highly expressed in both murine brown and white adipocytes, suggesting that these isoforms are the key drivers of murine adipocyte EpFA production. Furthermore, we show that CYP epoxygenase expression is higher in mature adipocytes vs. preadipocytes, except in the most strongly differentiated murine brown adipocytes, suggestive of a bell-shaped pattern in relation to adipocyte differentiation and epoxygenase expression levels. A previous study showed that *Cyp2j9* and *Cyp2c55* expression slightly decreased in mature white adipocytes compared to the preadipocytes [14]. It is possible that degrees of white adipocyte differentiation may be different between our model and the published study. Further studies are needed to confirm the expression patterns of *Cyp2j* and *2c* isoforms in murine brown and white adipogenesis.

We also demonstrated that *Cyp2j* mRNA levels showed an upward trend in the iBAT of HFD-fed mice. Given that obesity is associated with reduced BAT mass and activity [22], this finding seems to suggest that elevated *Cyp2j* expression is also associated with reduced BAT mass and activity, which seemingly aligns with our finding that *Cyp2j* mRNA levels were suppressed in more strongly differentiated brown adipocytes. Together, this implicates that *Cyp2j* may be a negative regulator for brown adipocyte development. This suspected impact of *Cyp2j* expression on brown adipogenesis was further validated by our studies of *Cyp2j13* overexpression and knockdown, which significantly suppressed and promoted brown adipocyte differentiation, respectively. Previous studies have shown that *Cyp2j13* is highly expressed in

mouse kidneys and hearts, but not detectable in the liver [64, 131, 132]. Overall, however, this epoxygenase isoform is largely unexplored, and we are the first to closely examine its expression in adipose tissue. Our data strongly indicates that *Cyp2j13* in adipose tissue plays a critical role in brown adipogenesis and may be a novel target for obesity treatment and prevention.

Sustained white adipogenesis is integral in the initiation and progression of obesity and its related diseases. EET analogs have been shown in multiple studies to suppress white adipocyte differentiation [14, 82, 86], and knockout of the major rat epoxygenase *Cyp2j4* in MSCs resulted in spontaneous adipogenesis, in that the MSCs became mature adipocytes without the addition of adipogenic media [87]. These data indicate that EpFAs inhibit white adipogenesis; however, it has been speculated that these lipid mediators elicit opposing effects on brown adipogenesis. We have demonstrated that sEH inhibition by *t*-TUCB dose-dependently promotes brown adipogenesis *in vitro* [112]. Another study utilizing a different sEH inhibitor also reported enhanced iBAT mass in *fat-1* mice which express an n-3 desaturase that elevates endogenous n-3 PUFAs [107]. It has been suggested that this enhanced brown differentiation was a result of EpFA stabilization by sEH inhibition. However, results of the current study suggest that perhaps the promotion of brown adipogenesis by *t*-TUCB and other sEH inhibitors may be primarily attributed to their off-target effects, and not simply the inhibition of the EpFA-degrading enzyme. It is worth noting that in our previous study, *t*-TUCB was also shown to activate both PPAR γ and PPAR α in the brown preadipocytes *in vitro* [112], so it cannot be ruled out that the promotion of cellular brown adipogenesis by *t*-TUCB was a direct result of its activation of the PPARs and their downstream target genes, and not due to EpFA actions.

Cyp2j13 epoxygenase overexpression presumably elevates EpFAs, implicating a similar suppressive effect of these lipid mediators on both brown and white adipogenesis. A comparable

influence of EpFAs on differentiation of both adipocyte types is certainly possible, considering that both brown and white adipocyte differentiation are under the master regulation of PPAR γ , and both cell types express similar CYP epoxygenase isoforms and exhibit an upregulation of sEH expression throughout differentiation [81, 112]. However, a limitation in our current study is that we did not measure the effect of *Cyp2j13* expression on EpFAs, so we cannot definitively state that the observed suppression of brown adipogenesis is attributed to enhanced EpFAs. Studies directly examining the effect of EpFAs and stable analogs on cellular brown adipogenesis are warranted to further elucidate the role of the CYP epoxygenase pathway in brown adipocyte development and activation.

It is also important to mention that it is unknown if differing EpFAs may elicit contrasting effects on brown adipocytes, which may impact the observed effects of CYP overexpression, since we are unsure of how the EpFA profile of the cells were affected in our studies. There are some discrepancies in functions between the n-6-derived EETs and the n-3 varieties. For example, EETs are recognized as more proliferative and angiogenic, while EEQs and EDPs exert opposing actions [67]. There is substantial evidence proposing a role of fish oil in the promotion of brown adipogenesis [114-121], and it has been speculated that many of the suggested benefits of fish oil in cardiovascular disease and other conditions may actually be attributed to their epoxide derivatives [123]. Perhaps, addition of EPA or DHA as substrates in conjunction with CYP overexpression may produce opposite effects on brown differentiation than what was observed in our study.

Ultimately, the exact mechanism through which *Cyp2j13* overexpression inhibits brown adipogenesis remains to be determined. It is likely this suppression occurs through a similar manner in which EETs inhibit white adipogenesis as shown in other studies. A previous study

postulated that EETs elicit anti-adipogenic effects by specifically targeting signaling events in the early phase of adipogenesis, as administration of EET analogs solely in the later phases diminished the suppressive effect [14]. In the early phases of preadipocyte differentiation, inhibition of the ERK signaling pathway and activation of the cAMP/PKA and AMPK signaling pathways have been shown to inhibit differentiation of 3T3-L1 cells with repression of PPAR γ , C/EBP α , and SREBP1c activation [133-135]. Increased EETs through epoxygenase overexpression or administration of stable analogs has been shown to inhibit ERK dephosphorylation in endothelial cells, activate AMPK in the liver, skeletal muscle, aorta, and kidney, and activate the cAMP/PKA pathway in the vasculature [15, 82, 94, 136, 137]. Thus, we can hypothesize that CYP-derived EETs and other EpFAs are involved in complex interactions with a variety of signaling pathways that ultimately suppress adipogenic transcription factor networks, particularly in the early phases of brown adipogenesis.

There is also question as to whether all *Cyp2j* isoforms possess the ability to inhibit brown differentiation, or if this is unique to certain isoforms, such as *Cyp2j13*. A previous study analyzed expression of all murine *Cyp2j* and *Cyp2c* isoforms in the liver, duodenum, kidney, and brain in response to LPS-induced inflammation, and reported that changes in mRNA expression levels were both isoform- and tissue-specific [132]. This strengthens the idea that although each of the *Cyp2j* and *Cyp2c* isoforms likely possess epoxygenase ability, their overall effect on cellular physiological processes may differ between isoforms and tissues. It would be beneficial to examine overexpression and knockdown of other CYP isoforms in brown adipocytes in order to compare effects on differentiation potential. *Cyp2j6* may be an interesting candidate to explore, as it is the most abundant murine *Cyp2j* isoform in adipose tissue based on our data and the official homolog to human *CYP2J2* [138]. Of the *Cyp2c* genes, analysis of *Cyp2c44*

overexpression could be noteworthy based on its unique expression patterns compared to the other *Cyp2c* isoforms, suggesting it may play a distinctive role in cellular processes.

