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Pref-1 Expressing Cells in Embryonic Adipose Development and Adipose Expansion in Adults

by

Carolyn Suzanne Sayre Hudak

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Biochemical Nutrition

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor Hei Sook Sul, Chair
Professor Jen-Chywan (Wally) Wang
Professor Daniel Nomura
Professor Astar Winoto

Pref-1 Expressing Cells in Embryonic Adipose Development and Adipose Expansion in Adults
Carolyn Hudak

Abstract

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by

Carolyn Suzanne Sayre Hudak

Doctor in Philosophy in Molecular and Biochemical Nutrition

University of California, Berkeley

Professor Hei Sook Sul, Chair

Obesity has become a worldwide epidemic, which has lead to a surge of interest in therapeutics to combat this condition. Characterized by an increase in adipose tissue mass, obesity is associated with increased risk of a variety of detrimental diseases such as cardiovascular diseases and diabetes. Despite major advances in uncovering the root of this problem, the underlying basic biological process of adipose tissue development, as well as its ability to expand, is not well understood. To this end, many transcription factors have been identified to play a role in promoting the differentiation of precursor cells, or preadipocytes, into fully mature and lipid-filled adipocytes. Attempts have been made to identify early precursors of adipocytes, however the origin of these adipocytes are not clear, and classic lineage tracing has not been performed extensively. The aim of this dissertation work was to identify adipose precursor cells using the preadipocyte marker, Pref-1, to investigate adipose precursors during embryogenesis and the expansion of adipose tissue in adults.

Chapter 1 reviews the adipose tissue organ and the role of Pref-1 in the regulation of adipocyte differentiation. In addition to being the main energy storage organ, adipose tissue is now well recognized as an endocrine organ of the body, and there are a variety of hormones and cytokines that are secreted by adipose tissue for many physiological functions including energy homeostasis. Pref-1 is expressed prior to differentiation into adipocytes, and becomes extinguished during differentiation. Pref-1 activates MEK/ERK to upregulate Sox9 which binds to the promoter and blocks expression of several important transcription factors that promote adipocyte differentiation.

Chapter 2 focuses on understanding white adipose tissue development and the identification and characterization of adipose precursors. To investigate adipose tissue development, I generated transgenic mouse models to label and ablate Pref-1 expressing cells *in vivo*, with the overall goal of uncovering details of adipose development during embryogenesis, and diet induced expansion of adipose tissue in adults. Pref-1 expressing cells represent adipose

precursors that are mesenchymal in origin, and not endothelial cells or pericytes. These precursors are capable of division and are required for the expansion of the adipose tissue both during embryogenesis and during high fat diet induced obesity.

Chapter 3 focuses on another type of fat, brown adipose tissue (BAT), which, unlike white adipose tissue (WAT), burns fatty acids to perform non-shivering thermogenesis, and which was recently recognized to be present even in adult humans. Brown adipocytes have a different morphological and gene expression profile compared to white adipocytes, and can be identified by the unique expression of Uncoupling Protein-1 (UCP-1). UCP-1 acts to uncouple respiration from ATP production, resulting in heat production. I generated transgenic mice to label UCP-1 expressing cells in vivo, and to characterize these cells during cold induced or β3-adrenergic agonist induced expansion of BAT.

Chapter 4, concludes this body of work, and discusses future directions and remaining questions.

Thus, these studies provide a better understanding of both white and brown adipose tissue development and characterization of the precursors responsible for adipose tissue development, and may offer future therapeutic targets for obesity.

To my husband, the only one who truly understands me and makes me laugh every day.
And to my friends and family, for their love and support.

Table of Contents

List of Figures

List of Tables

Acknowledgements

First I would like to thank my advisor, Hei Sook Sul, for believing in me and pushing me to achieve my potential. Without her support and persistence, my graduate career would have been very different. I would also like to thank the members of my dissertation committee, Daniel Nomura, Wally Wang and Astar Winoto for their guidance and for sharing their expertise with me.

Thanks to my husband, Jason Hudak, who pushes me to get stronger both physically and mentally, and to improve myself and appreciate my natural abilities. He showed me that I can achieve any goal that I set my mind to, and how to strive to become the best version of myself that I can be. He is there to make me laugh even through the most frustrating and upsetting moments of my life, and believes in me more than I believe in myself.

I would not be who I am today without my parents, who taught me to be independent and pursue my dreams, and that with hard work and perseverance, I could become the kind of person I wanted to be, and to always strive to find the good in the world. My mother showed me how to believe in myself, and my father showed me that being smart was cool. Also, my grandparents, who paved the way for our family and taught me about sacrifice and hard work. Thank you to my brother, Zach Drew, for being genuinely excited that his sister became a scientist, and for always being proud of me and believing in me. And thank you to Bonnie Drew, for being amazing and to Lily Drew for astounding me with a level of adorableness that I didn't think possible. Thank you to my sister-in-law, Jocelyn Hudak, for teaching me how to have fun. I'd like to thank my Uncle David and Aunt Jill, without Uncle David, my Mom wouldn't be here, and thank you to my Aunt Jill for all her love and support. Thank to my wonderful cousins, Monica Putt, Aaron Putt, Jordan Putt, Andy Sayre, Eric Sayre, Sara Putt and Gwendolyn Putt for the joy you have brought to my life.

I want to thank my best friend growing up, Sarah (Rasmussen) Anderson, for showing me that no matter how much time goes by or how far apart we live, we can still catch up on our lives and know each other as if we had never been apart. Also, where would I be without the influence, positive energy, and outright awesomeness of Katherine Allen. She convinced me to join the rowing team in college, introduced me to my now husband, and showed me not to be afraid of my own physical strength, and that despite everyone taking advantage of your generosity, you can still have a smile on your face and be true to yourself.

I will never be able to give enough thanks to my (our) best friend, Maggie Brown, for her support, for sticking with us over the years, and for generally being amazing. I am so lucky to have had such a good friend to go through all the ups and downs of graduate school together with.

When I first began working on my thesis project, I received an immeasurable amount of help from Robin Duncan and Yuhui (Dawn) Wang. Without their expertise and incredible scientific skills, I would not have been able to do the very first experiments. I also worked with very talented graduate students in the lab, Maryam Ahmadian, and Roger Wong, who helped to shape the scientist I am today. I have been beyond lucky to work with people who I call friends,

CHAPTER I

PREF-1, A GATEKEEPER OF ADIPOGENESIS

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Pref-1, a Gatekeeper of Adipogenesis

Abstract

Preadipocyte factor 1 (Pref-1, also called Dlk1/ FA1) is a molecular gatekeeper of adipogenesis by maintaining preadipocyte state and preventing adipocyte differentiation. Pref-1 is made as an epidermal growth factor-like repeat containing transmembrane protein, and is cleaved by TNFα-converting enzyme (TACE) to generate a soluble form, which acts as an autocrine/paracrine factor. Pref-1 upregulates Sox9 expression by activating the ERK/MAPK pathway and the Pref-1 interaction with fibronectin is required for inhibition of adipogenesis. Pref-1 also prevents brown adipocyte differentiation and its thermogenic function. Here, we highlight the recent evidence for the role of Pref-1 in adipogenesis.

Introduction

Obesity, characterized by an increase in white adipose tissue (WAT) mass, has become a major health problem on an epidemic scale and is associated with increased risk of various diseases, such as cardiovascular diseases and diabetes. Throughout the lifespan, adipose tissue not only expands through hypertrophy of existing adipocytes, but also through hyperplasia of preadipocytes differentiating into adipocytes. Various factors that affect differentiation have been identified. However, despite major advances in adipose biology, the underlying processes and mechanisms for adipose tissue development and its expansion are not fully understood. In addition, with the recent evidence of the presence of functional brown adipose tissue (BAT) in adults, implicating its potential as a therapeutic target for obesity, attention has been focused on understanding the development of BAT, which dissipates energy, in contrast to WAT that serves as the major energy storage organ.

Adipocyte differentiation

Adipose tissue contains heterogeneous cell types. In addition to lipid filled adipocytes, adipose tissue contains the so-called stromal vascular fraction (SVF) composed of preadipocytes, macrophages, endothelial cells, and other fibroblast-like cells. Primary preadipocytes and preadipocyte cell lines, such as 3T3-L1 and 3T3-FT442A cells, undergo differentiation in the presence of adipogenic media containing dexamethasone (DEX), isobutylmethylxanthine (IBMX) and insulin and the differentiation process has been extensively studied at the transcriptional level. Intricate coordination of various transcription factors is required for differentiation. CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPδ are induced early during differentiation and activate peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα. PPARγ, is necessary and sufficient to promote adipocyte differentiation, and treatment with PPARγ agonists, thiazolidinediones, can promote differentiation in vitro.[1] Since the discovery of PPARγ, many additional transcription factors that play a role in adipocyte differentiation have been identified and have been extensively reviewed elsewhere.[2–7] Recent research has been devoted to identifying unique markers of preadipocytes or adipose progenitors. In this regard, ZFP423 has been reported to be critical for the preadipocyte commitment process.[8] And, PRDM16 has been identified as a major player in the differentiation of brown adipocytes.[9]

Not only does adipose tissue play a critical role in energy metabolism by being the major energy storage organ, it also secretes and responds to a variety of molecules involved in energy homeostasis, immunity and vascular function. The role of leptin and adiponectin, two cytokines

that are secreted by adipocytes and regulate energy homeostasis, have been well described elsewhere.[10,11] Inflammatory cytokines such as TNF-α and IL-6 are also secreted from adipocytes.[12] TNFα may help recruit mesenchymal precursors from other organs to the adipose tissue, which may then undergo differentiation to form new adipocytes.[13] We originally identified Preadipocyte factor-1 [Pref-1; also called Dlk1 (Delta-like protein-1), fetal antigen-1 (FA-1)] as a preadipocyte factor that prevents adipocyte differentiation and Pref-1 expression is decreased during differentiation to allow adipogenesis.[14–16] While many of the adipose related cytokines are secreted from adipocytes, Pref-1 is produced by preadipocytes and acts in an autocrine/paracrine fashion. This review highlights recent advances in the knowledge of the role of Pref-1 in the regulation of adipocyte differentiation and its signaling mechanism, as well as its effects on brown adipose tissue.

Pref-1 structure and signaling

Pref-1 is synthesized as a transmembrane protein that contains six EGF-like repeats at the extracellular domain.[17,18] These repeats maintain the conserved spacing to form three disulfide bonds from six cysteine residues.[19] Upon proteolytic cleavage by TNF-α converting enzyme (TACE), multiple soluble forms of Pref-1 are generated and the larger 50 kDa form is biologically active. Pref-1 cleavage by TACE can be inhibited by GM6001, a broad metalloproteinase inhibitor, indicating that Pref-1 cleavage is dependent on metalloproteinase activity, and this activity is enhanced by phorbol ester treatment, showing that Protein Kinase C may regulate Pref-1 cleavage. Though dexamethasone (DEX) treatment down-regulates Pref-1 expression, regulation of Pref-1 activity may also occur at the proteolytic cleavage step.[20]

We demonstrated that Pref-1 rapidly induces Sox9 expression through activation of the MEK/ERK pathway.[21] Thus, Sox9 constitutive overexpression can inhibit adipocyte differentiation. Downregulation of Pref-1 during differentiation coincides with increased C/EBPβ and C/EBPδ, which occurs prior to C/EBPα and PPARγ induction. Induction of Sox9 by Pref-1 during adipocyte differentiation is at the very early stage, and Sox9 acts to maintain cells in a preadipocyte state by suppressing transcription of C/EBPβ and C/EBPδ by directly binding to their promoter regions.We also found that Pref-1 not only inhibits adipogenesis, but promotes chondrogenic commitment.[21] In this regard, Sox9 has been known to function in chodrogenesis, and Sox9 ablation in mice results in the prevention of cartilage formation. We have observed that, like Sox9, ablation of Pref-1 causes malformation of skeletons in mice. Pref-1 causes inhibition of osteoblast differentiation, also via its induction of Sox9 that in turn prevents expression of an osteogenic transcription factor, Runx2. These similar in vitro and in vivo effects observed further demonstrate that physiological effects of Pref-1 are mediated via its induction of Sox9 expression.

The mechanistic details of the Pref-1 signaling pathway have been recently uncovered. Although it has been proposed that Pref-1 may function in the Notch signaling pathway due to the presence of EGF repeats,[22,23] Pref-1 lacks the Delta:Serrate:Lin-12 (DSL) domain that is required to mediate activation of Notch. Furthermore, we found that there is no direct interaction between Pref-1 and Notch, nor were there any changes in the expression of a downstream target of Notch, Hes-1, by Pref-1. Though the putative Pref-1 receptor is yet to be identified, we found that Pref-1 interacts with fibronectin.[24] The Pref-1 interaction with the C-terminal domain of fibronectin is necessary for the Pref-1 inhibition of adipocyte differentiation. Several growth factors are known to bind various regions of fibronectin for modulation of growth factor function. Furthermore, fibronectin was shown previously to prevent adipocyte differentiation.[25,26]

3

We found that knockdown of fibronectin or addition of Pref-1 interacting domains of fibronectin prevent the Pref-1 mediated activation of MEK/ERK and Sox9 induction, resulting in enhancement of adipocyte differentiation. Furthermore, knockdown of the α5 of the major integrin in preadipocytes, α5β1, or treatment with RGD peptide which blocks the fibronectin interaction, prevent the Pref-1 inhibitory action on adipocyte differentiation. Pref-1-fibronectin-integrin interaction activates the FAK/Src complex and Rho-like GTPases, which can then activate the ERK/MAPK pathway.[27] Overall, Pref-1 directly binds fibronectin and activates integrin signaling through ERK/MAPK to inhibit adipocyte differentiation.[24]

Role of Pref-1 during adipogenesis

As described above, Pref-1 was originally identified as an inhibitor of adipocyte differentiation and its effect on WAT development has been well documented in vitro and in vivo. Overexpression of Pref-1 in 3T3-L1 preadipocytes drastically lowers the degree of adipocyte differentiation, and knocking down Pref-1 greatly increases differentiation.[14,28] Furthermore, we have generated Pref-1 null mice as well as transgenic mice overexpressing Pref-1.[29–31] Pref-1 null mice have increased adiposity and also higher mRNA levels of adipocyte markers. On the other hand, mice overexpressing Pref-1 have decreased adipose mass and decreased adipocyte marker expression. As observed in lipodystrophy models, these mice also have hypertriglyceridemia, decreased glucose tolerance, and lower insulin sensitivity.[31] We can conclude that Pref-1 clearly affects adipogenesis.

The role of Pref-1 in BAT has not been well explored. As in WAT, however, it is known that Pref-1 is downregulated during BAT development, being present at a very high level in fetuses but decreasing to an undetectable level several weeks after birth.[32] Though BAT appeared to be normal prior to birth, Pref-1 ablated mice show altered BAT morphology after birth, with potential thermogenic over-activation, indicated by the induction of thermogenic markers and enhanced lipid mobilization. Conversely, in lipodystrophy mouse models, Pref-1 is expressed at a higher level in BAT, which is associated with loss of thermogenesis and increased lipid accumulation.[33] An indirect mechanism of this effect may be through the DEX-mediated induction of C/EPBδ, as was recently demonstrated byArmengol, *et al*.[32] Taken together, these results demonstrate that Pref-1 plays an important role in the regulation of both WAT and BAT differentiation.

DEX suppresses Pref-1 expression in preadipocytes allowing for the eventual induction in PPARγ expression and differentiation of preadipocytes into adipocytes. Though the adipocyte differentiation process has been well studied, little is known about the transitions steps from embryonic stem cells (ESC) or mesenchymal stem cells (MSC) to preadipocytes.[34] A potential role of Pref-1 in the commitment stage of adipogenesis has been revealed via the use of mesenchymal stem cells (MSCs). These pluripotent MSCs can differentiate into adipocytes upon treatment with IBMX and DEX. It has recently been reported that IBMX increases Pref-1 expression initially, [35] followed by a DEX-mediated decrease in Pref-1 expression, showing a transient rise in the Pref-1 level in MSCs at the commitment stage prior to adipocyte differentiation. Similarly, during differentiation of human fetal MSCs into adipocytes, Pref-1 expression level rose early in the differentiation process but decreased in the later stages. Furthermore, epinephrine treatment in mouse embryonic stem cells was reported to induce Pref-1 expression and cell proliferation. Interestingly, the Pref-1 level was increased at the commitment stage, indicating that epinephrine may induce cells to become committed to the adipocyte lineage. This effect could be reversed by treatment with antagonists of the Neuropeptide Y

(NPY) receptor.[36] These observations together suggest that commitment to the adipocyte lineage involves Pref-1 induction,[37] and Pref-1 may be used as a marker for precursors of adipogenesis.

The identity of adipose precursors remains an active area of research. Lineage tracing has often been used to understand the developmental origin of various cell types.[38–40] By using the PPARγ locus, Tang *et al* first showed that adipose progenitors express PPARγ, and they reside near the vasculature. [41] However, PPARγ is expressed not only in preadipocytes but also in differentiated adipocytes, which makes it tricky to discern adipose precursors. Regardless, other recent reports also indicate that adipocytes may come from endothelial cells or pericytes.[8,42,43] Adipocyte precursor cells were also identified in the adipose stroma using a combination of fluorescence-activated cell sorting. These cells were found to have a unique cell surface signature, including the presence of CD34, Sca-1, and CD24.[44] And, we recently employed Pref-1 labeling to identify and characterize adipose precursors throughout development, as well as during diet induced expansion of WAT (Hudak and Sul, unpublished results).

