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Origin of White and Brown Adipose Cells From Vascular Endothelium: A Dissertation

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ORIGIN OF WHITE AND BROWN ADIPOSE CELLS FROM VASCULAR ENDOTHELIUM

A Dissertation Presented

By

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I dedicate this work in love and gratitude to my family

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ABSTRACT

Obesity is associated with insulin resistance, dyslipidemia, and cardiovascular disease. The current obesity epidemic is the result of surplus energy consumption. Excess energy is stored in expanding adipose tissue. Adipose tissue growth entails the enlargement of existing adipocytes, the formation of new fat cells from preexisting progenitors, and the coordinated development of supporting vasculature. Identifying adipocyte progenitors and the mechanism of adipose tissue expansion is crucial for the development of new strategies to combat obesity and its complications.

Though important progress has been made towards understanding the developmental origin of adipocytes, the identities of adipocyte progenitors are still not completely known. The main objective of this study is to determine whether endothelial cells of the adipose tissue can give rise to new adipocytes. Our results indicate that murine endothelial cells of adipose tissue are pluripotent and can potentially give rise to preadipocytes. Lineage tracing experiments using the VE-Cadherin-Cre transgenic mouse reveal localization of reporter genes in endothelial cells, preadipocytes and white and brown adipocytes. Moreover, capillary sprouts from human adipose tissue, which have predominantly endothelial cell characteristics, are found to express Zfp423, a preadipocyte determination factor. In response to PPARγ activation, endothelial characteristics of sprouting cells are progressively lost, and cells form structurally and biochemically defined adipocytes. Taken together, our data support an endothelial origin of a population of adipocytes. The ability of the vascular endothelium to give rise to

adipocytes may explain how angiogenesis and adipogenesis can be temporally and spatially coordinated.

Analysis of BAT and WAT revealed that adipose depots have distinct compositions of adipocyte progenitors. Of the CD45-CD29+Sca1+CD24+ progenitor population, only 17% and 52% express VE-Cadherin in WAT and BAT, respectively. Our data show that the number of these specific progenitors in BAT and WAT are highly variable and suggest that a considerable number of adipocytes progenitors may have a non-endothelial cell origin. Differences in composition and types of adipocyte progenitors may explain the differences in the adipocytes phenotypes that we observe in discrete depots.

In brief, we find that the vascular endothelium gives rise to a population of brown and white fat cells, and that the number of endothelial-derived adipocyte progenitors residing in BAT and WAT is highly variable. These results expand our current understanding of adipose tissue growth, and, we hope, will accelerate the development of treatments for obesity-related complications.

CHAPTER I: Adipose Tissue in Human Health and Disease

"Thou seest I have more flesh than another man, and therefore more frailty"

William Shakespeare. Henry IV, Part I

Introduction

Only recently in human history has the association between high body weight and poor health become readily apparent. For much of history, a combination of natural and artificial disasters caused mankind to be preoccupied with under-nutrition and disease-induced anorexia [1]. Drought, floods, blights, plagues, and human nature have made famine a frequent occurrence documented in ancient times through the writings of Plato, Thucydides, and Aristotle [2].

Famine has contributed to the downfall of many great civilizations [3]. The fall of the Roman Empire was partly attributed to famines brought on by severe transportation and communication challenges, which ultimately caused the deaths of thousands [4]. The infamous “Bread and Circuses” strategy of Roman emperors sprung from efforts to distribute free food in an attempt to avoid social unrest. Traditionally how well empires dealt with food shortage problems was directly correlated to their longevity [1].

To the same extent, how well an organism deals with periods of starvation is tied to its survival over time. It is conceivable that an organ would evolve to meet the energy demands of an organism during periods of ecological instability. In humans and other vertebrate animals, this organ is the adipose tissue. The accumulation of fuel in discrete adipose depots allow for a better regulation of energy balance. First, adipose depots can be regulated by the central nervous system (CNS) specifically and efficiently via molecular signaling. Second, the adipose tissue itself can act as a metabolic sensor and effector by signaling to the brain [5]. Formation of a peripheral organ that can store fuel and interact with the CNS to meet energy needs allows an organism to adapt to the reality of an uncertain food supply. This work aims to

provide insight on the mechanism of adipose tissue growth. To give context and establish the importance of fat cell biology, we will briefly discuss the current obesity epidemic and prevailing theories regarding the role of adipose tissue in human health and disease.