CYP2J2 overexpression did not significantly affect human brown adipocyte differentiation based on the markers analyzed. However, it is possible that *CYP2J2* overexpression produced effects on target genes that were not examined in the current study, such as those involved in inflammation and oxidative stress. *CYP2J2* overexpression has been shown to attenuate HFD-and aging-induced increases in body weight, adiposity, serum lipids, insulin resistance, and vascular dysfunction [89, 92, 93, 94, 96]. Primary mechanisms reported for these outcomes include activation of PPAR γ , AMPK, and PI3K/Akt pathways, resulting in reduced inflammation, increased glucose uptake, and enhanced lipid metabolism. Endothelial-specific *CYP2J2* overexpression has been shown to improve dyslipidemia through activation of AMPK and elevation of PPAR α expression [94]. In our study, however, there were no significant changes in *PPAR α* mRNA expression with *CYP2J2* overexpression in the human brown cells. Perhaps, this can be attributed to the lack of metabolic stress, as alterations in markers of lipid metabolism were only present in the *CYP2J2* overexpressing mice when fed a HFD [94]. In fact, many of the beneficial metabolic effects of *CYP2J2* overexpression previously reported are only apparent when subjects are placed under metabolic stress such as a HFD or aging, with no differences observed in the basal state. Much like white adipocytes, brown adipocytes are also capable of producing pro-inflammatory cytokines under circumstances of metabolic stress, and accumulating evidence suggests that inflammation can directly alter BAT development and thermogenic activity [139, 140]. We have previously shown that activation of Toll-like receptor (TLR)4, TLR2, or nucleotide-oligomerization domain-containing protein (NOD)1 in brown adipocytes induces inflammation and impairs UCP1 expression and

mitochondrial respiration [129]. Although *CYP2J2* overexpression did not remarkably affect the expression of brown marker genes in the basal state, it would be intriguing to examine if *CYP2J2* overexpression may help alleviate inflammation-induced suppression of brown adipocyte differentiation and activity.

It is also important to discuss the complex interactions of CYP epoxygenases and PPAR γ expression. Despite reported suppressive effects of EETs on white adipogenesis, their involvement in this process is not straightforward and some findings are contradictory to this notion. EETs have been shown to bind and activate PPAR γ , the master regulator of both brown and white adipocyte differentiation [77]. PPARs heterodimerize with RXR and bind to PPRES in target genes, such as *UCPI* in brown adipocytes, in order to regulate their expression [33]. Rosiglitazone, a synthetic ligand for PPAR γ , has been shown to enhance brown adipocyte differentiation [141]. Although EETs have demonstrated to be PPAR γ ligands, data regarding the effect of EETs and CYPs on PPAR γ expression are still unclear. EETs have been shown to repress PPAR γ expression in white adipocyte differentiation [14, 82, 93]. However, another study demonstrated that EET analogs in the presence of an sEH inhibitor activated PPAR γ and increased *Fabp4* mRNA expression in 3T3-L1 preadipocytes, a general marker of adipocyte differentiation [77], suggesting the effects of EETs on white adipogenesis are not straightforward and may depend on the cellular conditions and interactions with other signaling pathways. In our current study, *Cyp2j13* overexpression significantly suppressed PPAR γ mRNA and protein expression in the differentiated murine brown adipocytes. However, knockdown of this gene also resulted in significant reduction of *Ppar γ* mRNA expression, and protein levels were not affected. Conversely, *CYP2J2* overexpression did not affect PPAR γ mRNA levels, but significantly increased its protein expression. These findings highlight the complex nature of

CYP epoxygenase interaction with PPAR γ expression and indicate that the effect of CYPs on PPAR γ expression levels may be variable depending on the tissue microenvironment. Further studies are needed to define the effects of the CYP epoxygenase pathway on PPAR γ expression in different adipose tissue depots and under circumstances of metabolic stress.

In conclusion, the results presented in the current study help to characterize CYP epoxygenase expression in adipocytes and suggest that *Cyp2j13* is a critical epoxygenase involved in the regulation of murine brown adipogenesis. Our findings indicate that EETs and other EpFAs may suppress brown adipogenesis through a similar manner in which these lipid mediators suppress white adipogenesis. Although *CYP2J2* overexpression did not remarkably affect the expression of brown marker genes in the basal state, further studies are warranted to examine if *CYP2J2* overexpression may help alleviate inflammation-induced suppression of brown adipocyte differentiation and activity. Moreover, the roles of *CYP2J2* should be further investigated by gene knockdown and pharmacological antagonism. Additional studies analyzing other CYP epoxygenase isoform overexpression and knockdown are necessary to generate a complete picture on the role of CYP epoxygenases in brown adipogenesis.

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APPENDIX

Figure 1. CYP epoxygenase expression in murine brown adipocyte differentiation

Murine brown preadipocytes were induced to differentiate for 6 days. Total RNA samples were collected on day 0, day 2, day 4, and day 6. Upon initiation of differentiation, select cells received induction media for 24 hours (indicated as the day collected + I) to induce a stronger degree of differentiation. mRNA expression was quantified relative to the loading control *36b4* and is displayed as fold of the day 0 samples (**A**, **B**, **C**). (**A**) Relative mRNA expression of brown adipocyte marker genes *Ucp1*, *Pgc-1 α* , *Ppar γ* , *Prdm16*, and *Cidea*. (**B**) Relative mRNA expression of *Cyp2j* epoxygenase genes *Cyp2j6*, *Cyp2j8*, *Cyp2j9*, and *Cyp2j13*. (**C**) Relative mRNA expression of *Cyp2c* epoxygenase genes *Cyp2c37*, *Cyp2c44*, *Cyp2c50*, *Cyp2c55*, *Cyp2c65*, and *Cyp2c66*. (**D**) Total mRNA abundance of *Cyp2j* and *Cyp2c* genes on day 0 and day 6 of differentiation, relative to *36b4*. Data = Mean $\pm$ SEM (n=3). *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, differentiation only groups compared to day 0 and induction groups compared to day 0 (**A**, **B**, **C**) or between sample groups (**D**).