Regulation of Pref-1 expression during adipogenesis

Pref-1 is part of the imprinted gene cluster, Pref-1-Dio3.[45,46] Ten imprinted genes have been identified in this cluster. There are three paternally expressed genes, Pref-1, Paternally Expressed Gene 11 [Peg11, also called Retrotransposon-like 1(Rtl1)] and type 3 deiodinase (Dio3) , and seven maternally expressed genes coding for noncoding RNAs.[46,47] Interestingly, Pref-1 is located in the same imprinted region as *Dio3,*[48] an inhibitor of T3, and changes in *Dio3* expression levels during differentiation parallel that of Pref-1. Thyroid hormone is critical for BAT function and thermogenesis,[49,50] and T3 can stimulate brown adipocyte differentiation.[51] Accordingly, DEX can suppress expression of both Dio3 and Pref-1 in cultured preadipocytes. In this regard, high levels of Dio3 have been detected in differentiating primary brown preadipocytes. Together, the coordinated expression of Pref-1 and Dio3 further implicate the important role they may play during brown adipocyte differentiation.[52] Not only is DNA methylation important in the regulation of Pref-1 expression, but modification of histones also plays a role in expression of these imprinted genes. With regard to Pref-1 repression during adipogenesis, a recent study revealed that a decreased level of H3 K4 tri-methylation[53] leads to de-repression of Pref-1, and the Pref-1 locus exhibits lower levels of active chromatin makers, such as H3 K4 tri-methylation as well as H3 and H4 acetylation in 10T1/2 cells compared to committed 3T3-L1 preadipocytes. Furthermore, the Pref-1 gene showed a predominant enrichment of H3 K27 tri-methylation, demonstrating that these repressive marks play a role in silencing Pref-1 gene expression in 10T1/2 cells.[54,55]

Regulation of adipogenesis by certain growth factors has been reported to be through modulating Pref-1 levels. Thus, growth hormone has been previously reported to maintain high levels of Pref-1 in 3T3-L1 cells to prevent differentiation of preadipocytes.[56,57] Furthermore, growth hormone stimulates production of Insulin-like growth factor 1 (IGF-1), which is reduced by Pref-1 treatment, showing the ability of Pref-1 to regulate growth hormone levels.[58] It has also been suggested that IGF-1 may be able to bypass the inhibition of Pref-1 on adipocyte differentiation of 3T3-L1 cells.[59] Interestingly, in human patients with anorexia nervosa, there was an increased level of Pref-1, which correlated to an increased number of undifferentiated cells with a corresponding decrease in BAT mass.[60] Furthermore, these patients have low gonadal steroids and treating adolescent female anorexia nervosa with estradiol caused a significant decrease in Pref-1 levels.[61] In postmenopausal women, estrogen deficiency leads to increased serum levels of Pref-1, which is associated with bone loss, and could be normalized

with estrogen replacement therapy.[62] Overall, there seems to be evidence of estrogen regulation of Pref-1 expression, which plays a role in regulating homeostasis of adipocyte as well as osteocyte abundance.

Conclusion

Obesity is characterized by increased adipose tissue through both hypertrophy and hyperplasia, and much interest has been garnered to understand the regulation of this process. In this regard, Pref-1 has been used as a preadipocyte marker, and is notable in its ability to prevent adipocyte differentiation in an autrocrine/paracrine manner. Pref-1interacts with fibronectin and activates MEK/ERK to induce Sox9 expression, which blocks adipocyte differentiation by binding to the promoter regions of C/EBPβ and C/EBPδ and preventing activation of C/EBPα and PPARγ. Several hormones and other cytokines have been suggested to either regulate or be regulated by Pref-1, further suggesting the critical role of Pref-1 in the maintenance of the preadipocyte state. Pref-1 expression transiently increases early during differentiation of mesenchymal stem cells. Pref-1 may be a valuable tool as a marker of precursor cells and we are employing Pref-1 to label adipose precursors for lineage tracing and investigation of adipose tissue development. Additionally, given that imprinted genes function in fetal development and growth and with its effects on chondrogenesis and osteogenesis, Pref-1 most likely functions beyond the control of adipogenesis and mesenchymal cell fate. Uncovering the Pref-1-fibronectin interaction has begun to shed light on the molecular mechanism underlying the function of Pref-1 and Pref-1 signaling in adipogenesis.
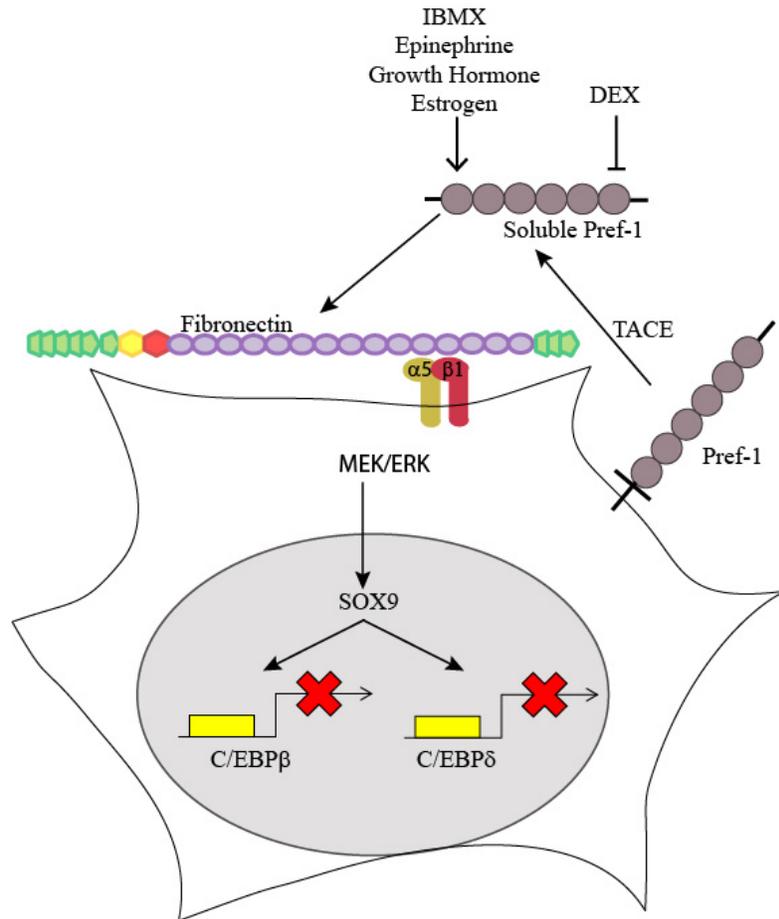
Figure 1. Pref-1 inhibition of adipogenesis. Membrane bound Pref-1 is cleaved by TACE to generate a 50 kDa soluble factor. Pref-1 interacts with fibronectin and via integrin α5β1 activates MEK/ERK, which increases Sox9, which then binds to the promoter regions of C/EBP β/δ to block adipocyte differentiation. Dexamethasone has been shown to decrease Pref-1 expression while IBMX, epinephrine, growth hormone and estrogen have been shown to increase Pref-1 expression.

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CHAPTER II

PREF-1 MARKS EARLY MESENCHYMAL PRECURSORS REQUIRED FOR ADIPOSE TISSUE DEVELOPMENT AND EXPANSION

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INTRODUCTION

Obesity has become a major health problem of epidemic scale, but the basic biological process of adipose tissue development and expansion is not well understood. Adipose tissue is organized into discrete depots; in rodents, white adipose tissue (WAT) is found in subcutaneous areas such as the inguinal depot, visceral areas such as epididymal depot, as well as brown adipose tissue (BAT) in the interscapular region.[1–4] While BAT dissipates energy as heat for thermogenesis, WAT is specialized for energy storage and releases fatty acids for other organs to use in times of energy shortage. WAT is generally thought to develop postnatally, [5,6] and upon excess energy intake, it can increase in mass by hypertrophy and hyperplasia throughout the lifespan. [7,8] Adipose stem cell populations have been isolated from the stromal vascular fraction (SVF) of adipose tissue and have been characterized using various markers. [9] It has been proposed that within WAT, progenitors with adipogenic potential may reside near the vasculature [10] and that they may be of endothelial and/or pericyte origin. [11] However, the definitive origin of adipocytes is yet to be understood. Furthermore, the identity, location, and emergence of adipose tissue early during development, are not known.

We originally identified Preadipocyte factor-1 (Pref-1, also called Dlk1) as an inhibitor of adipocyte differentiation.[12–15] Pref-1 is synthesized as a transmembrane protein that contains EGF-repeats at the extracellular domain.[16,17] Upon cleavage by TACE, Pref-1 generates a soluble factor which is biologically active and activates MEK/ERK to upregulate Sox9.[9,18–22] In this regard, the role of PPARγ, C/EBPs as well as other transcription factors in adipocyte differentiation have been extensively studied.[23–25] Also, ZFP423 has been reported to be critical for the preadipocyte commitment process. [26]

Adipose tissue has been presumed to form postnatally, but the location of the early precursors and the developmental process are not known. Lineage tracing has often been used in other systems, [10,27–30] and by using the PPARγ locus, Tang *et al* first showed that adipose progenitors express PPARγ and they reside near the vasculature.[10] In this regard, PPARγ is expressed not only in preadipocytes but also in differentiated adipocytes, which makes it tricky to discern adipose precursors. Furthermore, recent reports indicated that adipocytes may come from endothelial cells or pericytes.[11,26,31] Here, by marking or eliminating Pref-1 expressing cells, we show that adipose precursors are not endothelial cells or pericytes, but mesenchymal cells and, during embryogenesis, adipose tissue is formed from these precursors, first appearing in the mesenteric region at E10.5 to develop into adipocytes at E17.5. We also show Pref-1 expressing precursors are required for embryonic adipose tissue development as well as its expansion in adults.

RESULTS

Generation of Transgenic Mouse Models for Labeling or Deletion of Pref-1 Expressing Cells

Various proteins have been suggested as markers of adipogenic progenitors, but many of these markers are also expressed in other tissues. Pref-1 provided us with a unique tool to study

the origin and development of adipose tissue, because Pref-1 is mainly found in adipose tissue in adults and is not expressed in mature adipocytes. To mark and perform lineage analyses of Pref-1 expressing cells in an inducible manner, we first engineered Pref-1-reverse tet transactivator (rtTA) transgenic mice expressing rtTA under the control of the -6 Kb Pref-1 promoter (Figure 1A).[13,32] Although both tTA and rtTA are used in a variety of mouse models,[33] we chose the rtTA system due to its higher induction in response to tetracycline compared to the tTA system. Upon co-transfection of -6 Kb Pref-1 promoter-rtTA and TRE-Luc constructs, treatment of cells with a tetracycline analog, Doxicycline (Dox) increased luciferase activity by 9-fold. We obtained similar results using GFP as a reporter also.
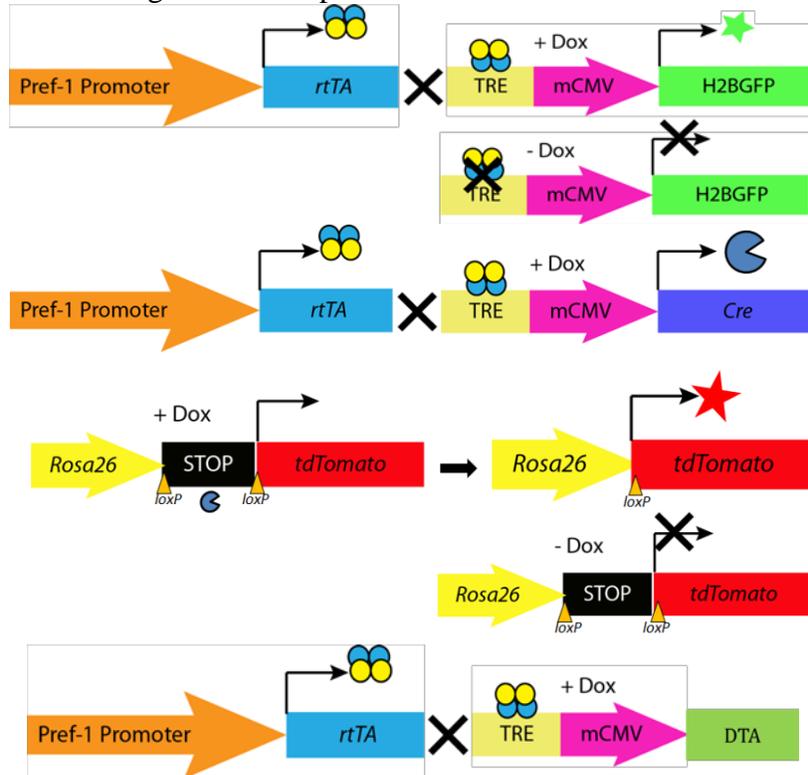
Figure 2. Generation of mouse models to label or delete Pref-1 expressing cells. Pref-1-GFP mice were generated by inserting reverse tet-transactivator (rtTA, Dox On) directly following -6Kb of the Pref-1 promoter, whereby the Pref-1 promoter drives rtTA expression and subsequent TRE-H2BGFP fluorescence transiently labels Pref-1 expressing cells only in the presence of Dox. For indelible and inducible lineage tracing, the Pref-1-rtTA and (TRE)-Cre transgenic lines were crossed with a ROSA26-flox-stop-flox-tdTomato (tdTomato), a knock-in line that contains tdTomato 3' of a floxed transcriptional stop cassette. Cre activation permanently excises the transcriptional stop and tdTomato irreversibly marks Pref-1 expressing cells and all descendants. In the absence of Dox, the rtTA protein cannot bind to the TRE, the stop remains intact, and tdTomato is not expressed. Pref-1-DTA mice were generated by crossing Pref-1-rtTA mice with a Dox inducible TRE-DTA mouse line.

We then generated three different Pref-1 mouse models using our Pref-1-rtTA transgenic mice. For the Pref-1-GFP mouse model, a tet-responsive histone 2B (H2B) green fluorescent protein (GFP) [tetracycline response element-H2BGFP (TRE-H2BGFP)] [34,35] was introduced into the Pref-1-rtTA line, thus creating the Pref-1-GFP mice whereby the Pref-1 promoter directs expression of GFP localized in the nucleus. In this transient labeling mouse model, GFP is stable in postmitotic cells but becomes diluted in proliferating cells upon removal of Dox. For

permanent labeling of Pref-1 expressing cells, we introduced Pref-1-rtTA and TRE-Cre into the ROSA26-flox-stop-flox-tdTomato knock-in line.[36] In this permanent labeling, Dox treatment causes expression of tdTomato in the cytoplasm of Pref-1 expressing cells and tdTomato expresses in all of the progeny even after cell division. We also developed a transgenic mouse model to deplete Pref-1 expressing cells by crossing our Pref-1-rtTA mice with the TRE-DTA mouse line for expression of cytotoxic diphtheria toxin A chain (DTA) in an inducible fashion to examine their role in adipose development and expansion (Figure 2).[37]

First, to test the efficacy of our mouse models, we compared the expression of the fluorescent reporters in adipose tissue of the Pref-1-GFP and Pref-1-tdTomato mice upon Dox treatment (Figure 3). Laser scanning confocal (LSC) microscopy of adipose tissue sections showed the presence of GFP positive cells only in Dox-treated, but not untreated animals. GFP positive cells were not detected in control GFP only mice even in the presence of Dox. GFP positive cells could easily be detected by fluorescence microscopy even in whole mount adipose tissue from Dox treated mice (data not shown). Similarly, tdTomato was detected in WAT sections from Dox treated mice. However, TdTomato was not detected in non-treated control Pref-1-tdTomato mice, or in Dox treated control mice lacking the Pref-1-rtTA allele.
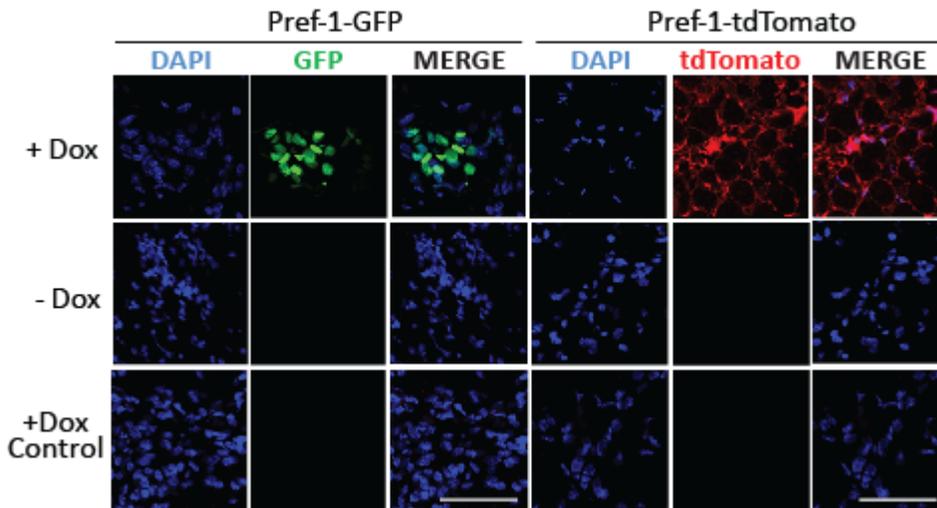
Figure 3. Pref-1-GFP mice or Pref-1-tdTomato mice were treated with or without Dox and TRE-H2BGFP or Rosa26-tdTomato control mice were treated with Dox from E0 to P21 and inguinal and epididymal WATs were excised, cryosectioned, and examined for GFP (green) or tdtomato (red). GFP and tdTomato were not detected in the absence of Dox or in control adipose tissue. Scale bars = 200 μm.