Obesity Epidemic

Through 19th century, the significance of adipose tissue was most apparent in times of under-nutrition [1]. However, it is evident in the present obesity epidemic that adipose tissue function is also of particular importance in time of over-nutrition. In 2008, the World Health Organization (WHO) estimated that 1.5 billion adults worldwide were either obese or overweight. Obesity and overweight status are defined in terms of body mass index (BMI), the ratio between weight (kg) and the square of height (m^2). Those with BMI ≥ 30 are obese and those with BMI ≥ 25 are overweight. According to Center for the Disease Control and Prevention (CDC), 35.7% of adults in the US are obese (Figure 1.1). Approximately 12.5 million children are obese and it is forecasted that life expectancy will either decrease or no longer increase due to the significant disease burden associated with increase adiposity [6]

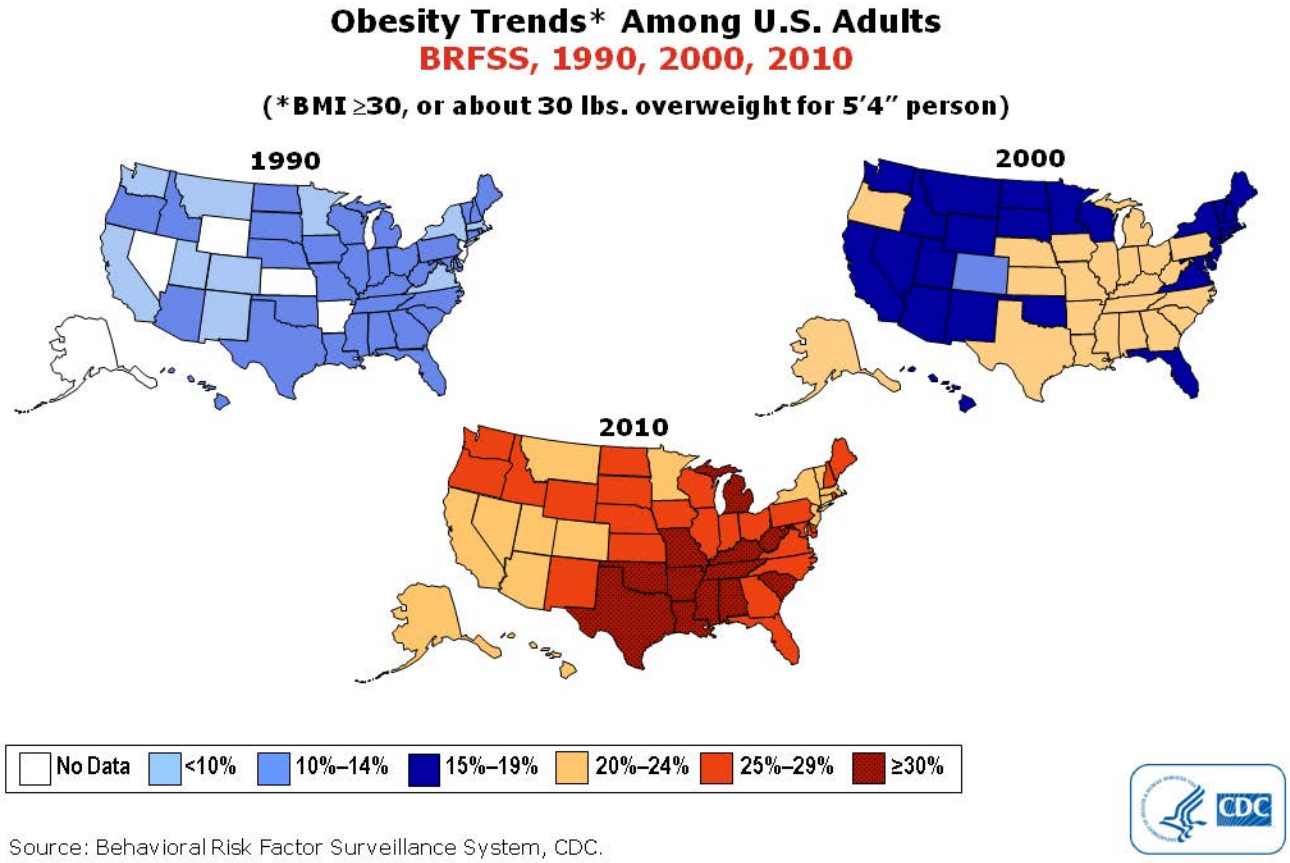


Figure 1.1. **Rapid rise in the prevalence of obesity in the United States**. Statistics reported by the CDC on the prevalence of obesity in adults in the US. Adapted from the CDC website (www.cdc.gov/obesity).

It is well established that obesity increases the risk of type II diabetes (T2D), hypertension, hyperlipidemia, ischemic heart disease and cancer [7-10]. The correlation between obesity and T2D is clearly mapped by epidemiological data (Figure 1.2). An increase of 2 in BMI in overweight males can potentially reduce their lifespan by approximately one year [11-13]. Currently, our nation spend 147 billion dollars to treat obesity-related illnesses with the poor being most affected [14]. Thus, the obesity epidemic, like famine is a significant social and biological crisis that must be addressed.