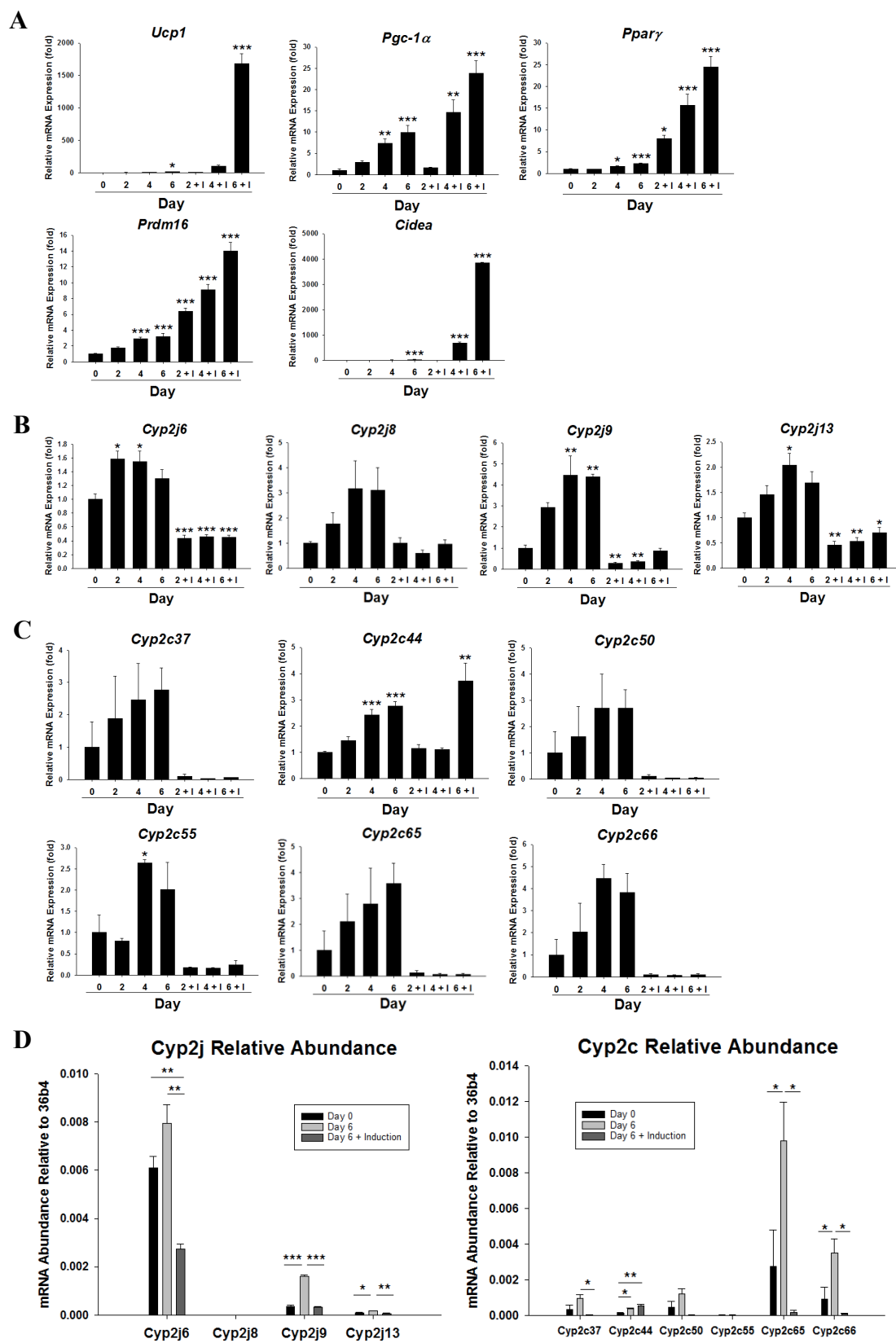
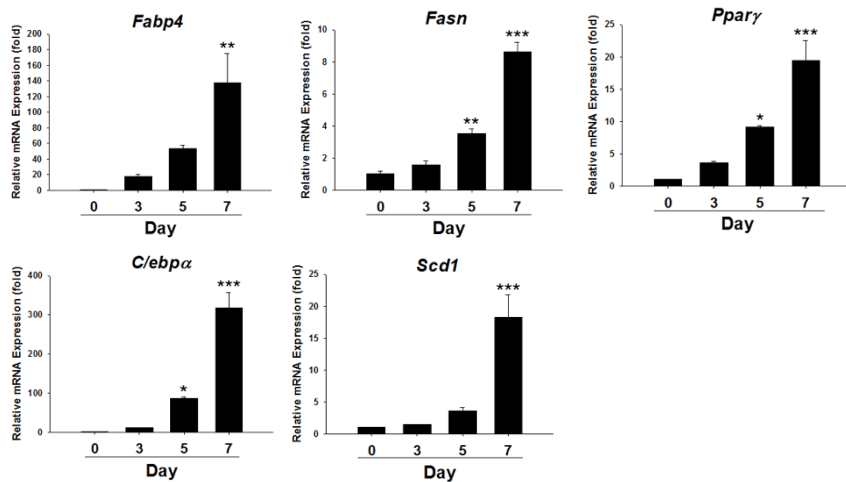


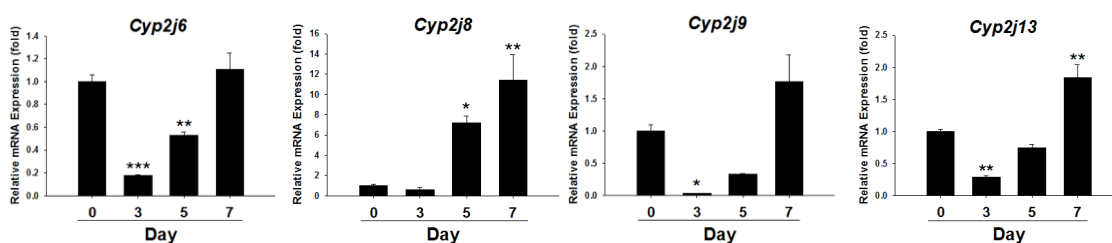
Figure 2. CYP epoxygenase expression in 3T3-L1 adipocyte differentiation

3T3-L1 preadipocytes were induced to differentiate for 7 days. Total RNA samples were collected before each media change on day 0, day 3, day 5, and day 7. mRNA expression was quantified relative to the loading control *36b4* and is displayed as fold of the day 0 samples (**A**, **B**, **C**). (**A**) Relative mRNA expression of adipocyte differentiation marker genes *Fabp4*, *Fasn*, *Ppar γ* , *C/ebp α* , and *Scd1*. (**B**) Relative mRNA expression of *Cyp2j* epoxygenase genes *Cyp2j6*, *Cyp2j8*, *Cyp2j9*, and *Cyp2j13*. (**C**) Relative mRNA expression of *Cyp2c* epoxygenase genes *Cyp2c37*, *Cyp2c44*, *Cyp2c50*, *Cyp2c55*, *Cyp2c65*, and *Cyp2c66*. (**D**) Total mRNA abundance of *Cyp2j* and *Cyp2c* genes on day 0 and day 7 of differentiation, relative to *36b4*. Data = Mean $\pm$ SEM (n=3). *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the day 0 sample.