Next, to examine if expression of GFP and tdTomato in our mouse models can mimic endogenous Pref-1 expression, we first measured the mRNA levels of Pref-1-rtTA in various tissues by RT-qPCR. Similar to endogenous Pref-1 expression, Pref-1-rtTA was not expressed in other tissues but was highly expressed in WAT and to a lower level in BAT in adult mice, demonstrating that rtTA expression driven by the -6 Kb Pref-1 promotor mimics endogenous Pref-1 expression. We next examined GFP and tdTomato fluorescence upon cryosection of WAT from Pref-1-GFP and Pref-1-tdTomato mouse models, respectively. In Pref-1-GFP mice, Dox was administered throughout embryogenesis until 3 weeks of age before various tissues were harvested for sectioning. In Pref-1-tdTomato mice, Dox was given at birth, since we previously reported that Pref-1 is found in a variety of tissues during embryogenesis but it is restricted to adipose tissue after birth. Indeed, GFP fluorescence in the nucleus was detected in 30-40% of

total cells observed by DAPI staining in WAT, and a lower percentage of cells were GFP positive in BAT. In Pref-1-tdTomato mice, more than 90% of the cells were tdTomato positive in WAT, and, similar to Pref-1-GFP mice, much fewer tdTomato positive cells were detected in BAT. Overall, we detected GFP and tdTomato only in adipose depots, including inguinal, epididymal, and interscapular BAT, but not in other tissues such as liver, kidney, and muscle (Figure 4). These data show that Pref-1 expressing cells are restricted to adipose tissues.
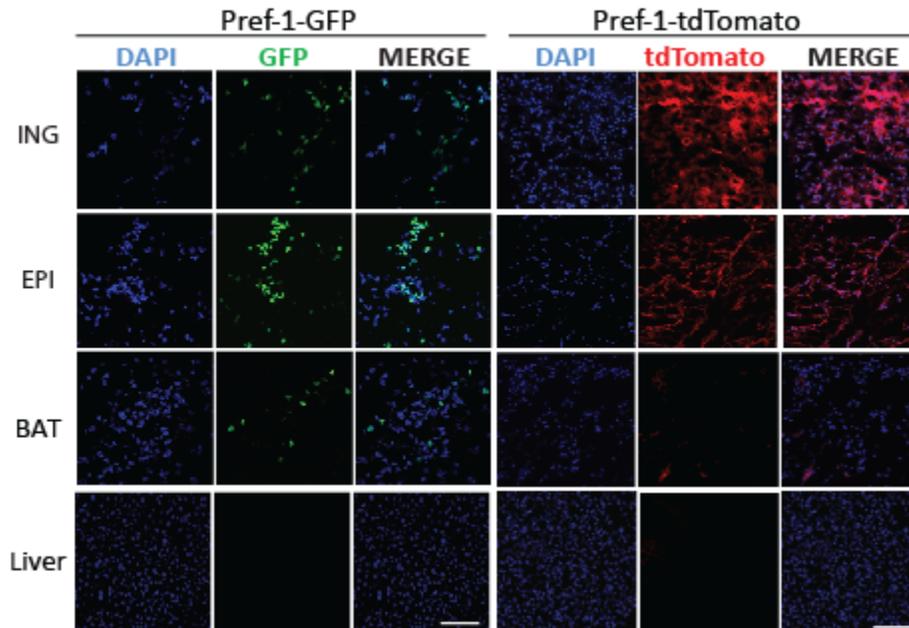
Figure 4. Pref-1-GFP and Pref-1-tdTomato mice were Dox treated from P1 to P21 and various tissues were cryosectioned and imaged for GFP (green) or tdTomato (red). Scale bars = 200 μm.

Since Pref-1 is not expressed in adipocytes, we next compared the expression of Pref-1-rtTA and GFP between SVF and the adipocyte fraction of inguinal and epididymal WAT from Dox treated mice (Figure 5). As expected, Pref-1 mRNA was specifically detected in the SVF only, whereas FAS was expressed in the adipocyte fraction only. Similar to the Pref-1 expression pattern, Pref-1-rtTA levels were much higher in the SVF compared to the adipocyte fraction and GFP also showed a similar pattern. The expression pattern of Pref-1-rtTA and GFP we observed clearly show that the -6 Kb Pref-1 promoter is sufficient to mimic tissue-specific and developmental pattern-specific expression of the Pref-1 gene.

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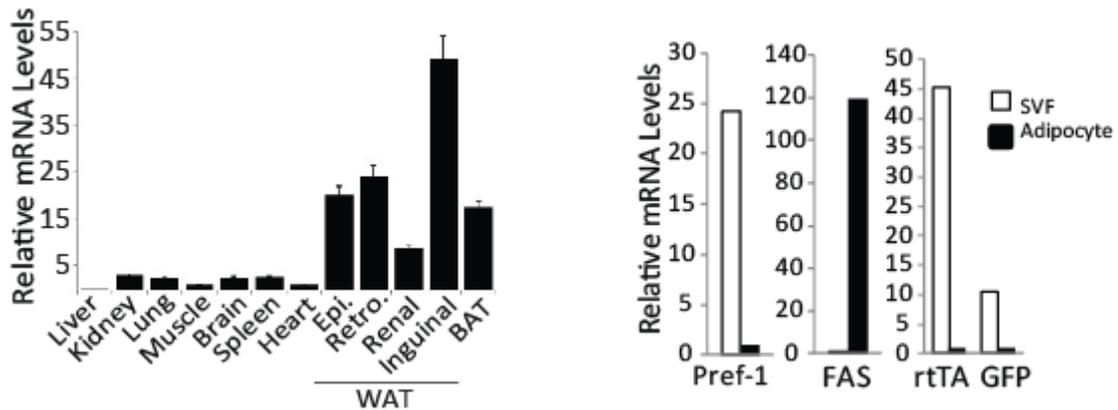
Figure 5. (Left) Total mRNA from various tissues of Pref-1-rtTA mice was used for RT-qPCR. rtTA was not expressed in non-adipose tissues. (Right) Pref-1-GFP mice were treated with Dox from E0 to P21 and WAT was dissected, fractionated by centrifugation, and total mRNAs were extracted from the SVF and adipocyte fractions; mRNA levels were determined by RT-qPCR.

By single cell mRNA analysis, we further verified that GFP expression mimics endogenous Pref-1 expression (Figure 6). Cells of SVF from Dox treated Pref-1-GFP mice were plated in microwells so that each well contained a single cell. The cells were labeled by DAPI and GFP fluorescence. We found that approximately 50% of the total cells stained by DAPI were GFP positive also. We measured Pref-1 mRNA level *in situ* using Taqman (VIC) probes and found that those cells that were positive for GFP fluorescence precisely overlapped with those that expressed Pref-1 (Figure 7).[41] Taken together, these results clearly demonstrate that reporter expression driven by the -6 Kb Pref-1 promoter faithfully recapitulates endogenous Pref-1 expression and that these mice are valid mouse models for characterizing Pref-1 expressing cells to study adipose tissue development.
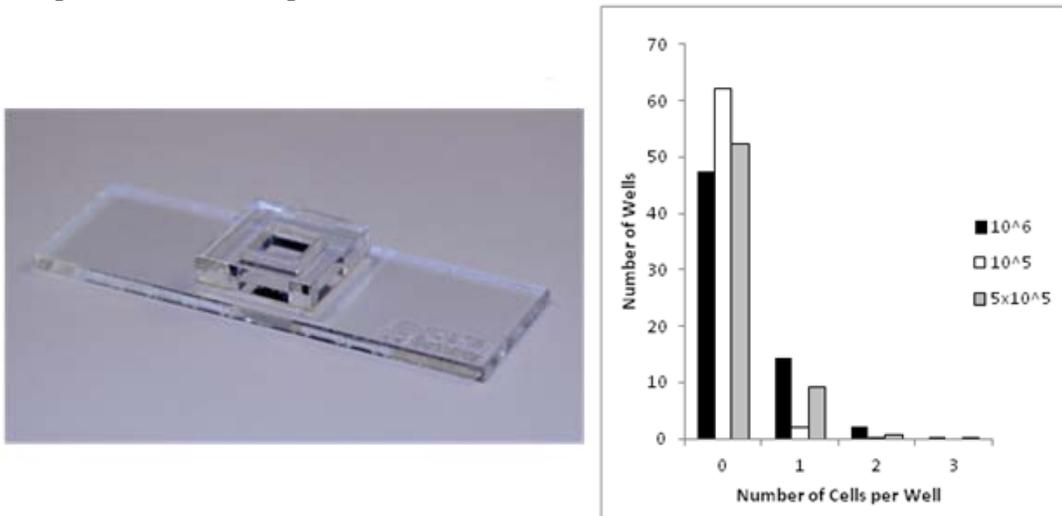
Figure 6. Multi-well devices were designed and adhered to a glass slide for cell plating. Cells were suspended and placed directly onto the device. Cells were diluted to indicated concentrations to maximize the number of cells that contained only one cell per well. The concentration of 5x10^5 was used for all single cell experiments.
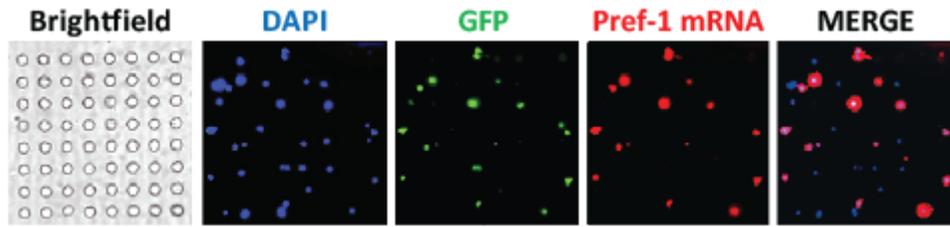
Figure 7. Analysis of Pref-1 mRNA at the single cell level demonstrated expression of Pref-1 specifically in GFP positive cells.

Pref-1 Expressing Cells are of Mesenchymal, but not Endothelial or Pericyte, origin

Recently, it has been reported that adipose precursors may be localized near the vasculature and that they may be of an endothelial or pericyte origin. We therefore examined the Pref-1 expressing cells for various endothelial or pericyte markers. First, we employed GFP positive cells from fluorescence activated cell sorting of the freshly isolated SVF of WAT of Dox-treated Pref-1-GFP mice. We compared gene expression levels of various markers between GFP positive and GFP negative cells by RT-qPCR (Figure 8). As expected, the GFP mRNA levels were greatly higher in sorted GFP positive cells compared to GFP negative cells. Similarly, Pref-1 expression was greatly higher in GFP positive cells, and Pref-1 was barely detectable in GFP negative cells. However, we did not detect a significant expression of any of the endothelial markers we tested, including VE-cadherin, PDGFRβ, CD31 or αSMA in GFP positive cells. Nor did we detect significant expression of pericyte markers, such as CD146 and CD90, or the hematopoetic marker, CD45. Importantly, expression of the mesenchymal marker, CD105, was not detectable in GFP negative cells, but was markedly high in GFP positive cells. Also, Sox9 expression was significantly higher in GFP positive cells compared to GFP negative cells. Similarly, expression of CD34, which was shown to mark adipose progenitors by sorting of SVF cells, was significantly higher in GFP positive cells.[38] GFP positive cells also expressed CD24, which has been reported to represent adipose precursors with higher proliferative capacity, but CD24 was not detectable in GFP negative cells.
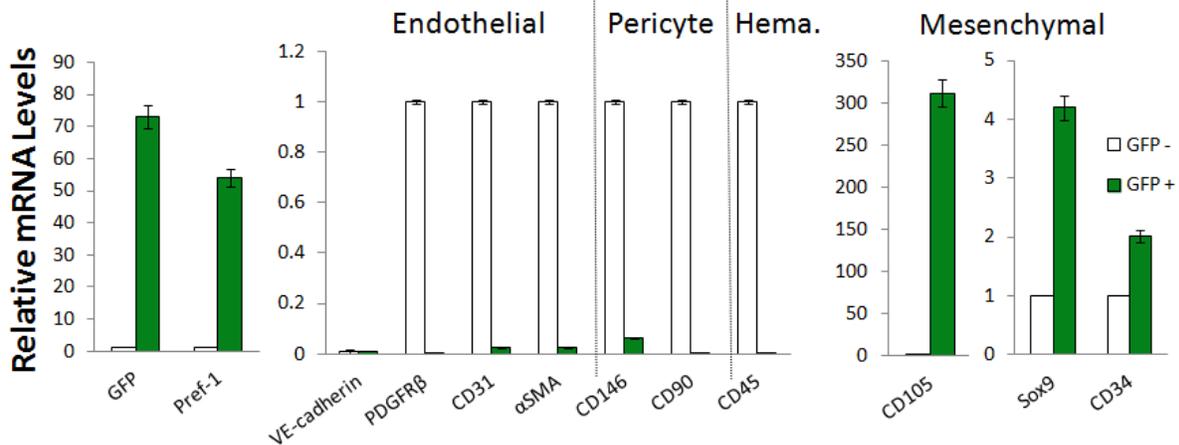
Figure 8. GFP positive and negative cells were sorted via FACS from freshly isolated SVF from WAT of Dox treated Pref-1-GFP mice, total mRNA was extracted and expression levels of various endothelial, pericyte, hematopoietic and mesenchymal markers were measured.

Next, by immunostaining of cryosections and LSC microscopy, we examined a variety of markers for colocalization with GFP positive cells employing Pref-1-GFP mice at E19.5, a time point when an abundance of GFP positive cells were detectable. As expected, GFP positive cells were colocalized with Pref-1 staining, indicating that these cells expressed Pref-1. However, GFP positive cells did not colocalize with endothelial markers, such as CD31 (PECAM), VE-cadherin, or PDGFRβ. Furthermore, GFP positive cells did not express a pericyte marker, CD146. Nor did we detect the presence of a hematopoietic marker, CD45 [39] in GFP positive cells. And, GFP positive cells colocalized with Sox9 expression, which we previously have shown to be a downstream target of Pref-1 and to affect mesenchymal cell fate. In this regard, immunostaining of the WAT sections from Dox treated Pref-1-tdTomato mice with permanent labeled Pref-1 expressing cells showed that all tdTomato positive cells colocalized with CD34. We next examined various adipocyte markers by immunostaining of tissue sections and found that GFP positive cells of Pref-1-GFP mice did not colocalize with adipocyte markers such as PPARγ or FABP4, clearly showing that they did not represent mature adipocytes. These cells also did not colocalize with ZFP423 that has been reported to be a commitment marker for adipocytes.[26] GFP positive cells were colocalized with CD24, indicating the proliferative capacity of Pref-1 expressing cells which was also further confirmed by the colocalization of both GFP and tdTomato with Ki67 in both Pref-1-GFP and Pref-1tdTomato mice (Figure 9). Overall we conclude that Pref-1 expressing cells are not of an endothelial, pericyte, or hematopoietic origin, but show characteristics of mesenchymal cells and have proliferative capacity.
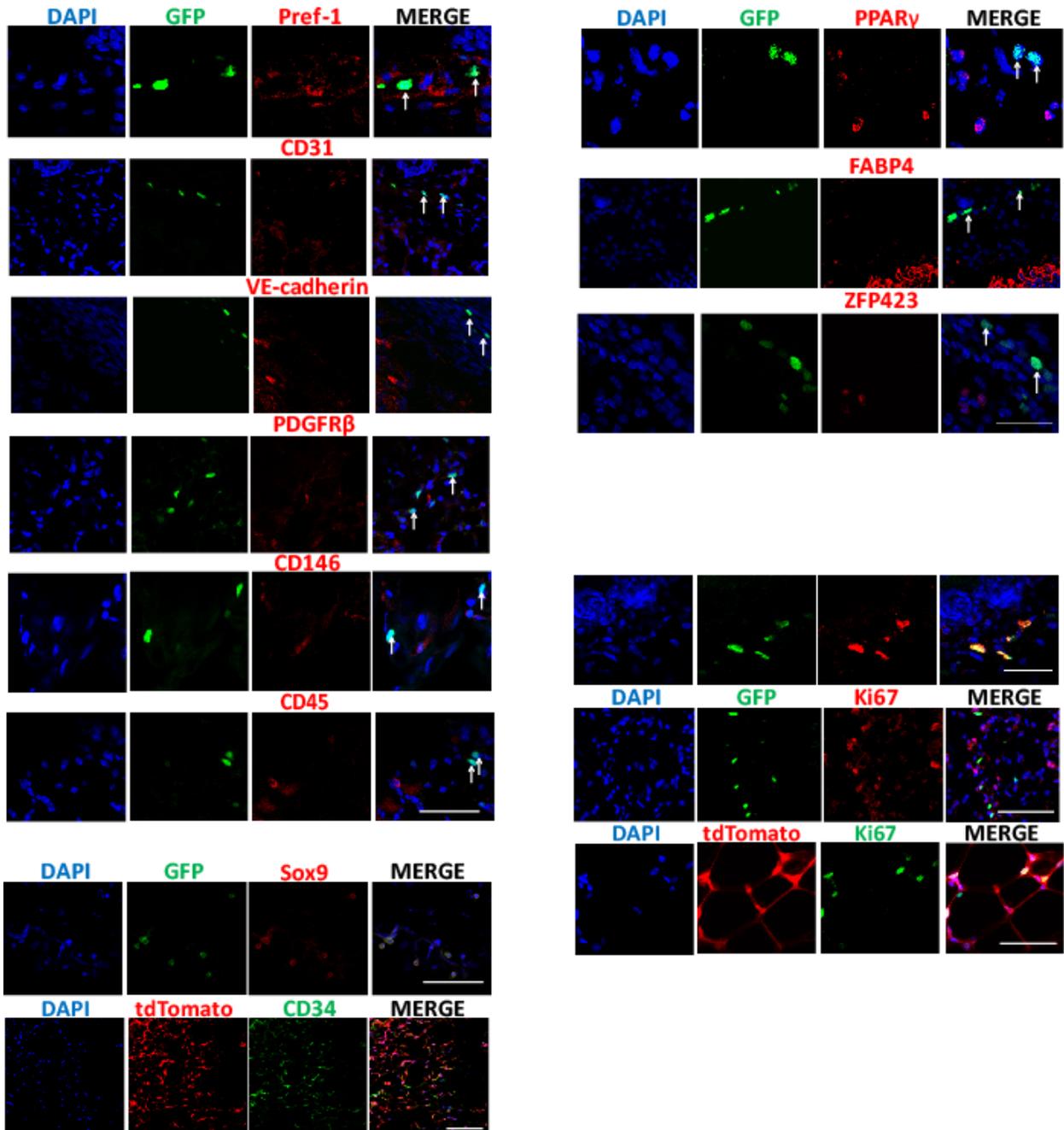
Figure 9. Confocal images of whole embryos for GFP (green) and indirect immunofluorescence for Pref-1, CD31(PECAM), VE-cadherin and PDGFRα, CD146, CD45, Sox 9 and CD34, PPARγ, FABP4, Zfp423, CD24 and Ki67. Scale bars: (Left) 100 μm, (Right lower middle) 200 μm, (Right lower lower) 150 μm.

Pref-1 Expressing Cells are Adipose Precursors

Since Pref-1 expressing cells are proliferative but did not express adipocyte markers, we postulated that Pref-1 expressing cells may represent early adipose precursors. We first tested the adipogenic potential of GFP or tdTomato labeled Pref-1 expressing cells in vitro. We cultured cells of the SVF from WAT of Dox treated Pref-1-GFP mice. We then subjected these cells to adipogenic protocol, and stained with DAPI and lipidTox (Figure 10). At Day 0, neither GFP positive nor negative cells showed any lipids detected by lipidTox staining. At Day 5 upon differentiation, GFP positive cells identified by the nuclear GFP fluorescence showed lipid staining in the cytoplasm. In contrast, GFP negative cells did not show any lipidTox staining. In a similar experiment using Dox treated tdTomato mice, at Day 0, we clearly detected the tdTomato fluorescence in the cytoplasm but did not detect lipidTox staining. At Day 5, most of the tdTomato positive cells could be seen with numerous lipid droplets in the cytoplasm, but tdTomato negative cells did not show any lipid accumulation. Upon differentiation, the FAS mRNA level was greatly increased while the Pref-1 mRNA level was greatly decreased, verifying that these cells differentiated into adipocytes. These data demonstrate that Pref-1 expressing cells have the capability of adipocyte differentiation, and therefore represent adipose precursors.
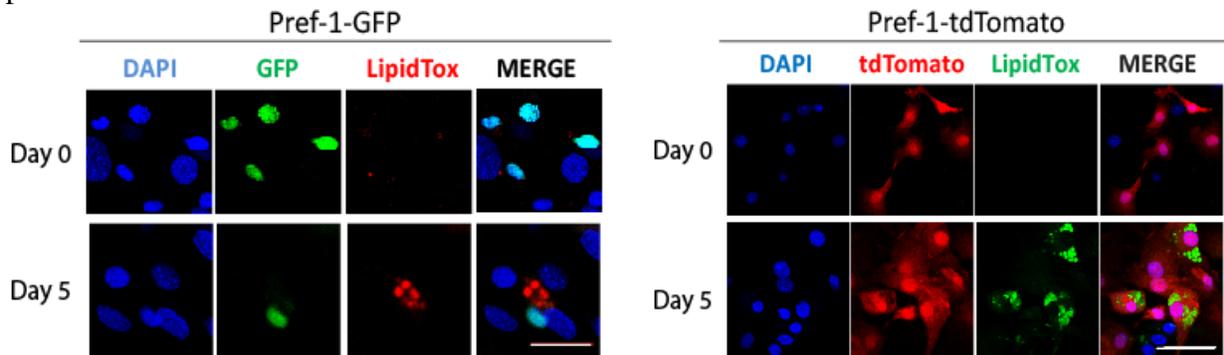
Figure 10. Cultured SVF from Dox treated (E0-P21) Pref-1-GFP mice were DAPI stained, and examined for GFP expression and adipocyte differentiation was assessed by lipidTox staining (red) at Day 0 and Day 5 after exposure to an adipogenic cocktail. Lower Left Panel; Cultured SVF from Dox treated (P1-P21) Pref-1-tdTomato mice were DAPI stained, and examined for tdTomato expression and adipocyte differentiation was assessed by lipidTox staining (red) at Day 0 and Day 5 after exposure to an adipogenic cocktail. Scale bars = 100 µm.

To further verify the adipogenic potential of Pref-1 expressing cells, by fluorescence activated cell sorting, we separated GFP positive from GFP negative cells of the SVF from the inguinal and epididymal WAT of Pref-1-GFP mice. The same number of GFP positive and GFP negative cells were then plated and these cells were subjected to adipogenic differentiation. Bright field microscopy of adipocyte morphology with multiple lipid droplets confirmed that GFP positive cells were undergoing differentiation, but GFP negative cells were unable to differentiate into adipocytes (Figure 11). At Day 5, mRNA levels of adipocyte markers, such as PPARγ and FAS were significantly higher upon differentiation of GFP positive cells in comparison to GFP negative cells. In contrast, Pref-1 mRNA level was significantly lower upon differentiation. Furthermore, GFP positive cells accumulated robust lipid droplets detected by lipidTox staining even without treatment with adipogenic cocktail, whereas GFP negative cells did not (data not shown). These results unequivocally demonstrate that Pref-1 expressing cells

are capable of differentiating into adipocytes in vitro and thus they represent the adipose precursor cells.
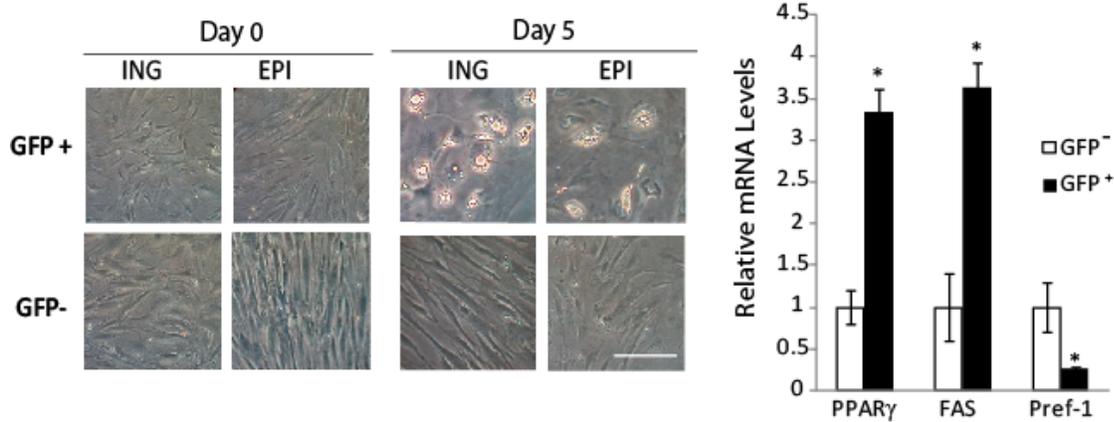
Figure 11. Brightfield microscopy of cultured sorted GFP positive and negative cells in adipogenic media, only GFP positive cells accumulate lipid. Right panel; RT-cPCR of adipogenic genes from total mRNA of cultured sorted GFP positive and negative cells at Day 0 and Day 5 of differentiation, p value <0.05. Scale bar = 200 µm.

Next, to demonstrate that Pref-1 expressing cells are adipose precursors in vivo, we employed our Pref-1-tdTomato mice with permanently labeled Pref-1 expressing cells. In this study, we treated Pref-1-tdTomato mice with Dox from birth and examined cryosections of inguinal or epididymal WAT for the presence of tdTomato labeled cells and for expression of a variety of adipocyte markers. Indeed, all lipidTox stained cells were positive for tdTomato fluorescence detected in the cytoplasm. In addition, the tdTomato fluorescence was colocalized with adipocyte markers, such as PPARγ and FABP4. We also found that tdTomato labeled cells in the adult WAT were positive for ZFP423 (Figure 12). Overall, these results show that Pref-1 expressing cells represent very early adipose precursors.
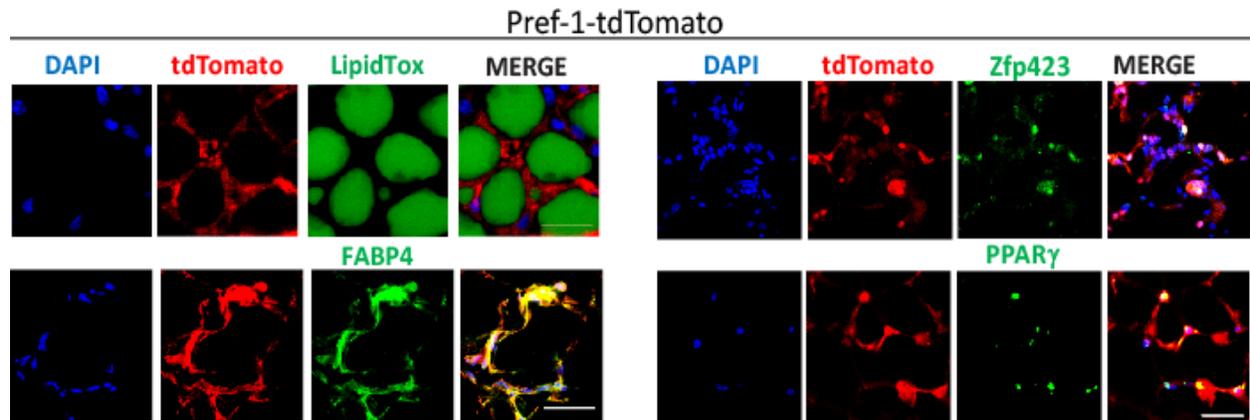
Figure 12. Confocal images of cryosectioned WAT from Dox treated Pref-1-tdTomato mice examined for tdTomato (red) and stained for Upper Left; Lipidtox (green). Immunostaining for FABP4 (Lower Left), ZFP423 (Upper Right), PPARγ (Lower Right). Scale bars = 100 µm..

Evidence and Requirement of Pref-1 expressing cells for Embryonic WAT Development and its Expansion in Adults

Unlike other tissues, WAT development during embryogenesis is not known, and the presumed notion that WAT development occurs postnatally has not been explored. For this purpose, our Pref-1-GFP mice allowed us to examine embryonic WAT development in vivo. Dox was given to the mothers at mating so that all Pref-1 expressing cells would be labeled during embryogenesis. Whole embryo cryosections were examined by LSC microscopy and lipidTox staining. Prior to E10.5, no obvious GFP positive cells nor lipid staining were detected, as shown at E8.5. At E10.5, GFP positive cells were easily detected in two distinct groups in the dorsal mesenteric layer between the vertebrae and the skin, poised for adipose development in the presumptive inguinal and dorsal subcutaneous depots. At E13.5, GFP positive cells were detected in a distinct line at the dorsal edge of the embryo close to the skin, but lipid staining was still not detected. Finally, at E17.5, lipid staining representing early WAT was clearly detected in this region and GFP positive cells were localized near the outer edge of the early WAT. Thus, unlike what has been believed, WAT is formed during embryogenesis, as shown by the clear detection of lipid staining at this stage. At E19.5, the number of lipid staining cells were increased by 2.5 fold compared to E17.5, indicating hyperplasia of WAT during embryogenesis (Figure 13).
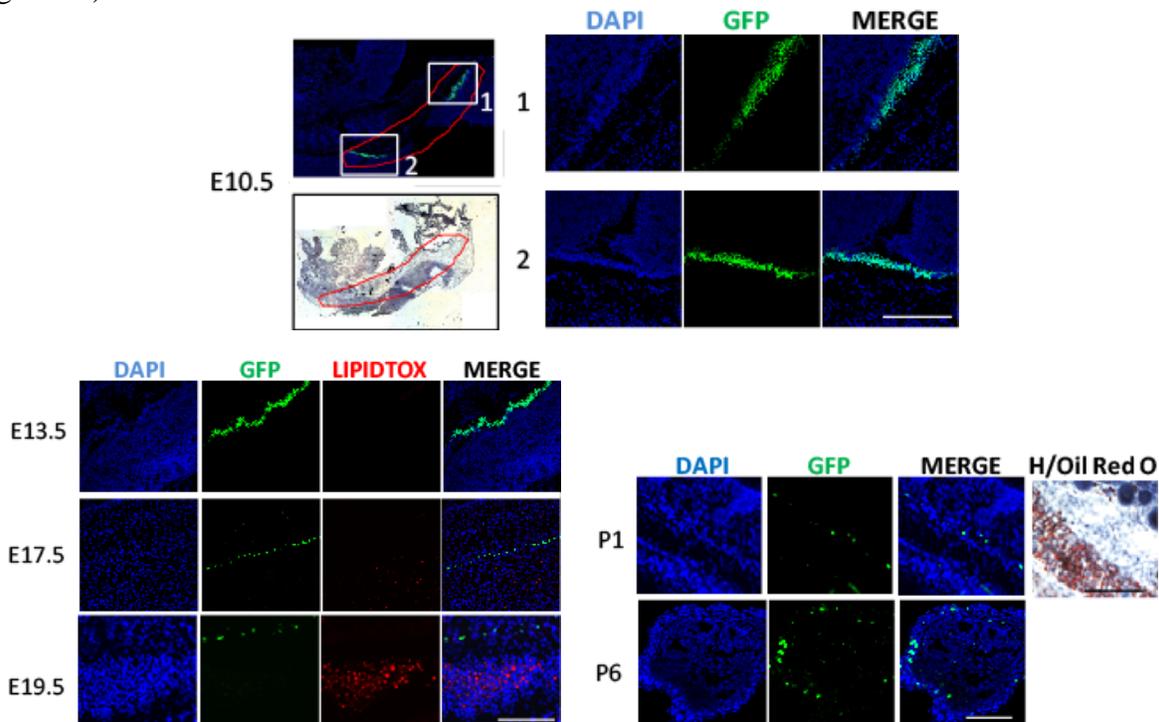
Figure 13. Female Pref-1 GFP mice were treated with Dox at mating and whole embryos were cryosectioned at different time points throughout embryogenesis for GFP and DAPI and lipidTox staining. Detection of direct GFP fluorescence identified two areas of GFP positive cells, H&E staining to show mesenteric region (red outline), and overlay. Whole embryos at E13.5, E17.5 and E19.5; GFP positive cells were detected near the outer edge of the developing embryo, and no lipid staining or lipid droplets were detected until E17.5 by lipidTox(red). Direct fluorescence of GFP in a newborn mouse, with GFP positive located near the edge of the developing adipose tissue as shown by overlay with hematoxymal and Oil Red O staining. P6 epididymal adipose tissue showed GFP positive cells along the outer edge of the adipose tissue, but were not detected in visceral areas prior to this time point. Scale bars = 400 μm.