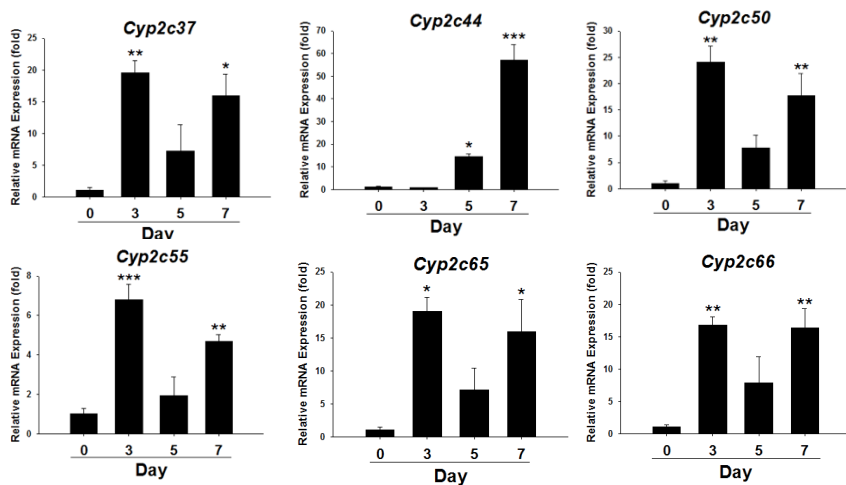
A



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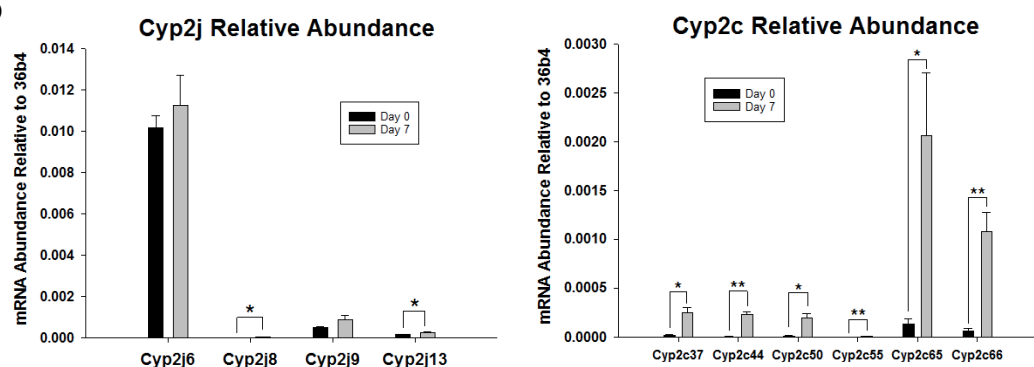


Figure 3. CYP epoxygenase expression in human brown adipocyte differentiation

Human brown preadipocytes were induced to differentiate for four weeks. Total RNA samples were collected on day 0, week 1, week 2, week 3, and week 4 of differentiation. mRNA expression was quantified relative to the loading control *36B4* and is displayed as fold of the day 0 samples (**A, B**). (**A**) Relative mRNA expression of brown adipocyte marker genes *UCP1*, *PGC-1 α* , and *PPAR γ* . (**B**) Relative mRNA expression of human CYP epoxygenase genes *CYP2J2*, *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19*. (**C**) Total mRNA abundance of CYP epoxygenase genes on day 0 and week 4 of differentiation, relative to *36B4*. Data = Mean $\pm$ SEM (n=3). *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the day 0 sample.

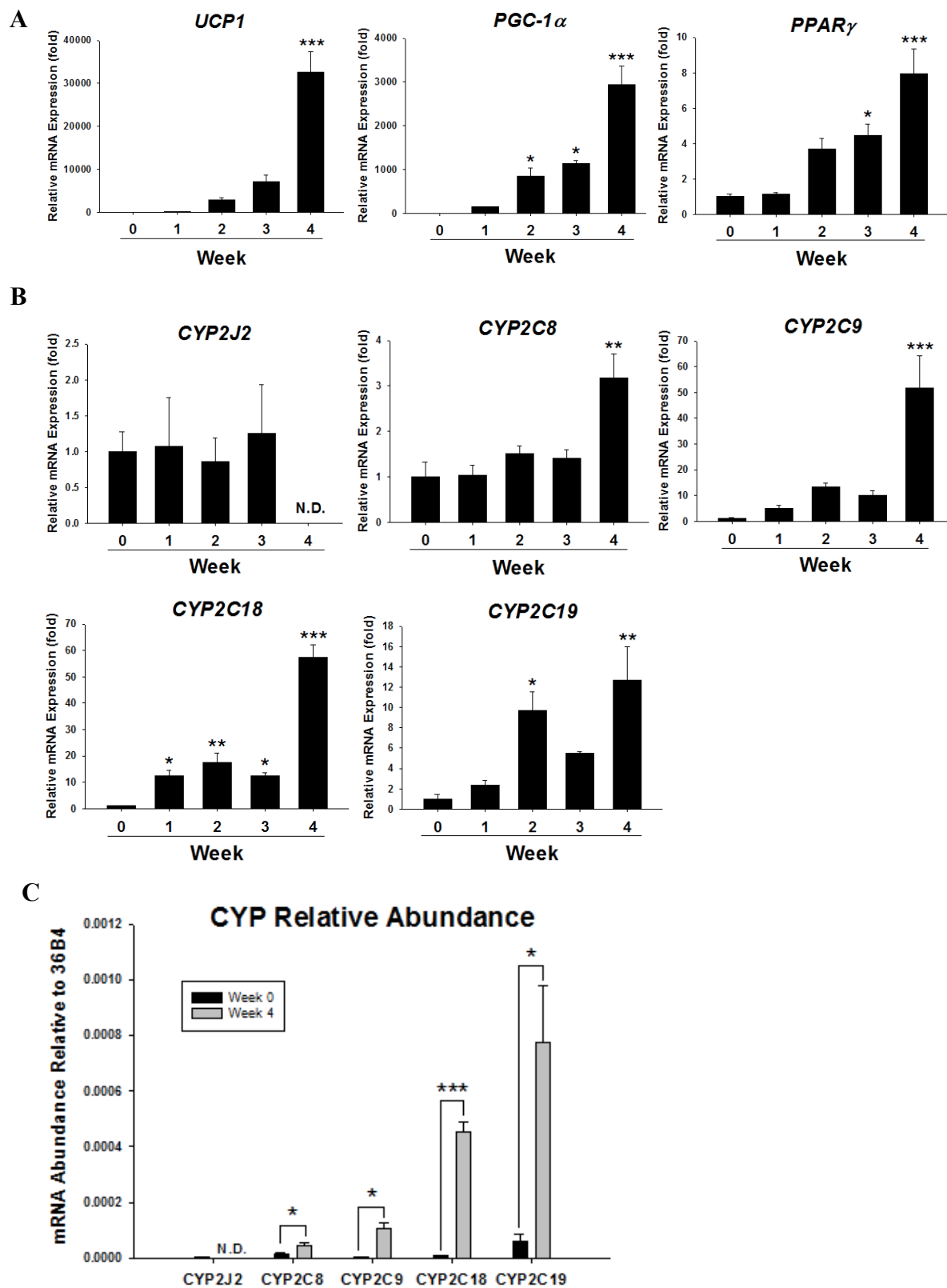


Figure 4. CYP epoxygenase expression in diet-induced obesity

Six-week old C57BL/6J mice were fed with either a high-fat diet (60% kcal from fat) or a regular chow diet for 20 weeks, before being sacrificed at 26 weeks of age. iBAT, eWAT, and iWAT were collected and total RNA was isolated. mRNA expression was quantified relative to the loading control *36b4* and is displayed as fold of the regular chow fed controls. **(A)** Relative mRNA expression of *Cyp2j* genes in iBAT (n=7). **(B)** Relative mRNA expression of *Cyp2c* genes in iBAT (n=7). **(C)** Relative mRNA expression of *Cyp2j* genes in eWAT (n=6). **(D)** Relative mRNA expression of *Cyp2c* genes in eWAT (n=6). **(E)** Relative mRNA expression of *Cyp2j* genes in iWAT (n=6). **(F)** Relative mRNA expression of *Cyp2c* genes in iWAT (n=6). Data = Mean $\pm$ SEM. *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the regular chow fed controls.

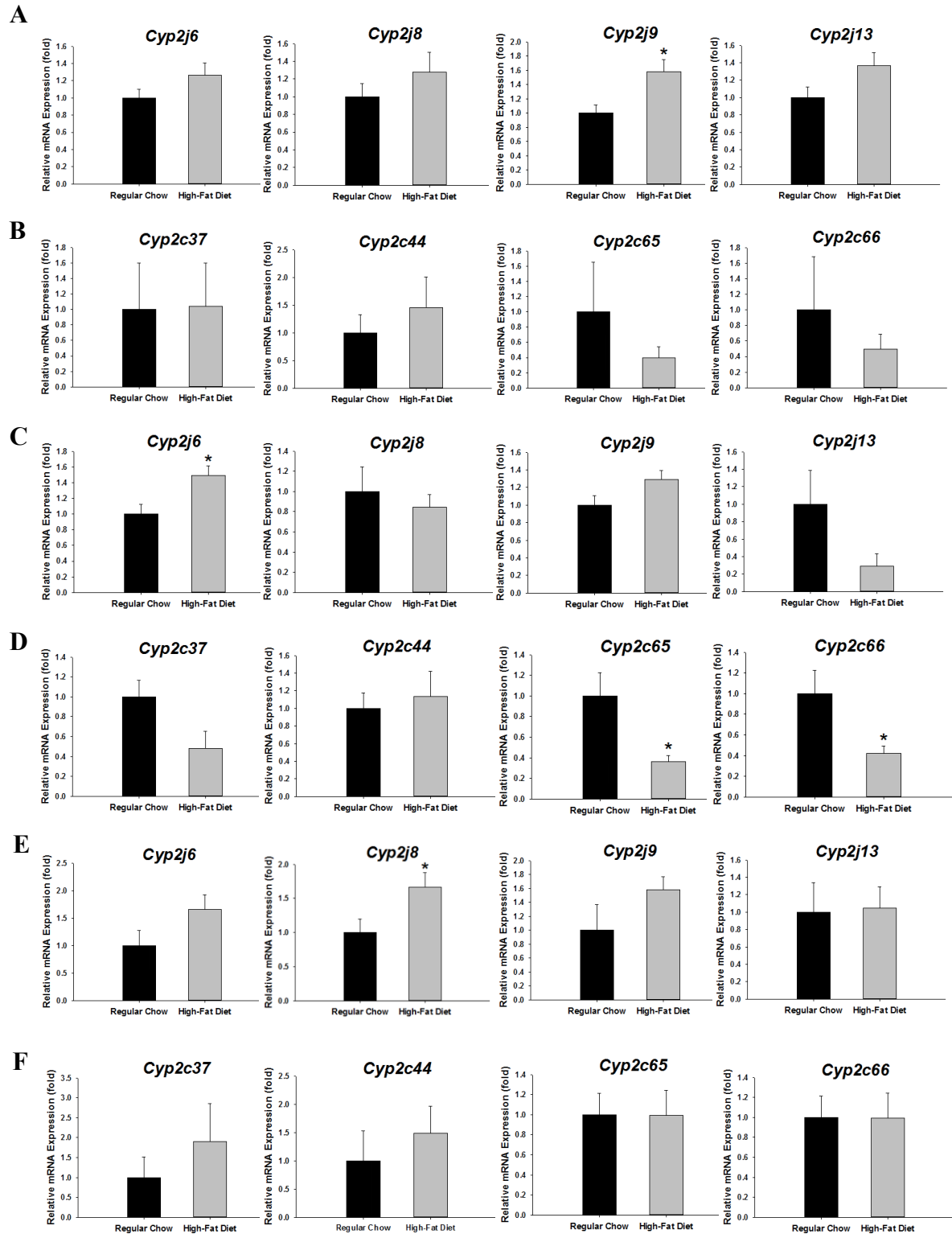


Figure 5. *Cyp2j13* overexpression suppresses murine brown adipocyte differentiation

Murine brown preadipocytes were stably transfected with the Myc-DDK-tagged *Cyp2j13* expression plasmid or the empty vector (pCMV6). Stably transfected cells were selected for two weeks with neomycin. Individual clones were selected and expanded to assess differentiation potential. Cells were stained and total RNA and protein samples were collected on day 6 of differentiation for analysis. mRNA expression was quantified relative to the loading control *36b4*. Densitometry of protein bands were normalized to the loading control ERK1/2 or non-specific bands. Data are displayed as fold of the empty vector controls. **(A)** Protein bands and densitometry for Myc-tagged CYP2J13 in the murine brown preadipocytes (n=1). **(B)** ORO stained cell morphology and ORO absorbance for the differentiated adipocytes (n=3). **(C)** Relative mRNA expression of *Cyp2j13*, *Ucp1*, *Pgc-1 α* , *Cidea*, *Ppar γ* , *Fabp4*, *Fasn*, and *Ppar α* in the differentiated adipocytes (n=6). **(D)** Protein bands and densitometry for UCP1, PGC-1 α , PPAR γ and ERK1/2 in the differentiated adipocytes (n=3). Data = Mean $\pm$ SEM. *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the empty vector controls.