24

To confirm that GFP-positive cells detected at E10.5 in our Pref-1-GFP embryos became adipocytes, we employed our Pref-1-DTA mice to delete Pref-1 expressing cells at E12.5 and examined the embryos for lipid staining at E17.5. Indeed we detected almost no lipid staining in Pref-1-DTA embryos compared to controls cells that showed obvious lipidTox staining, demonstrating the requirement of Pref-1 expressing cells for the generation of adipocytes during embryogenesis (Figure 14). At birth, GFP positive cells were detected at the edge of the subcutaneous WAT, which was clearly identified by the presence of lipid droplets staining by Oil Red O. In contrast to subcutaneous WAT, we could not detect any GFP or lipid droplets in areas that are presumed to be visceral adipose depots during embryogenesis (data not shown). And, it was not until P6 that we could detect epididymal WAT where GFP positive cells were localized mostly around the outer edge of this developing visceral WAT (Figure 13). These results indicate that subcutaneous depots develop during embryogenesis, while visceral adipose depots develop postnatally and that Pref-1 expressing cells are required for the development of both of these WAT.
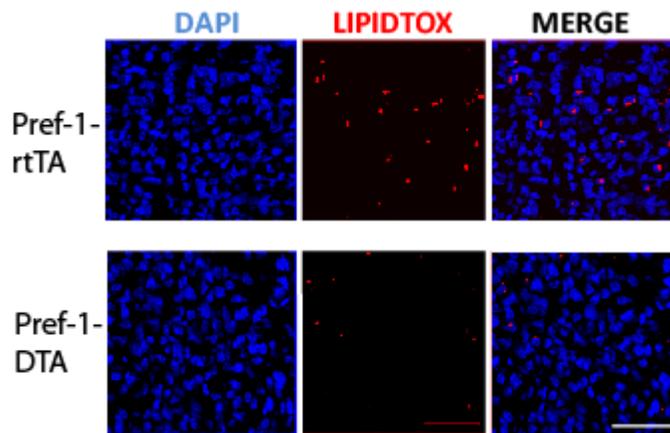
Figure 14. Depletion of Pref-1 expressing cells from E12.5 to E17.5 drastically reduced lipid staining compared to control. Scale bars = 200 μm.

Since Pref-1 expressing cells are early adipose precursors and are required for embryonic WAT development, we next sought to investigate whether Pref-1 expressing cells are also required for and contribute to WAT expansion in adults. When we examined adult WAT, we could detect a cluster of Pref-1 positive cells near the vasculature, but they were not positive for αSMA or CD31, commonly used markers of vasculature and pericytes. Clusters of GFP positive cells were also detected throughout the adipose tissue also (Figure 15). This varied distribution of GFP positive cells may be due to either proliferation of Pref-1 expressing cells or recruitment of these cells from the surrounding stroma.
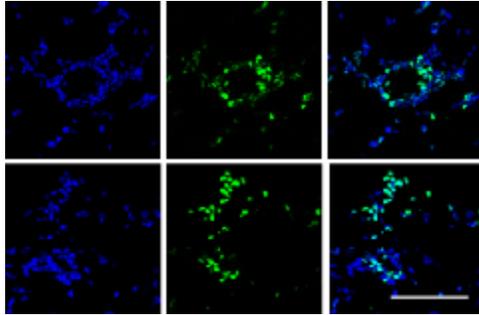
Figure 15. Clusters of GFP positive cells could be seen near vasculature, and also distributed throughout adult adipose tissue. Scale bar = 100 µm.

We next subjected Pref-1-GFP mice with transiently labeled Pref-1 expressing cells to high fat diet. Pref-1-GFP mice were maintained on Dox, which was removed upon weaning, and mice were maintained on chow or high fat diet for 3 weeks without Dox treatment. As predicted, weights of inguinal, epididymal, renal adipose depots were increased by approximately 40%, whereas other organs such as kidney were not. While WAT weights were increased, the number of GFP positive cells was decreased drastically as shown in Figure 15, indicating the division of Pref-1 expressing cells as evidenced by the loss of GFP fluorescence. The decrease in number of GFP positive cells was more significant in the epididymal compared to the inguinal depot. Quantification by cell sorting showed that the abundance of GFP positive cells was decreased in epididymal and inguinal WAT by 90% and 75%, respectively (Figure 16). It is clear that Pref-1 expressing adipose precursors contribute to high fat diet induced WAT expansion via hyperplasia. Incidentally, due to the nuclear localization of the GFP, we could not clearly detect changes in the adipocyte size by high fat feeding. However, a similar experiment with Pref-1-tdTomato mice with cytoplasmic labeling of tdTomato showed a larger adipocyte size, especially in inguinal WAT, upon high-fat feeding (Figure 17).
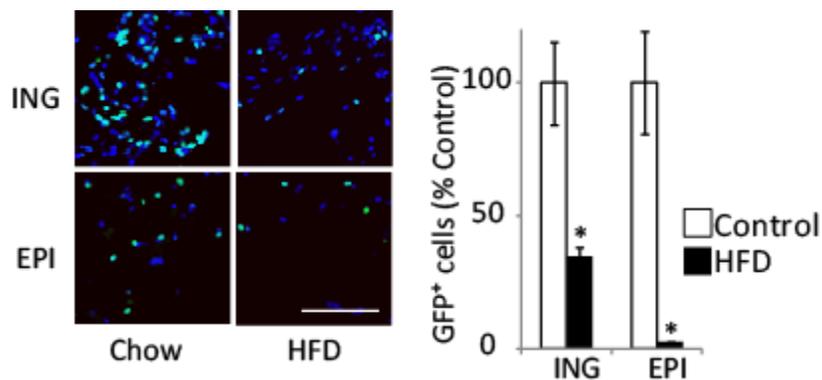
Figure 16. (Left) Confocal microscopy of cryosectioned inguinal and epididymal adipose tissue from Pref-1-GFP mice after 3 weeks of high fat feeding were examined for DAPI staining and direct fluorescence for GFP. (Right) FACS analysis of GFP positive cells of epididymal and inguinal adipose tissues from chow and high fat diet fed Pref-1-GFP mice. Scale bar = 100 µm.
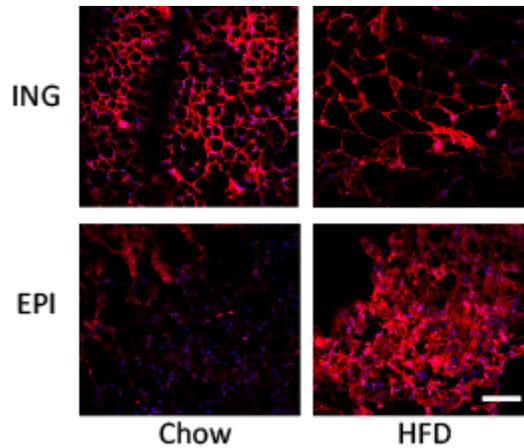
Figure 17. Confocal microscopy of cryosectioned inguinal and epididymal adipose tissue from Pref-1-tdTomato mice after 3 weeks of high fat feeding were examined for DAPI staining and direct fluorescence for tdTomato.Scale bar = 100 µm.

We next employed our Pref-1-DTA mice for inducible deletion of Pref-1 expressing cells. Mice were given Dox at birth, and WAT was examined at 3 weeks of age. We found a significant decrease in the WAT mass expressed as percent of body weight, with inguinal and epididymal fat depots decreasing by 20% and 50%, respectively (Figure 18). We further examined by staining for FABP4 and lipid staining of cryosections of WAT from these mice. In control mice, both inguinal and epididymal WAT showed high levels of lipid staining, although the inguinal depot had somewhat higher lipid content (Figure 18). However, in both inguinal and epididymal sections from Pref-1-DTA mice with depleted of Pref-1 expressing cells, there was a drastically decreased lipid staining, to an almost undetectable level in the epididymal depot (Figure 17). Quantitation of lipid containing cells showed a 60% and more than 95% reduction in the inguinal and epididymal fat depots, respectively.[42] Overall, these data clearly show that Pref-1 expressing cells undergo division and are required to contribute to adipose expansion in adults.
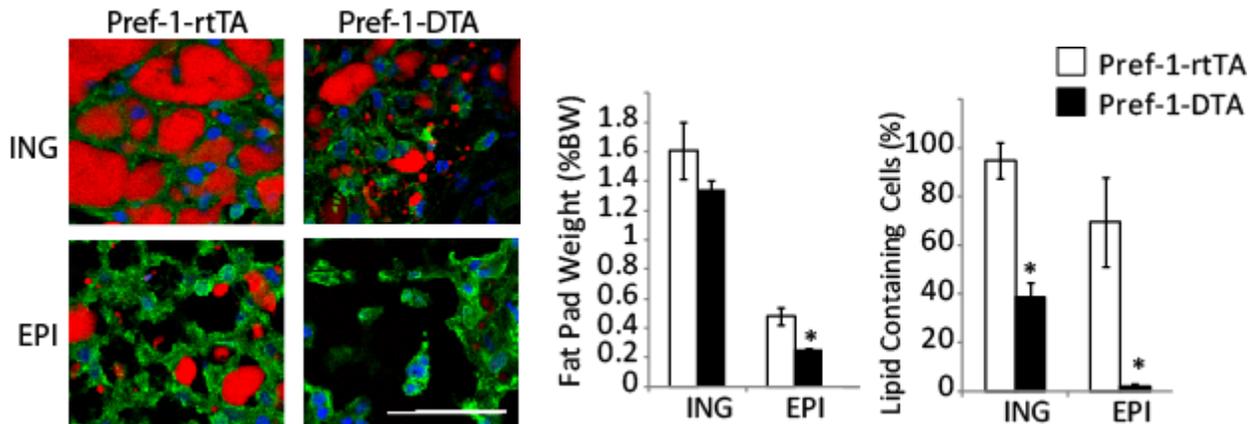
Figure 18. (Left) Cryosection of inguinal and epididymal WAT from Pref-1-rtTA control and Pref-1-DTA mice, stained with lipidTox (red) and immunostained for FABP4 (green). Depletion of Pref-1 expressing cells drastically reduced lipid content of adipose tissue. (Middle) Fat pad weight was measured in control and Pref-1-DTA mice and was decreased upon depletion of Pref-1 expressing cells. (Right) Quantification of lipid containing cells in control and Pref-1depleted inguinal and epididymal WAT, p value<0.05. Scale bar = 100 µm.

DISCUSSION

With the current epidemic of obesity and its related disorders, it is critical to understand the developmental process of adipose tissue, and to identify and characterize adipose precursors.[40] In this regard, though the SVF of adipose tissue contains various cell types including preadipocytes and macrophages, adipose precursors have not been well characterized. Furthermore, although some insight has been provided with the recent report of ZFP423 as a commitment marker, embryonic as well as early postnatal development of adipose tissue and adipose expansion have not been explored in vivo. Pref-1, which is expressed only in adipose, but not other tissues, and whose expression is extinguished during adipocyte differentiation, provided us a unique tool to investigate adipose tissue development and expansion. Here, by generating Pref-1-GFP and Pref-1 tdTomato mice that transiently and permanently label Pref-1 expressing cells, respectively, we show that Pref-1 expressing cells become adipocytes, indicating that Pref-1 expressing cells are the adipose precursors. Furthermore, we demonstrate the requirement of Pref-1 expressing cells for the formation of adipose tissue. We found that deletion of Pref-1 expressing cells significantly reduced adipose tissue mass in embryonic stage as well as diet-induced expansion of adipose tissue mass in adults. Here, by employing our Pref-1-GFP and Pref-1-DTA mouse models, we clearly show that adipocytes are formed by E17.5, demonstrating the establishment of subcutaneous WAT during embryogenesis, while visceral WAT develops postnatally, all produced from Pref-1 expressing cells.

Adipose progenitors have been reported to be near the vasculature, residing at the mural compartment, or to be of endothelial or pericyte origin [10,11,26]. Here, we show that Pref-1 expressing cells are near the dorsal edge of the embryo, which is the presumptive subcutaneous adipose tissue, but they are not localized to vasculature. In addition, during adipose expansion upon high fat feeding in adults, Pref-1 expressing cells are present throughout the adipose tissue. Although small clusters of Pref-1 expressing cells can be found near the vasculature, they also are present throughout the adipose tissue. It is possible that Pref-1 expressing cells proliferate or are recruited to vasculature due to nutrient and oxygen availability. In this regard, the Pref-1 expressing cells do not express endothelial markers, such as CD31, VE-cadherin, PDGFRβ, or αSMA, nor do they express pericyte markers such as CD146, indicating that these Pref-1 expressing cells are not of endothelial or pericyte origin. Importantly, Pref-1 expressing cells are positive for mesenchymal marker, CD105, as well as Sox9, a transcription factor known to determine mesenchymal cell fate. Overall, our studies show a mesenchymal origin of white adipocytes.

Our indelible marking of Pref-1 expressing cells shows that these cells become adipocytes expressing all of the known adipocyte markers including PPARγ, as well as the reported commitment gene, ZFP423. However, Pref-1 expressing cells do not express these markers, demonstrating that Pref-1 marks very early adipose progenitors before they express ZFP423 or PPARγ. In this regard, Pref-1 expressing adipose progenitors are positive for Ki67, and these cells can increase in number during adipose expansion. Therefore, it is likely that Pref-1 expressing cells undergo cell division prior to undergoing adipocyte differentiation, as has been shown in vitro.[8] In this regard, Pref-1 expressing cells are also CD24 positive. This is in agreement with a report that CD24 positive cells are an adipogenic population capable of proliferating and reconstituting adipose tissue in mouse models of lipodystrophy.[38] Thus, Pref-1 marks very early adipose precursors prior to ZFP423 or PPARγ expression, and these cells have proliferative capacity.

Overall, we conclude that Pref-1 expressing cells are the adipose progenitors and these cells represent very early precursors prior to the expression of ZFP423 or PPAR γ1. Pref expressing adipose precursors first appear early during embryogenesis at E10.5 at the subcutaneous region and adipocytes are present at this region as early as E17.5. We also show that these Pref-1 expressing adipose progenitors are not endothelial or pericyte in origin but are mesenchymal cells. Furthermore, Pref-1 expressing cells are proliferative and contribute to adipose tissue expansion during high fat feeding in adult stage.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Animals

The Pref-1-rtTA mouse was generated via insertion of the reverse tetracycline transactivator protein (rtTA, or Tet-On) directly downstream of -6.0 Kb of the Pref-1 promoter. The construct containing the Pref-1 promoter sequence used previously to generate Pref-1 transgenic mice was employed [19,20]. C57Bl/6J (C57Bl6/J; stock no. 000664) tetO-HIST1H2BJ/GFP)47Efu/J; (TRE-H2BGFP, stock no. 005104, B6.Cg-Tg(tetO-Cre)1Jaw/J; (TRE-CRE, stock no. 006234), and B6;129S-Gt(ROSA)26Sortm34.1 (CAG-Syp/tdTomato)Hze/J; (Rosa26-flox-stop-flox-tdTomato, stock no. 012570), B6.Cg-Tg(tet)-DTA)1Gfi/J; (TRE-DTA, stock no. 008468) mice were purchased from the Jackson Laboratory. All animal studies were performed under the guidance of UC Berkeley ACUC and OLAC regulations. Unless otherwise noted, all mice used were age-matched male littermates, with the exception of embryonic studies during which the gender was not determined. Dox was provided in the drinking water (1 mg/mL in 3% sucrose) and water was refreshed twice per week.

Cell Culture

For adipocyte differentiation, 2 day post-confluent cells were treated with differentiation inducing media containing 1 μM DEX, 0.5 mM MIX and 1.67 μM insulin in DMEM with 10% FBS and maintained for 2 days, after which cells were cultured in DMEM with 10% FBS for further experiments. For Dox treatment, 1 μg/mL Dox was added to the media.

FACS Analysis and Sorting

FACS experiments were carried out on a FACSAria flow cytometer. Briefly, fresh adipose tissue was fractionated, and the SVF was washed three times, and resuspended in 5% FBS/PBS at a concentration of 10^6 cells/mL, and strained through a 40 µm mesh. For quantification, cells were initially selected by size on the basis of forward scatter (FSC) or side scatter (SSC). Dead cells were excluded based on propidium idodide uptake. SVF from control mice were used to determine background fluorescence levels. Sorting experiments were carried out on a Cytopeia INFLUX Sorter. A similar protocol was followed as above for culture and maintenance, however, after sorting, cells were collected in 5% FBS in Ham's F12 media, and plated in 6 cm dishes.

Immunostaining and Immunocytochemistry

Whole embryos or tissues were flash frozen in O.C.T. (Sakura) and sectioned on a Leica CM3050S Cryostat in 10 or 12 μm thick sections, and collected on Superfrost + coated glass slides (Fisher). Frozen sections were stored at -20˚C until staining. Briefly, slides were allowed to warm to room temperature for 5 minutes, fixed in 4% formalin phosphate buffered saline for

30 minutes at room temperature, blocked in 5% BSA in PBST (PBS with 0.025% Triton X-100), and incubated with the appropriate antibody diluted in 2.5% BSA in PBST overnight at 4˚C. Slides were washed 3 times in PBST, and incubated with secondary antibody for 2 hours at room temperature. Slides were then washed twice in PBST, and then stained with DAPI for three minutes, washed once, and mounted in 15% glycerol in PBS with glass coverslips. Antibodies used for immunostaining can be found in the Supplemental Experimental Procedures.

RT-PCR and RT-qPCR

Total RNA prepared with Trizol (Invitrogen) was reverse transcribed using ImpromII reverse transcriptase (Promega). Primer sets used for RT-PCR are listed in the supplemental data section. The cDNAs were mixed with Maxima SYBR Green mastermix (Fisher), specific primers and for Sox9, Pref-1, CD24, PECAM, VE-cadherin, FAS, GFP, Zfp423, C/EBPα and PPARγ and analyzed by using ABI7900 (Applied Biosystems). The obtained CT values for these genes were normalized to those of rodent 18S RNA by the ΔΔCT method. The mean CT was converted to relative expression value by the equation, 2-ΔΔCt, and the range was calculated by the equation, 2^-(ΔΔCT +stdevΔΔCT). SYBR primer probe sequences can be found in Table 1.

Single Cell RT-qPCR

Freshly isolated WAT was fractionated by centrifugation and the SVF was resuspended in 2% FBS/PBS at a concentration of 10^6 cells/mL. Cells were fixed in 4% formalin in buffered PBS for 15 min at room temperature, and washed three times in DPBS (without Mg^{+2} or Ca^{+2}). Cells were applied to the single cell device and centrifuged at 3,000 rpm for 10 min twice. The device was then dried at 37˚C for 30 minutes prior to application of SuperScript III with Platinum *Taq* DNA Polymerase and Taqman probes (Life Technologies). Pref-1 was detected using Taqman probe (Mm00494477_m1) with VIC. A Biorad CFX96 Real-time PCR was used for thermocycling.

Microscopy

Laser Scanning Confocal Microscopy was performed using a Zeiss LSM710 running Zen 2010 software equipped with 10X and 20x air objectives, and 40X, 63X and 100X oil immersion objectives using standard excitation wavelengths for DAPI, GFP (or Alexafluor 488), tdTomato (or Alexafluor 594). Raw data was processed using NIH imageJ software (http://imagej.nih.gov/ij/, 1997-2012).

Antibodies

Primary antibodies for immunostaining were: aSMA (rabbit, Abcam Lot # GR100205-1,1:100), CD24 (rat, BD Pharmingen Lot# 04877,1:100), CD31 (rat, BD Pharmingen Lot# 73117,1:100), CD34 (rat, BD Pharmingen Lot# 23141, 1:100), CD45 (rat, BD Pharmingen Lot # 2243975, 1:100), CD146 (mouse, Abcam Lot# GR94980-1, 1:100), FABP4 (goat, Santa Cruz Lot# G2110,1:500), Ki67 (rabbit, Abcam Lot# GR34956-2, 1:300), PDGFR ☐ (rabbit,Santa Cruz Lot# C2312,1:100), PPAR ☐ (goat, Santa Cruz Lot# L1709 Lot# F2911, 1:100), Sox9 (rabbit, SantaCruz, Lot# I1807, 1:100), VE-cadherin (rabbit ,Abcam Lot# GR77655,1:100), ZFP423 (OAZ) (goat, SantaCruz Lot# L1306, 1:100).   Secondary antibodies were goat-anti-rabbit Alexafluor488/594, chicken-anti-rat Alexafluor488/594, goat-anti-mouse Alexafluor 488/594, chicken-anti-goat Alexafluor488/594 (Invitrogen, 1:1000).

Table 1. Primer Sequences Used

Gene	Forward (5'-3')	Reverse (5'-3')
aSMA	GCATCCACGAAACCACCTA	CACGAGTAACAAATCAAAGC
CD24	ACCGGGTGCTGTTCTATAAGG	TCACCTCGTACTCAATCGTGG
CD31	GTGAAGGTGCATGGCGTATC	CACAAAGTTCTCGTTGGAGGT
CD34	CTGGGTAGCTCTCTGCCTGAT	TGGTAGGAACTGATGGGGATATT
CD45	GAACATGCTGCCAATGGTTCT	TGTCCCACATGACTCCTTTCC
CD90	ATGAACCCAGCCATCAGCG	GGGTAAGGACCTTGATATAGG
CD105	AGGGGTGAGGTGACGTTTAC	GTGCCATTTTGCTTGGATGC
CD146	CCAAGGCAACCTCAGCCATGTC	CTCGACTCCACAGTCTGGGACGACT
C/EBPa	TGGACAAGAACAGCAACGAG	AATCTCCTAGTCCTGGCTTG
FABP4	CAATGGGGGCCAGATCAT	CAATGGGGGCCAGATCAT
FAS	TGCTCCCAGCTGCAGGC	GCCCGGTAGCTCTGGGTGTA
GFP	TCTGTCTCCGGTGAAGGTGAAG	GGCATGGCAGACTTGAAAAAG
PDGFRα	AGGACAACCGTACCTTGGGTGACT	CAGTTCTGACACGTACCGGGTCTC
PPARγ	ACTGCCTATGAGCTCTTCAC	CAATCGGATGGTTCTTCGGA
Pref-1	GACCCACCCTGTGACCCC	CAGGCAGCTCGTGCACCCC
Sox9	AGACCTTTGGGCTGCCTTAT	TAGCCTCCCTCACTCCAAGA
VE-cadherin	CCGGCGCCAAAAGAGAGA	CTGGTTTTCCTTCAGCTGGAAGTGGT

ACKNOWLEDGEMENTS

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CHAPTER III

UCP-1 EXPRESSION DURING DEVELOPMENT AND COLD-INDUCED ACTIVATION OF BROWN ADIPOSE TISSUE

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UCP-1 Expression During Development and Cold-Induced Activation of Brown Adipose Tissue

INTRODUCTION

Obesity is characterized by an increase in adipose tissue mass, and is associated with increased risks of a variety of detrimental health problems such as cardiovascular disease, cancer, and diabetes.[1–4] In mammals, adipose tissue is organized into two major types, white and brown. White adipose tissue (WAT) is the major site for energy storage, and is characterized by the presence of a single large lipid droplet within the cell. Brown adipose tissue (BAT) on the other hand, appears brown due to the abundance of mitochondria which contain multiple heme-coordinating metalloproteins.[5,6] While WAT performs lipolysis for the provision of fatty acids for other tissues during times of energy need, BAT is capable of burning fatty acids for thermogenesis.[7,8] This function is achieved through the expression of uncoupling protein 1 (UCP-1), a mitochondrial membrane protein that uncouples oxidative phosphorylation from ATP production by leaking protons across the mitochondrial membrane.[9–11] The recently appreciated presence of metabolically active brown adipose tissue (BAT) in humans, which is capable of dissipating energy as heat, has generated much interest in the anti-obesity potential of BAT.[12,13] Indeed, an increase in BAT mass in mice has been shown to have both anti-obesity and anti-diabetic effects.[5,14–16]

Identifying the cellular origins of BAT will provide further understanding of BAT biology, and in this regard, a shared origin of muscle and BAT was recently identified, however a unique origin of WAT is still hypothesized.[17–21] UCP-1, a specific marker of brown adipocytes, provides a unique ability to isolate and characterize brown fat cells. Lineage tracing of the muscle precursor cells has revealed that brown adipocytes arise from $Myf5^+$/Sca-1-$^+$/ $Pax7^+$ cells, coming from the same origin as skeletal muscle, cartilage and dermis.[22–25] A unique population of brown fat cells have been identified in white adipose tissue and arise during chronic cold exposure or β3-adrenergic stimulation. Because of their inducible nature, these cells are coined as brown-in-white or "brite cells", and function as brown-like cells within the white adipose tissue.[26] These inducible brown cells were found to have a different origin from classical brown cells and origin from $Myf5^-$ population.[27–29] Unlike classical brown fat cells, inducible brown cells have very low levels of UCP-1 until activated.[30,31] Though a few markers and characteristics of inducible brown cells are known, the origin and molecular mechanisms of their recruitment and activation are not fully understood.

Although many factors that activate UCP-1 have been identified in cell culture experiments, less is known about the process of thermogenic activation in vivo. Cold exposure activates sympathetic release of norepinephrine which binds to β3-adrenergic receptors, and promotes increased UCP-1 expression and activity.[28,32,33] Administration of β3 agonists also increases BAT mass, however, how this expansion occurs in vivo is not fully understood. In WAT, it is known that expansion occurs through hypetrophy of existing adipocytes as well as hyperplasia of preacipocytes differentiating into adipocytes.[34,35] In BAT, however, some reports have shown that inducible brown cells pre-exist in white adipose until activated, while others claim that this process occurs by transdifferentiation.[6,27,36,37] It may also be possible that cells at an early stage of differentiation can expand BAT mass. Overall, the mechanisms involved in increasing BAT mass, activation of UCP-1, and inducing brown adipocytes in white adipose are not well understood. Here we label UCP-1 expressing cells to uncover the mechanistic details of

embryonic development of brown adipose tissue, as well as investigate the activation of the UCP-1 promoter during cold exposure and β3-adrenergic stimulation.

RESULTS

Generation of Transgenic Mouse Models for Labeling of UCP-1 Expressing Cells

Several transcription factors, such as PPARγ and PRDM16 have been identified to play a role in differentiation of brown adipocytes, but these are expressed in other tissues. UCP-1 is uniquely expressed in brown adipocytes, and is considered to be the hallmark of activation of a brown adipocyte. To mark and perform lineage analysis of UCP-1 in an inducible manner, we first engineered UCP-1-reverse tet transactivator (rtTA) transgenic mice expressing rtTA under the control of -5.5 Kb of the mouse UCP-1 promoter (Figure 19). We used -5.5 Kb of the UCP-1 promoter due to the evidence that a region spanning -4.5 Kb has been shown to activate brown fat specific expression.[38] For the UCP-1-GFP mouse model, a tet-responsive histone 2B (H2B) green fluorescent protein (GFP) [tetracycline response element-H2BGFP (TRE-H2BGFP)][39–41] was introduced into the UCP-1-rtTA line, thus creating UCP-1-GFP mice whereby the UCP-1 promoter directs expression of GFP localized in the nucleus. In this transient labeling mouse model, GFP is stable in postmitotic cells but becomes diluted in proliferating cells upon removal of Dox. For permanent labeling of UCP-1 expressing cells, we introduced UCP-1-rtTA and TRE-Cre into the ROSA26-flox-stop-flox-tdTomato knock-in line.[42] In this permanent labeling, Dox treatment causes expression of tdTomato in the cytoplasm of UCP-1 expressing cells and tdTomato is expressed in all of the progeny even after cell division.
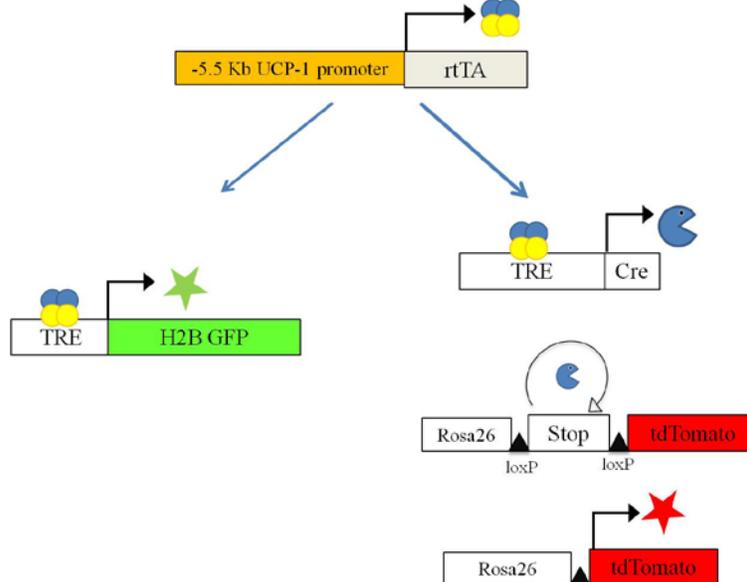
Figure 19. Generation of mouse models to label UCP-1 expressing cells. UCP-1-GFP mice were generated by inserting reverse tet-transactivator (rtTA, Dox On) directly following -5.5Kb of the UCP-1 promoter, whereby TRE-H2BGFP fluorescence transiently labels UCP-1 expressing cells only in the presence of Dox. For indelible and inducible lineage tracing, the UCP-1-rtTA and (TRE)-Cre transgenic lines were crossed with a ROSA26-flox-stop-flox-tdTomato (tdTomato) knock-in line. Cre activation permanently excises the transcriptional stop and tdTomato irreversibly marks UCP-1 expressing cells and all descendants. In the absence of Dox, the rtTA protein cannot bind to the TRE, the stop remains intact, and tdTomato is not expressed.

First, to test the efficacy of our mouse models, we compared the expression of the fluorescent reporters in BAT of the UCP-1-GFP mice upon Dox treatment. Laser scanning confocal (LSC) microscopy of brown adipose tissue sections showed the presence of GFP positive cells only in the BAT of Dox-treated, but not untreated animals (Figure 20). GFP positive cells were not detected in control GFP only mice even in the presence of Dox. Next, to examine if expression of GFP and tdTomato in our mouse models can mimic endogenous UCP-1 expression, we used immunostaining to label UCP-1 protein. We found that UCP-1 expression clearly overlapped with reporter expression in BAT of Dox-treated mice (Figure 21). We also found that Cidea expression overlapped with reporter expression in BAT (Figure 22). Indeed, GFP fluorescence in the nucleus was detected in a very small percentage (~1.5%) of total cells observed by DAPI staining in BAT. In UCP-1-tdTomato mice, more than 90% of the cells were tdTomato positive in BAT, as expected. Overall, we detected GFP and tdTomato only in BAT, but not in other tissues such as liver, kidney, and muscle. These data show that our reporter expressing cells are restricted to brown adipose tissue. Also, it is surprising that such a small percentage of cells were GFP positive, indicating that UCP-1 promoter activation is transient.
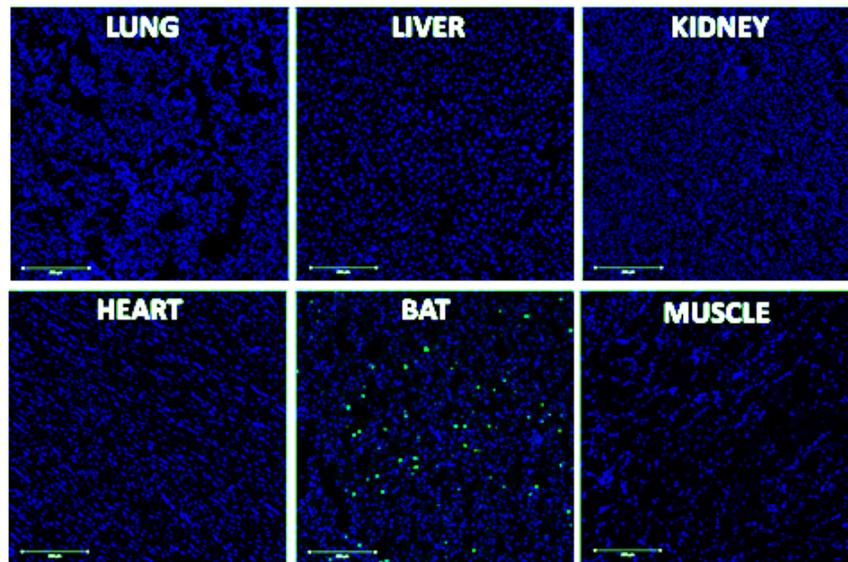
Figure 20. UCP-1-GFP mice were Dox treated from P1 to P21 and various tissues were cryosectioned and imaged for GFP (green). Scale bars = 200 μm
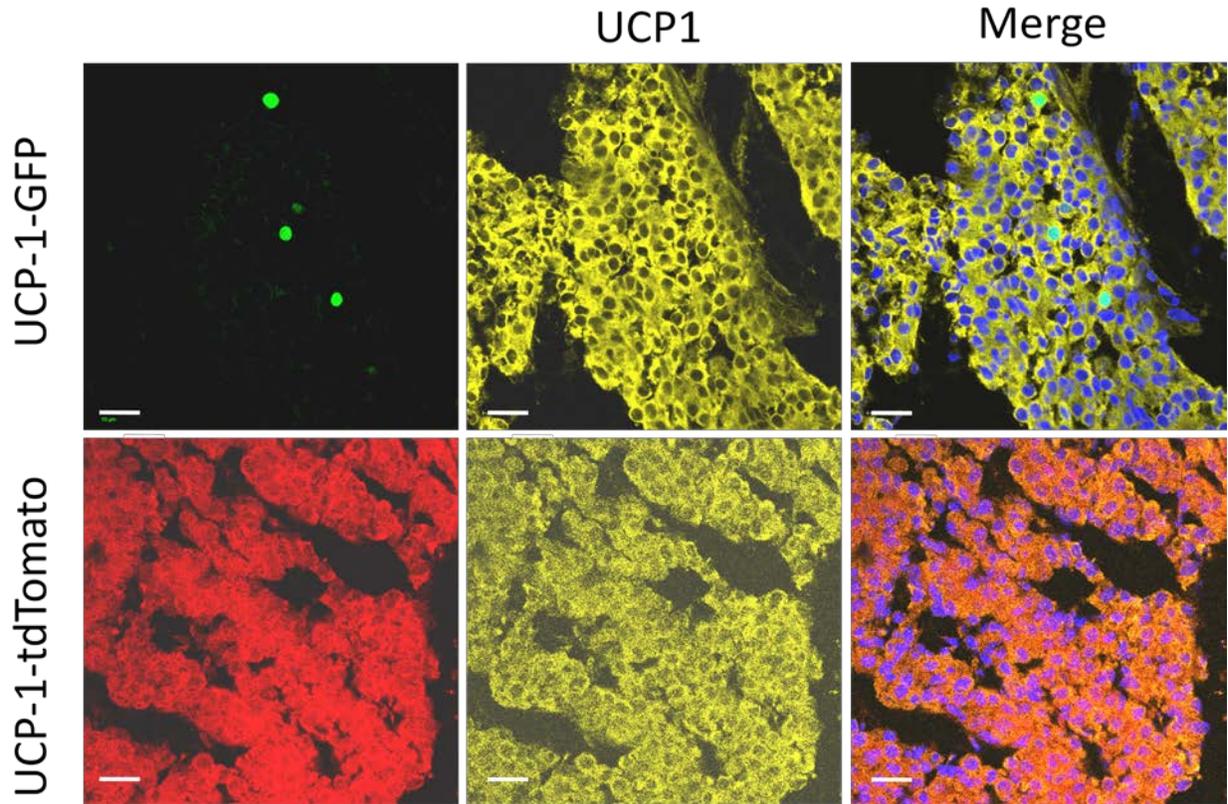
Figure 21. UCP-1 immunostaining of BAT cryosections from Dox-treated UCP-1-GFP and UCP-1-tdTomato mice. Colocalization of GFP and tdTomato reporters with UCP-1 is shown by overlap of UCP-1 expression in yellow, GFP in the nucleus (upper panels) and tdTomato in the cytosol of brown adipocytes (lower panels). Scale bars = 20µm.
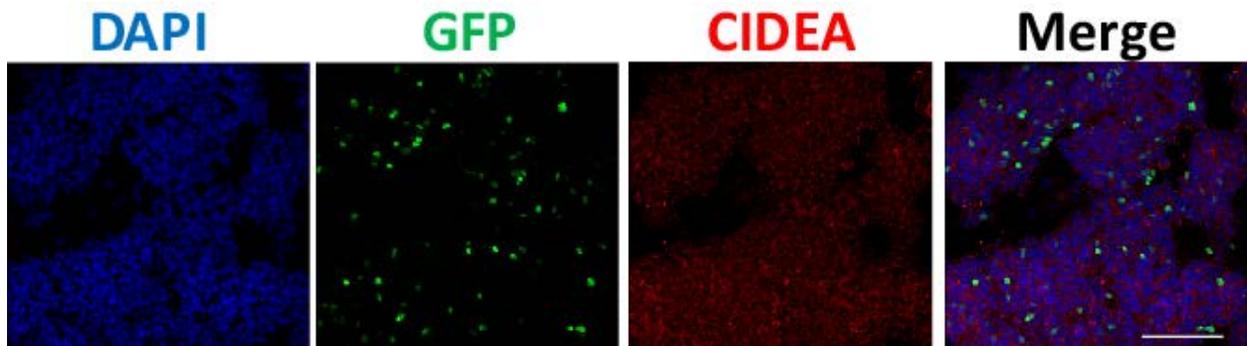
Figure 22. Cidea immunostaining of BAT cryosections from Dox-treated UCP-1-GFP and UCP-1-tdTomato mice. Colocalization of GFP and tdTomato reporters with Cidea is shown by overlap of Cidea expression in yellow, GFP in the nucleus. Scale bar = 100µm.