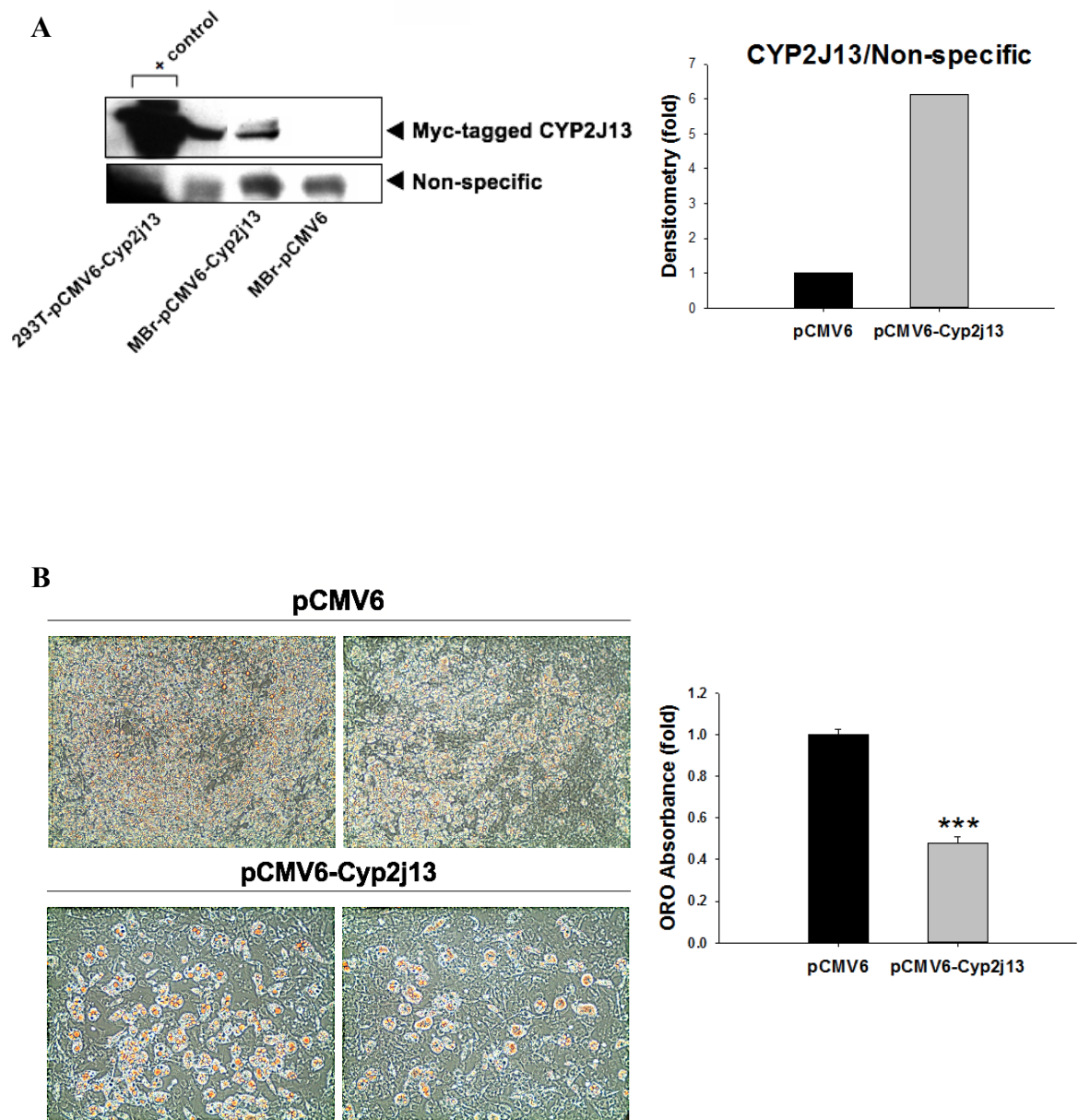
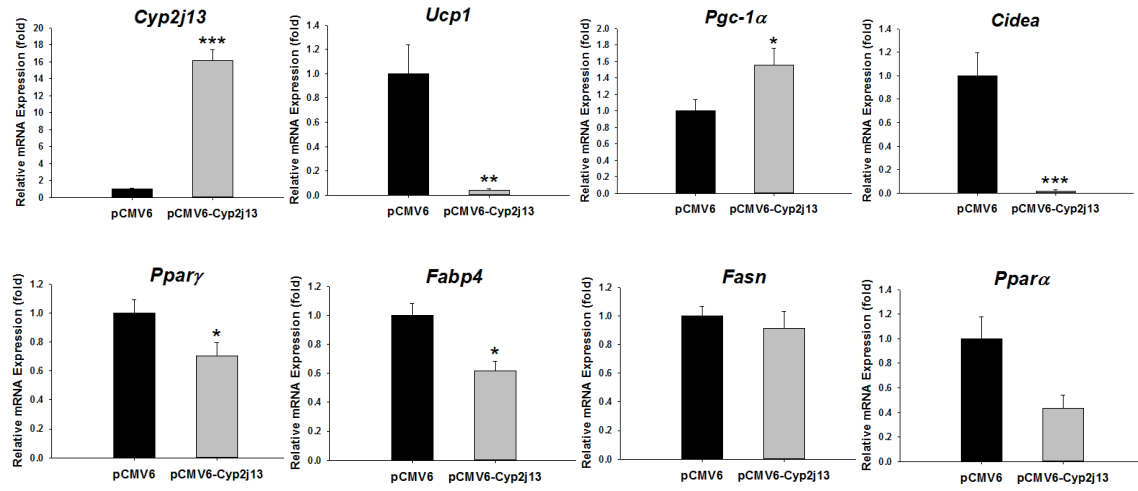


Figure 5 Continued

C



D

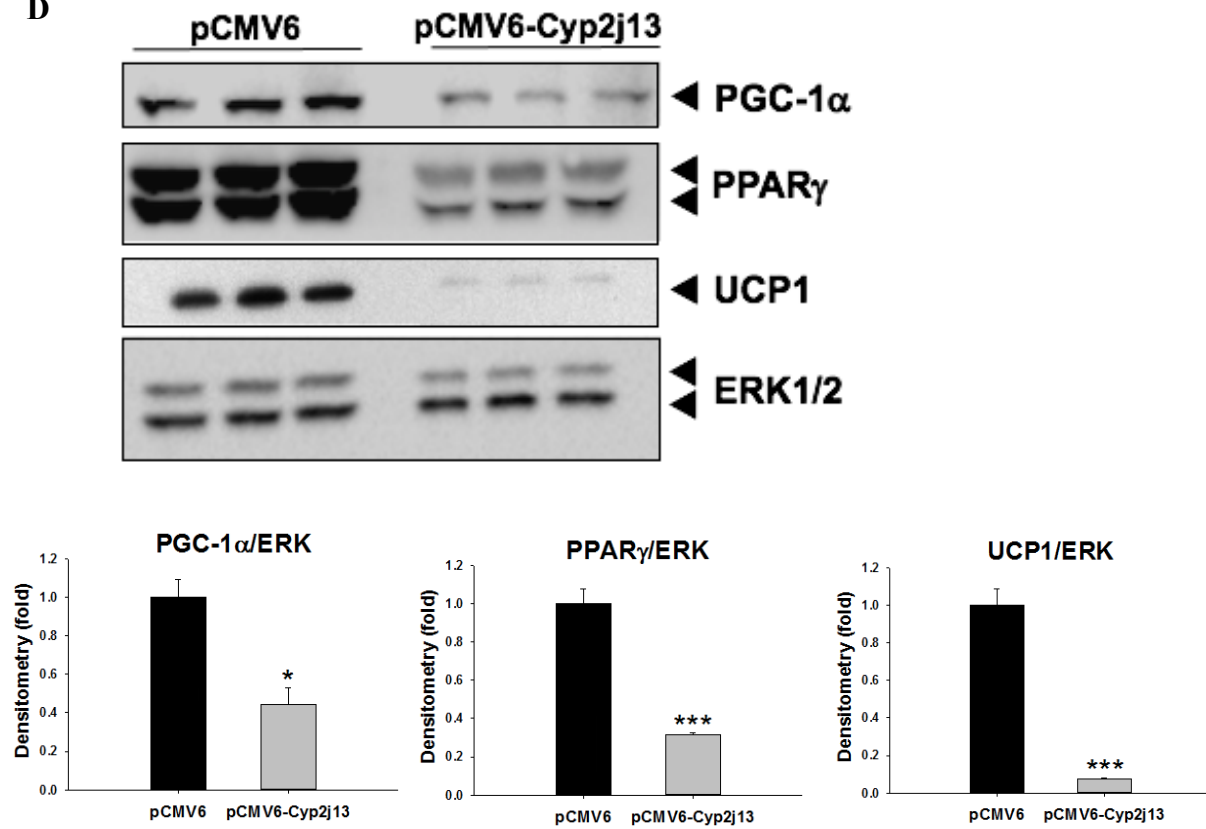
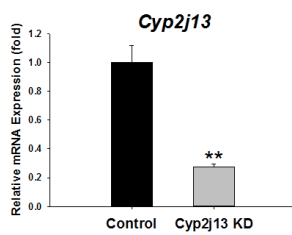