Embryonic Development of BAT

BAT mass is highest in newborns and decreases with age, and the precise timing of UCP-1 activation during development is not clearly known. It has been shown that UCP-1 expression is highest at embryonic day 16.5 (E16.5) which would indicate that BAT development is occurring rapidly at this stage. We investigated brown adipose tissue development by labeling UCP-1 expressing cells with GFP and cryosectioning of whole embryos at different timepoints during development (Figure23). We further investigated the pattern of UCP-1 promoter activation and UCP-1 protein accumulation. We could not detect any GFP positive cells prior to E14.5, and a few begin to appear at this timepoint. The number of GFP positive cells peaks at E16.5 and seems to decrease over time. This increase of UCP-1 promoter activation at E16.5 correlates with the high gene expression of UCP-1 that has been reported at this stage of development. We also detected UCP-1 protein by immunostaining at an earlier stage than has been reported previously, and UCP-1 protein accumulated throughout embryogenesis. Overall we show that the UCP-1 promoter activation occurs early during embryogenesis and once the protein is expressed, the tissue continues to accumulate UCP-1 protein.

Figure 23. GFP positive cells, representing UCP-1 promoter activation, peak at embryonic day 16.5 during embryonic development. The number of GFP positive cel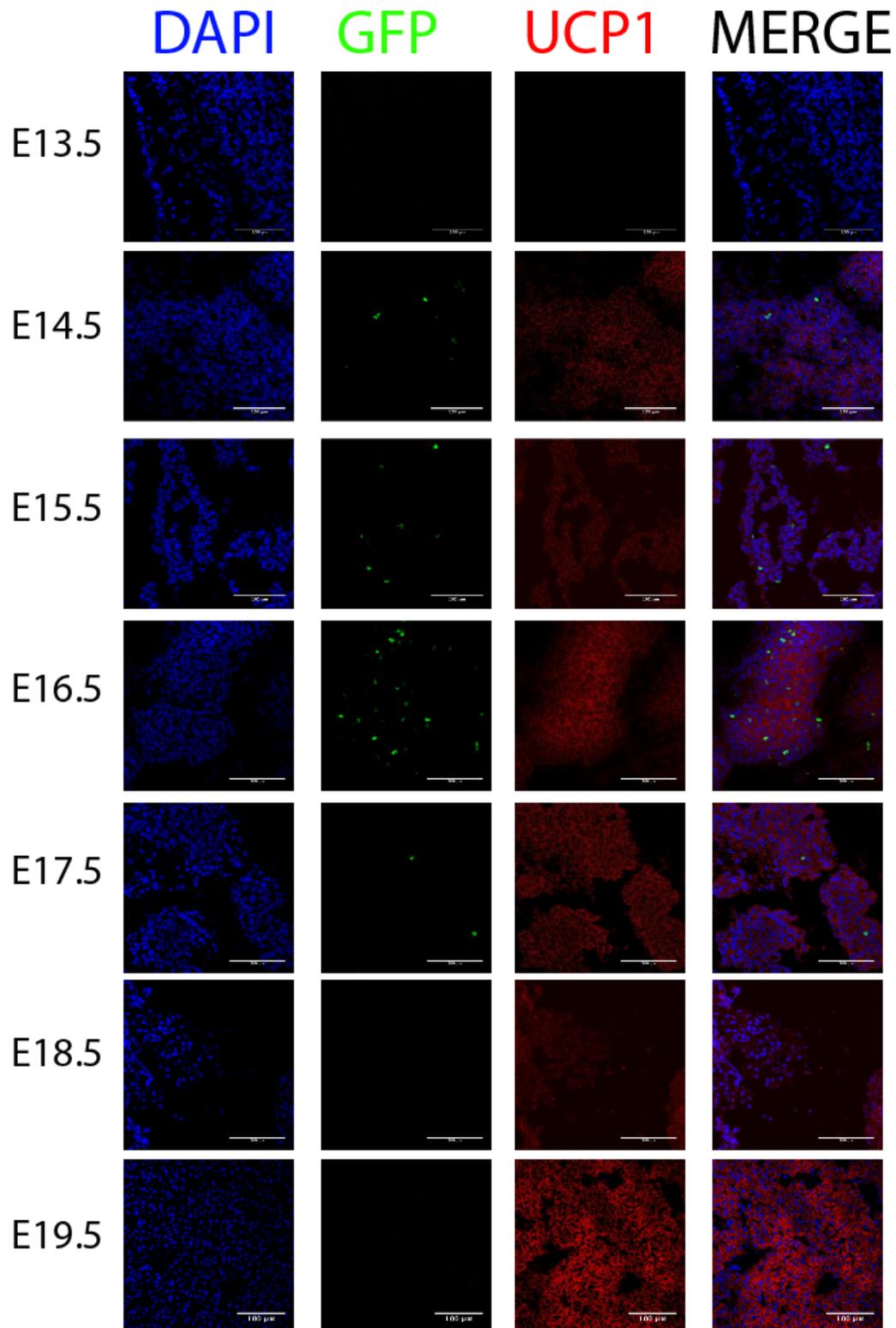ls peaks at E16.5, and UCP-1 protein expression follows a similar pattern and continues to increase. Scale bars = 100µm.

Cold-induced and β3-adrenergic stimulated activation of UCP-1 and BAT expansion

Cold exposure can expand BAT mass in adults, but the molecular mechanisms underlying this process are not fully understood in vivo. To investigate activation of the UCP-1 promoter during cold exposure, we exposed UCP-1-GFP mice to cold with and without doxycycline treatment. In dox-treated mice exposed to cold for 5 days, we saw a drastic increase in the total number of GFP positive cells in BAT, and an even greater increase in mice exposed to cold for 10 days. Mice who were exposed to cold with dox removed show a great decrease in the number of GFP positive cells, indicated that UCP-1 postive cells are dividing during cold exposure and the GFP signal is lost. Further loss of GFP positive cells was observed after 10 days of cold exposure (Figure 24).

To examine whether GFP positive cells that are activated during cold exposure are proliferating or not, we only treated mice with Dox during the cold exposure time period. In these mice, all GFP positive cells were labeled with the proliferation marker, Ki67, and interestingly, no other cells in the BAT were positive for this marker (Figure 25). These results indicated that GFP positive cells are the only cells that are proliferating during cold exposure, and therefore the UCP-1 promoter is activated first and then these cells divide. We also injected the mice with 5-bromo-deoxyuridine (BrdU) and found that GFP positive cells colocalize with BrdU staining, indicating that UCP-1 positive cells are dividing. To look at the effect of β3 adrenergic stimulation, we treated mice with a β3-adrenergic agonist, CL-316,243, for 5 and 10 days and compared the number of GFP positive cells to saline treated mice. We observed and increase in GFP positive cells in mice treated for 5 days, and then an even greater increase after 10 days of treatment (Figure 26). Whether the total cell number is increasing in BAT needs to be determined.
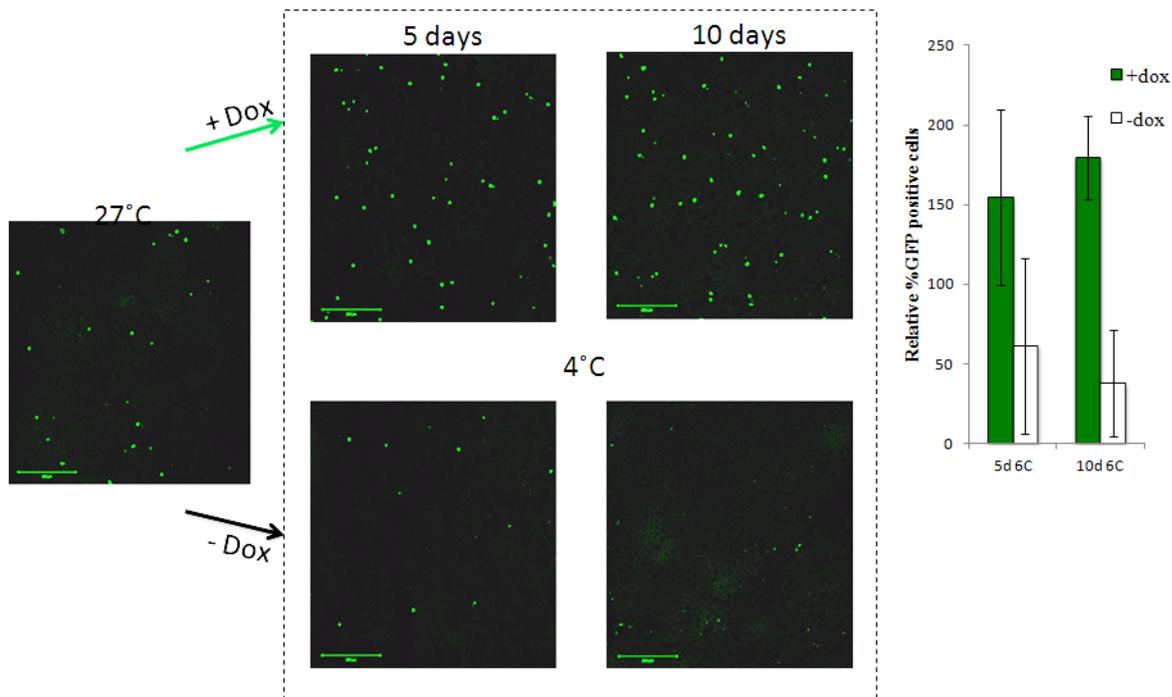
Figure 24. GFP positive cells increase in number during cold exposure in mice maintained on dox, in mice without dox, GFP positive cells decrease in number as they divide. Scale bars = 100µm.
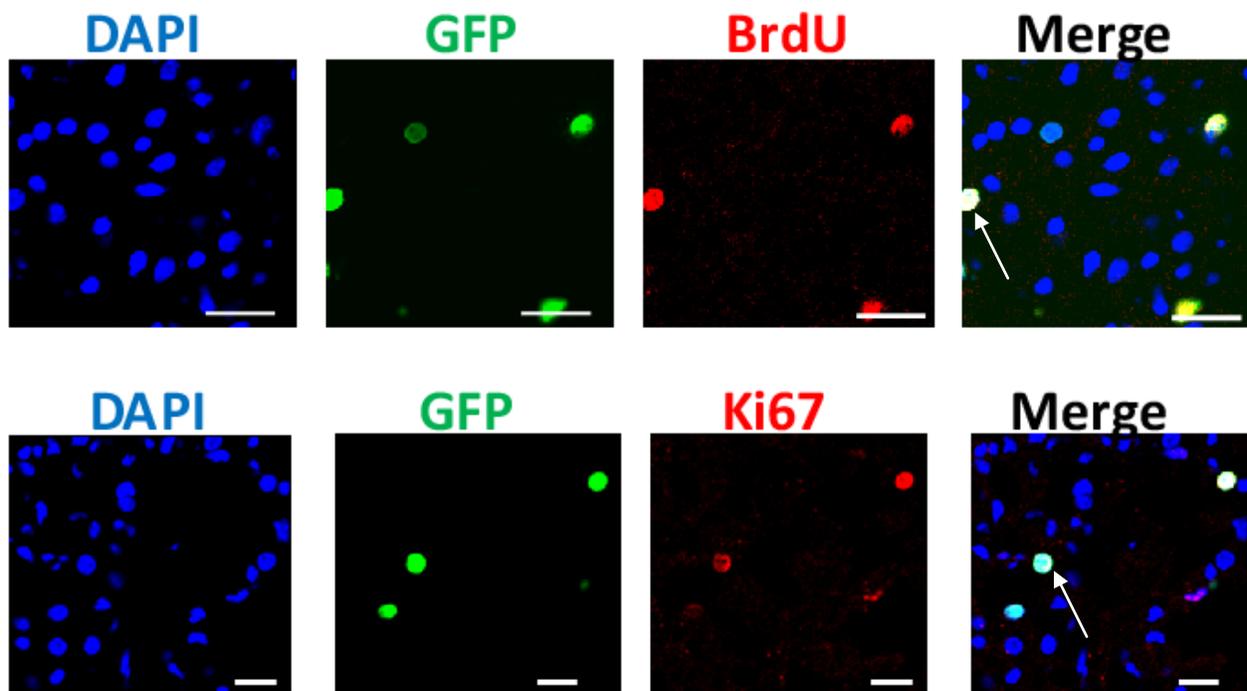
Figure 25. GFP positive cells proliferate in the cold following activation of the UCP-1 promoter as demonstrated by Ki67 colocalization with GFP. GFP positive cells also colocalize with BrdU staining, indicating that these cells are proliferating during cold exposure. Scale bars = 100μm.
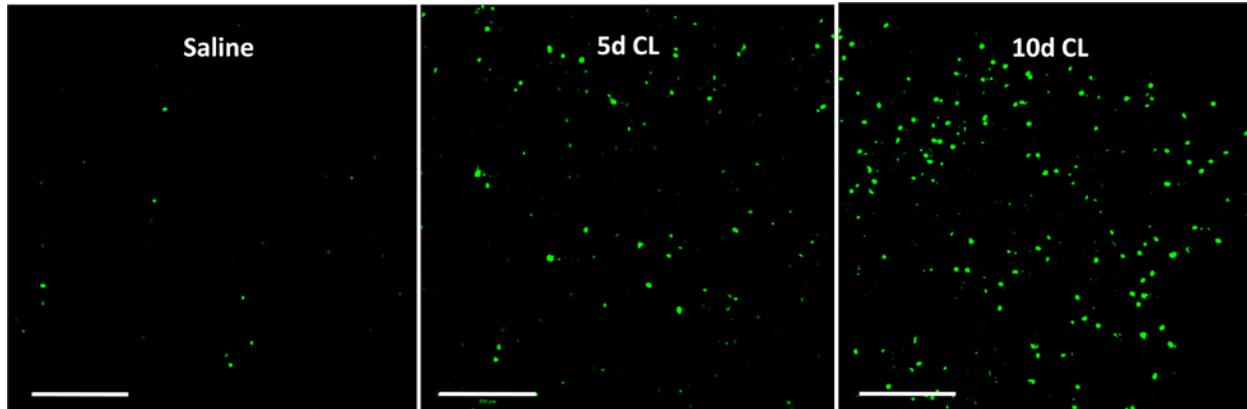
Figure 26. β3-adrenergic agonist treatment with the compound, CL-316,243, in UCP-1-GFP mice increases the number of GFP positive cells, demonstrating activation of the UCP-1 promoter. Scale bars = 100μm.