Figure 5 Continued

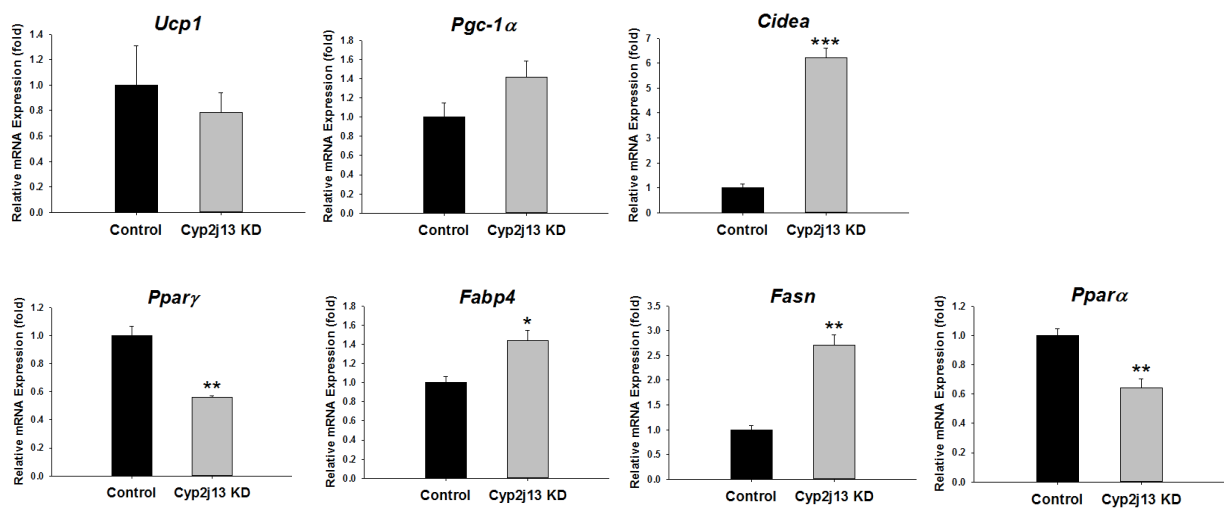
Figure 6. *Cyp2j13* knockdown enhances murine brown adipocyte differentiation

Murine brown preadipocytes were transduced with lentiviral particles against *Cyp2j13* or a scrambled non-targeting control. Stably infected cells were selected for two weeks with neomycin. Individual clones were selected and expanded to assess differentiation potential. Total RNA and protein samples were collected on day 6 of differentiation for analysis. mRNA expression was quantified relative to the loading control *36b4*. Densitometry of protein bands were normalized to the loading control ERK1/2. Data are displayed as fold of the non-targeting controls. **(A)** Relative *Cyp2j13* mRNA expression in the murine brown preadipocytes. **(B)** Relative mRNA expression of *Ucp1*, *Pgc-1 α* , *Cidea*, *Ppar γ* , *Fabp4*, *Fasn*, and *Ppar α* in the differentiated adipocytes. **(C)** Protein bands and densitometry for UCP1, PGC-1 α , PPAR γ , and ERK1/2 in the differentiated adipocytes. Data = Mean $\pm$ SEM (n=3). *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the controls.

A



B



C

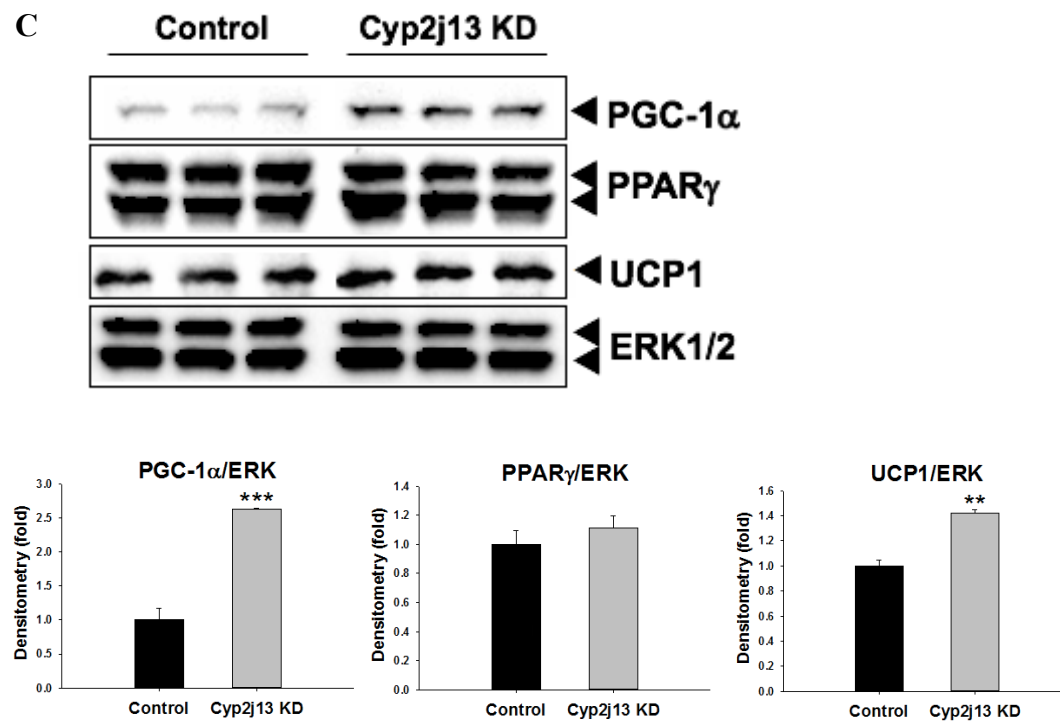


Figure 7. *CYP2J2* overexpression does not affect human brown adipocyte differentiation

Human brown preadipocytes were transfected with the *CYP2J2* expression plasmid or the empty vector (pSBtet). Stably transfected cells were selected for two weeks with puromycin. At 48 hours prior to confluency and at every media change thereafter, cells received either 1 $\mu\text{g/ml}$ doxycycline to induce stronger expression of *CYP2J2*, or the vehicle control (water). Total RNA and protein samples were collected on week 3 of differentiation for analysis. mRNA expression was quantified relative to the loading control *36B4*. Densitometry of protein bands was normalized to the loading control ERK1/2. Data are displayed as fold of the empty vector controls without doxycycline. **(A)** Validation of *CYP2J2* overexpression in human brown preadipocytes after 48 hours treatment with doxycycline or water. **(B)** Relative mRNA expression of *CYP2J2*, *UCP1*, *PGC-1 α* , *PPAR γ* , *FABP4*, *FASN*, and *PPAR α* in the differentiated human brown adipocytes **(C)** Protein bands for UCP1, PGC-1 α , PPAR γ , *CYP2J2* and ERK1/2 in the differentiated human brown adipocytes. Densitometry for UCP1, PGC-1 α , and PPAR γ is shown. Data = Mean $\pm$ SEM (n=3). *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the empty vector controls without doxycycline.

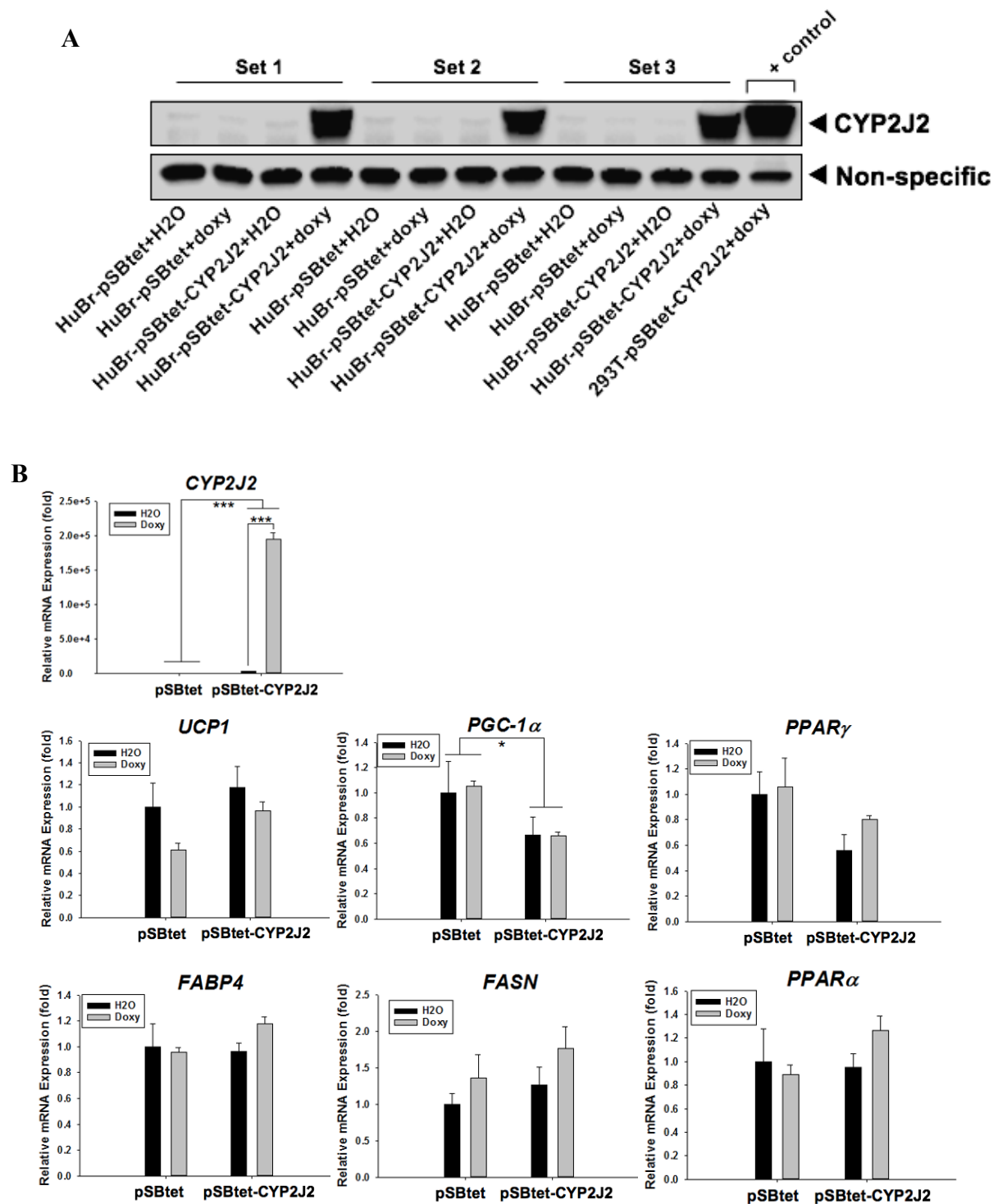


Figure 7 Continued

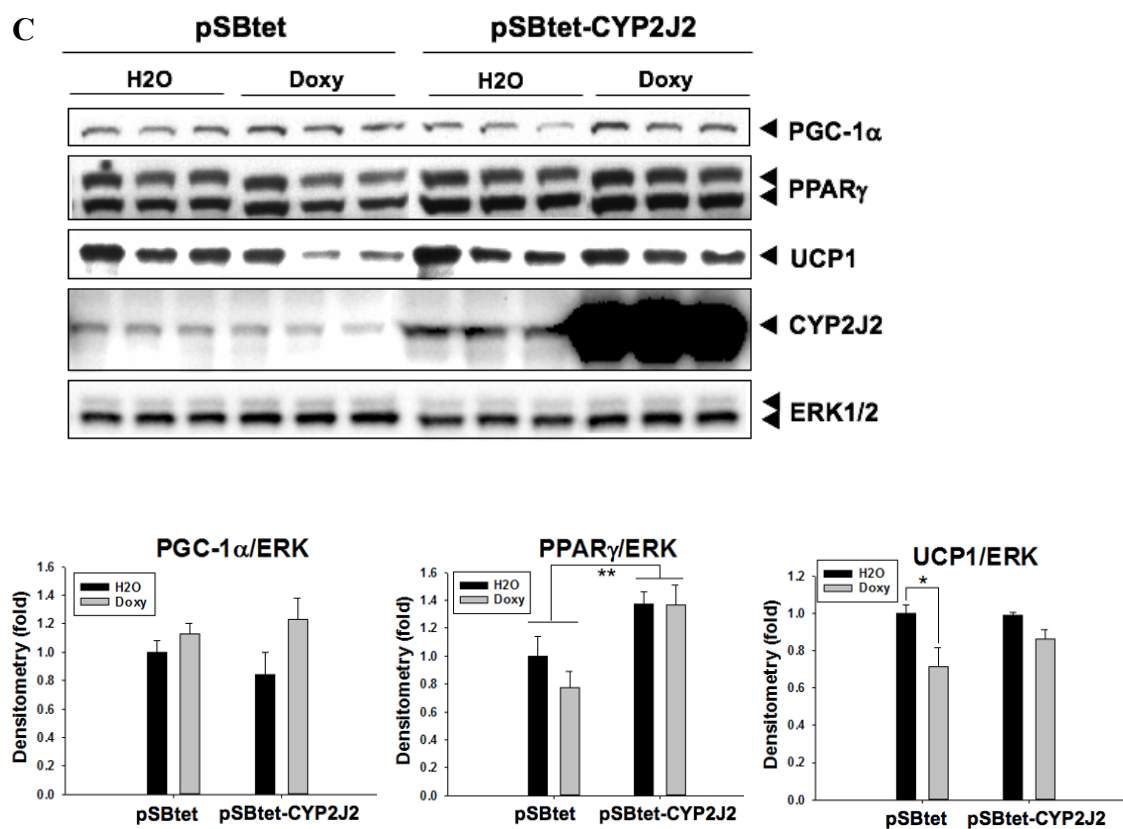


Figure 7 Continued

VITA

Katherine Marie Graham was born to Douglas and Christine Graham in Grosse Pointe Park, Michigan. In 2014, she graduated from Grosse Pointe South High School. She attended the University of Georgia where she obtained her bachelor's degree in Dietetics with a minor in Exercise and Sport Science in May 2018. Upon graduation, Katherine was accepted into the combined Master's/Dietetic Internship program with a concentration in Cellular and Molecular Nutrition at the University of Tennessee, Knoxville. She worked under the guidance of Dr. Ling Zhao to complete her thesis research. During her time at UTK, she acted as a teaching assistant for Nutrition 100. She completed her dietetic internship in July 2020. She defended her thesis on November 11, 2020. Following graduation, Katherine intends to sit for the Commission of Dietetic Registration exam and pursue a career as a Registered Dietitian Nutritionist.