DISCUSSION

With the current epidemic of obesity and its related disorders, it is critical to understand the developmental process of brown adipose tissue, and to characterize the cells that are capable of increasing brown adipose tissue mass.[43] UCP-1, which is expressed only in brown adipose, provided us a unique tool to investigate brown adipose tissue development and expansion. Here, by generating UCP-1-GFP and UCP-1 tdTomato mice that transiently and permanently label UCP-1 expressing cells, respectively, we show that UCP-1 expressing cells appear as early as E14.5. Furthermore, we demonstrate that the UCP-1 promoter is activated during cold exposure

and β3- adrenergic stimulation, and that UCP-1 positive cells proliferate. Here, by employing our UCP-1-GFP mice, we clearly show that brown adipocytes are formed by E14.5, demonstrating the establishment of BAT during embryogenesis.

During embryogenesis, UCP-1 expression has been reported starting at E16.5, the presumptive beginnings of BAT development. We show that UCP-1 expressing cells are present as early as E14.5, and UCP-1 protein continues to accumulate leading up to birth. Interestingly, the peak of GFP positive cells, representing activation of the UCP-1 promoter, occurs at E16.5 and decreases drastically until birth. These results indicate that the UCP-1 promoter is highly activated during the largest period of brown adipocyte differentiation that occurs at E16.5. Following this timepoint, only newly proliferating ells need to activate transcription of UCP-1. GFP positive cells are also expressing other transcriptional activators such as PPARγ and PGC1α.

In mammals, WAT mass increases throughout life, whereas BAT decreases. However, BAT mass can increase in response to chronic cold exposure or β3-adrenergic stimulation. Whether BAT mass increases by proliferation of preadipocytes, or proliferation of existing UCP-1 expressing brown adipocytes has not been fully determined. During cold exposure, we found that UCP-1 expressing cells were capable of cell division, an unexpected finding as these cells are considered to be the most differentiated, and therefore the least plastic. We also found that only those cells with active expression of UCP-1 were dividing during the cold exposure time course. This indicates the new brown adipocytes are producing UCP-1 protein and dividing, but existing brown adipocytes do not increase expression of UCP-1. These results show that the increase in UCP-1 expression that has been reported during cold exposure is due to an increase in the number of new brown adipocytes, not due to an increase in the UCP-1 protein concentration in existing brown adipocytes.

Overall, we conclude that UCP-1 expressing cells are capable of proliferation and act to expand BAT mass during cold exposure or β3-adrenergic stimulation. UCP-1 expressing cells, labeled by GFP, first appear early during embryogenesis at E14.5 and increase until E16.5 during a presumably rapid expansion of BAT. The number of GFP positive cells then decreases in neonates and adults. When stimulated by cold exposure or β3-adrenergic agonist treatment, the number of GFP positive cells increases greatly in a concentration dependent manner. Overall, UCP-1 positive cells divide to expand BAT mass during development as well as in adults. Further uncovering the gene expression profile of these proliferative UCP-1 positive cells will help to uncover the molecular mechanisms underlying BAT expansion and may lead to potential obesity therapeutics.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Animals

The Pref-1-rtTA mouse was generated via insertion of the reverse tetracycline transactivator protein (rtTA, or Tet-On) directly downstream of -6.0 Kb of the Pref-1 promoter. C57Bl/6J (C57Bl6/J; stock no. 000664) tetO-HIST1H2BJ/GFP)47Efu/J; (TRE-H2BGFP, stock no. 005104, B6.Cg-Tg(tetO-Cre)1Jaw/J; (TRE-CRE, stock no. 006234), and B6;129S-Gt(ROSA)26Sortm34.1 (CAG-Syp/tdTomato)Hze/J; (Rosa26-flox-stop-flox-tdTomato, stock no. 012570) mice were purchased from the Jackson Laboratory. All animal studies were performed under the guidance of UC Berkeley ACUC and OLAC regulations. Unless otherwise noted, all mice used were age-matched male littermates, with the exception of embryonic studies during which the gender was not determined. Dox was provided in the drinking water (1 mg/mL in 3%

sucrose) and water was refreshed twice per week. For β3-adrenergic stimulation, CL-316,243 (0.25 µg/g BW) was dissolved in saline and injected introperitoneally once per day for 5 or 10 days. Control mice received the same volume of saline as treated mice.

Immunostaining and Immunocytochemistry

Whole embryos or tissues were flash frozen in O.C.T. (Sakura) and sectioned on a Leica CM3050S Cryostat in 10 or 12 µm thick sections, and collected on Superfrost + coated glass slides (Fisher). Frozen sections were stored at -20˚C until staining. Briefly, slides were allowed to warm to room temperature for 5 minutes, fixed in 4% formalin phosphate buffered saline for 30 minutes at room temperature, blocked in 5% BSA in PBST (PBS with 0.025% Triton X-100), and incubated with the appropriate antibody diluted in 2.5% BSA in PBST overnight at 4˚C. Slides were washed 3 times in PBST, and incubated with secondary antibody for 2 hours at room temperature. Slides were then washed twice in PBST, and then stained with DAPI for three minutes, washed once, and mounted in 15% glycerol in PBS with glass coverslips.

Microscopy

Laser Scanning Confocal Microscopy was performed using a Zeiss LSM710 running Zen 2010 software equipped with 10X and 20x air objectives, and 40X, 63X and 100X oil immersion objectives using standard excitation wavelengths for DAPI, GFP (or Alexafluor 488), tdTomato (or Alexafluor 594). Raw data was processed using NIH imageJ software (http://imagej.nih.gov/ij/, 1997-2012).

Antibodies

Primary antibodies for immunostaining were: Ki67 (rabbit, Abcam Lot# GR34956-2, 1:300), PPARγ (rabbit, SantaCruz Lot# L1709, 1:100), UCP-1 rabbit, Sigma-Aldrich, 1:1000). Secondary antibodies were goat-anti-rabbit Alexafluor488/594, chicken-anti-rat Alexafluor488/594, goat-anti-mouse Alexafluor 488/594, chicken-anti-goat Alexafluor488/594 (Invitrogen, 1:1000).

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CHAPTER IV

CONCLUSION

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Conclusion

Until recently, the development of adipose tissue was relatively unknown, this has changed due to the increase in obesity, which is associated with increased risk of a variety of detrimental health problems such as cardiovascular disease, cancer, and diabetes.[1–4] The perception of adipose tissue as merely a storage space for excess energy has drastically changed, and adipose is now well recognized to be a major endocrine organ, capable of plasticity, adaptation, and cytokine release, all necessary for metabolic homeostasis.[5–7] The adipose organ is organized into two major types of tissue, white and brown. White adipose tissue (WAT) is the major site for energy storage, and accumulates triglyceride in large lipid droplets. While WAT performs lipolysis for the provision of fatty acids for other tissues during times of energy need, BAT is capable of burning fatty acids for thermogenic activity.[8,9] Non-shivering thermogenesis in BAT is achieved via the uncoupling protein 1 (UCP-1), which permeabilizes the inner mitochondrial membrane to allow inter-membrane protons to leak back into the mitochondrial matrix, thus uncoupling respiration from ATP synthesis.[10] A recently appreciated presence of brown adipose tissue (BAT) in humans, which is capable of dissipating energy as heat, has generated much interest in the anti-obesity potential of BAT.[11,12] Despite major advances in the field, the basic biological process of adipose tissue development, as well as its ability to continuously expand, is not fully understood (Figure 27).

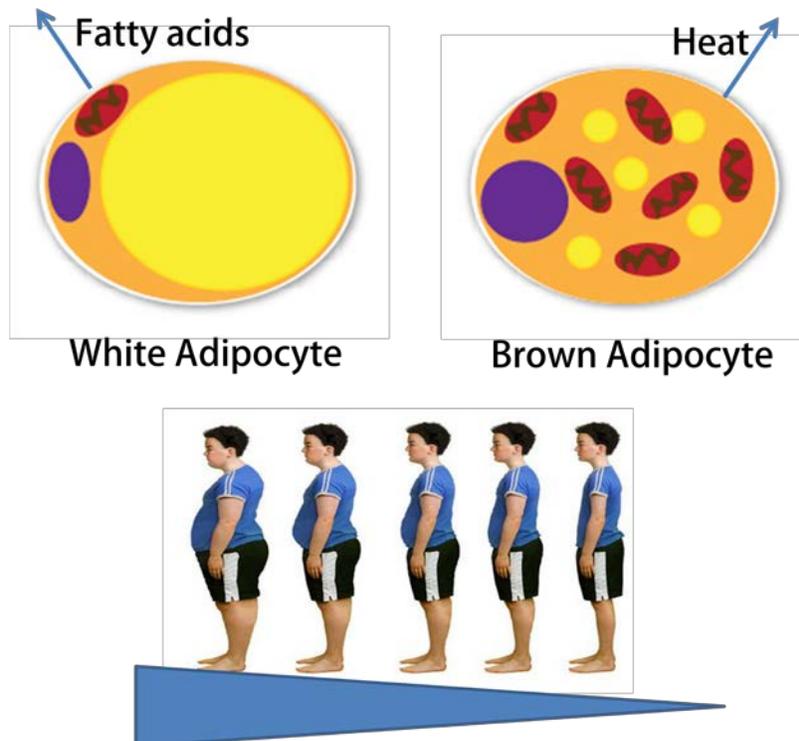**Figure 27.** White adipocytes store excess energy as triglycerides in a large lipid droplet and can release fatty acids through lipolysis during times of need. Brown adipocytes perform non-shivering thermogenesis by uncoupling respiration from ATP synthesis. Obesity is caused by increased white adipose tissue mass whereas increasing brown adipose tissue mass may be a potential anti-obesity therapeutic target.

Adipose tissue is capable of expansion through hypertrophy of existing adipocytes as well as hyperplasia of preadipocytes differentiating into adipocytes. The process of differentiation of preadipocytes into adipocytes has been studied in detail, and many factors have been identified that prevent or promote differentiation.[13–21] Furthermore, the origin of WAT and BAT is still being elucidated, and recent evidence points to a shared origin of muscle and BAT, but a unique origin of WAT.[22,23] Adipose stem cell populations have been isolated from the stromal vascular fraction (SVF) of adipose tissue and have been characterized using various markers.[24] It has been proposed that within WAT, progenitors with adipogenic potential may reside near the vasculature and that they may be of endothelial and/or pericyte origin.[25,26] However, the definitive origin of adipocytes is yet to be understood. Furthermore, the identity, location, and emergence of adipose tissue early during development, are not known. Lineage tracing has often been used in other systems,[27–30] and, by using the PPARγ locus, Tang *et al* first showed that adipose progenitors express PPARγ and they reside near the vasculature.[31] In this regard, PPARγ is expressed not only in preadipocytes but also in differentiated adipocytes, which makes it tricky to discern adipose precursors. Though recent reports indicated that adipocytes may come from endothelial cells or pericytes,[32] the definitive origin of adipocytes is highly debated. In this regard, Pref-1 has been used as a preadipocyte marker, and is notable in its ability to prevent adipocyte differentiation in an autocrine/paracrine manner. Pref-1 may be a valuable tool as a marker of precursor cells and we have employed Pref-1 to label adipose precursors for lineage tracing and investigation of adipose tissue development (Figure 28).
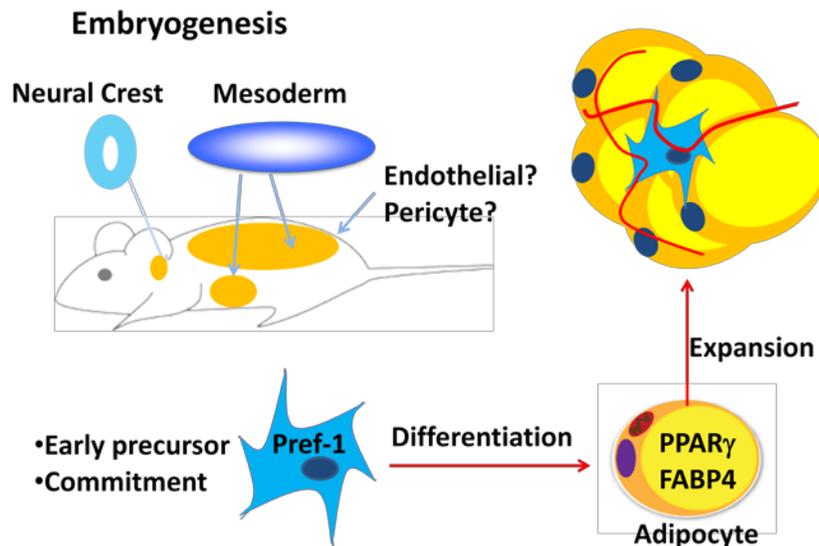

Figure 28. Adipose precursors are thought to be of mesodermal origin, though some may develop from the neural crest, and precursors isolated from adipose tissue have been found to be potentially endothelial or pericyte in origin. Early precursors undergo commitment to preadipocytes, and Pref-1 is expressed in undifferentiated cells in the adipose tissue. Preadipocytes undergo differentiation in a well-characterized manner, and express markers such as PPARγ and FABP4. In adults, adipose tissue is capable of expansion through hypertrophy of existing adipocytes or hyperplasia of preadipocytes differentiating into adipocytes.

Labeling of Pref-1 cells provided insight into the development of white adipose tissue, but there are still questions that remain about the definitive origin of adipocytes. By permanently labeling Pref-1 expressing cells, all adipocytes were labeled indicating that all adipocytes come

from a Pref-1 expressing cell origin. However, it is possible that Pref-1 expressing cells are playing a unique role during embryogenesis. Cells with an endothelial or pericyte origin have been demonstrated to differentiate into adipocytes following isolation from adult adipose tissue. Potentially, adipose precursors express Pref-1 prior to commitment to a perivascular origin, or there is another population of precursors that begin as perivascular cells and later express Pref-1. Pref-1 is expressed at a low level in brown adipose tissue as well, and may play a role in brown adipose tissue development. Since classical brown fat cells come from a myogenic precursor population, it is possible that inducible brown cells come from a unique population of precursors and pre-exist in the white adipose tissue before they are activated by cold exposure or β3-adrenergic agonist treatment. Inducible brown cells may come from the same origin as white adipocytes, and therefore, Pref-1 precursors may differentiate into this unique type of adipocyte. Overall, it is important to fully characterize the gene expression profile of the Pref-1 expressing precursors as well as the UCP-1 expressing cells that differentiate into adipocytes to understand and potentially regulate their differentiation. By combining these transgenic mouse models, we can potentially uncover the developmental origins of both white and brown adipose tissue.

Understanding the developmental process of brown adipose tissue and characterizing the cells that are capable of increasing brown adipose tissue mass are major goals of current adipose biology.[33] In mammals, WAT mass increases throughout life, whereas BAT decreases. However, BAT mass can increase in response to chronic cold exposure or β3-adrenergic stimulation. Whether BAT mass increases by proliferation of preadipocytes, or proliferation of existing UCP-1 expressing brown adipocytes has not been fully determined. Overall, we conclude that UCP-1 expressing cells are capable of proliferation and act to expand BAT mass during cold exposure or β3-adrenergic stimulation. UCP-1 expressing cells, first appear early during embryogenesis at E14.5 and increase until E16.5 during a presumably rapid expansion of BAT. Overall, UCP-1 positive cells divide to expand BAT mass during development as well as in adults. Further uncovering the gene expression profile of these proliferative UCP-1 positive cells will help to uncover the molecular mechanisms underlying BAT expansion and may lead to potential obesity therapeutics.

The prevalence of obesity has been increasing at a rapid rate over the past few decades, and many have sought adipose tissue as a potential anathema for obesity. Though it is well understood that diet and energy imbalance play a huge role in obesity progression, the regulation of whole body metabolism is highly complex. The role that adipose tissue plays in energy balance is just beginning to be appreciated. The massive expansion of adipose tissue that occurs during obesity is associated with a loss of normal regulation of both the function and adipokine secretion by adipose tissue, leading to dysregulation of energy balance. Furthermore, fatty acid synthesis and triacylglycerol synthesis play a major role in the accumulation of lipid within adipose tissue, and it is crucial to determine the molecular mechanisms of these processes. Although the field of adipose tissue biology has made great progress, we still lack consensus on the origin of bona fide adipocyte precursors, as well as markers that can distinguish mesenchymal stem cells from preadipocytes. There are also no known markers to identify the various stages of adipocyte differentiation. The lineage differences between brown and white adipocytes, and the recently identified inducible brown adipocytes, are newly emerging areas of research. Controlling the numbers of each adipocyte type within a specific depot and their differentiation could provide potent obesity treatment options. By defining and distinguishing adipocyte lineage, we could uncover the developmental origins of adipose tissue, and make great strides in treating the obesity.

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