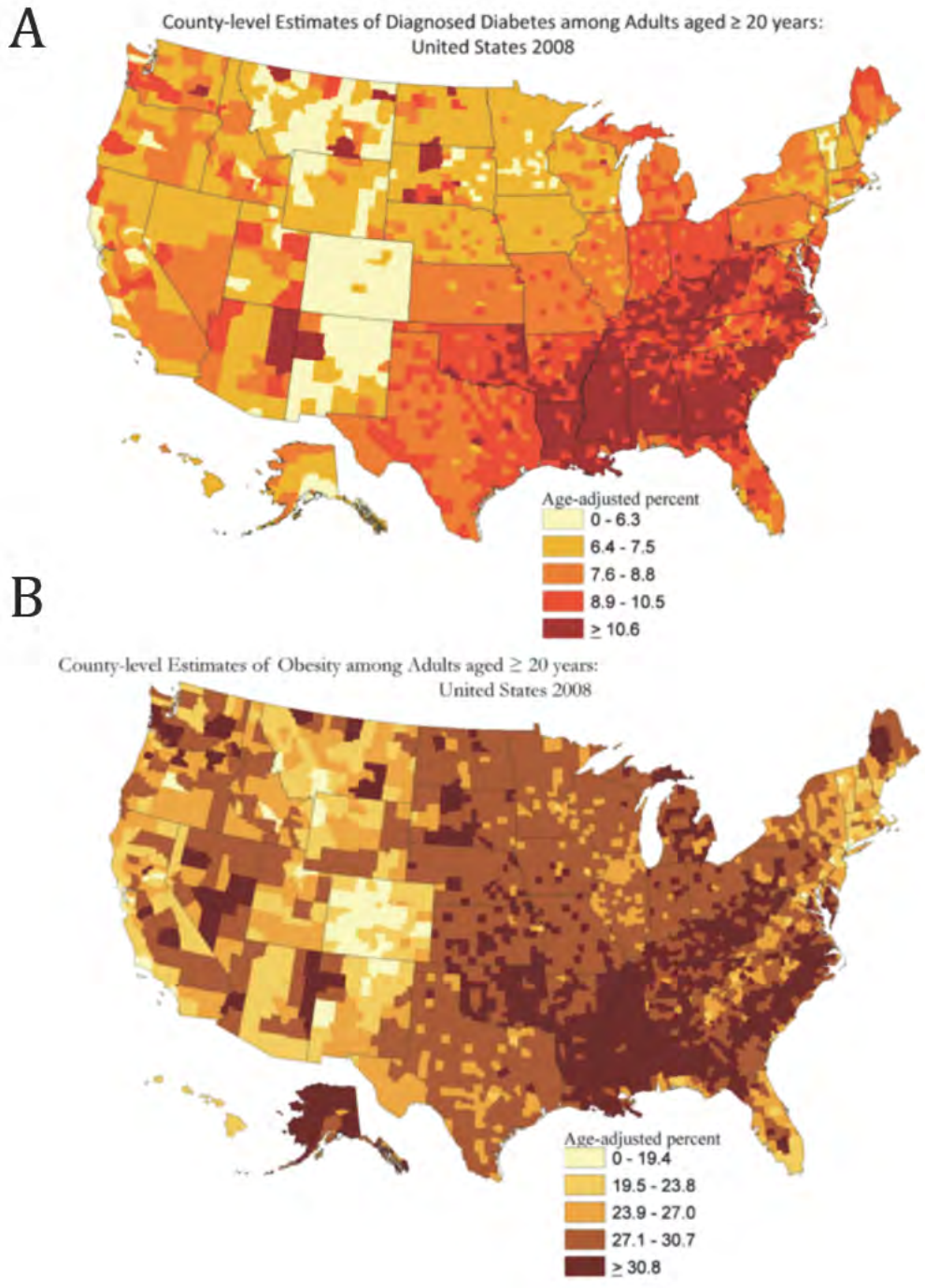


Figure 1.2. Correlation of obesity and diabetes. Statistics reported by the CDC about the concurrent rise of obesity and diabetes. Adapted from the CDC website (www.cdc.gov/diabetes).

Obesity from an evolutionary perspective

Thrifty genotype: There are several schools of thought regarding the cause of the growing obesity epidemic. In 1962, James Neel wrote a seminal paper suggesting that 'genetic thrift' was the driving force behind our currently pandemic [15, 16]. He argued that perhaps people who were more efficient at storing energy were better suited for survival in times of 'feast and famine'. According to Neel, natural selection would favor those with thrifty genotypes.

The agricultural and technological revolutions drastically changed *homo sapiens* environment and lifestyle [17]. We are now able to consume more energy and exert less using technological advances. Furthermore, improvements in heating and clothing have drastically reduced our need to expend energy to keep ourselves warm via adaptive thermogenesis [18-21]. In the new environment, the thrifty genotype is maladaptive as it carries a higher risk of developing T2D. Neel pointed out that perhaps obesity was like sickle cell anemia, except multifaceted and is not traced to a single allele. Much like obesity, at some point it was evolutionarily advantageous to carry the sickle cell gene, however, this trait has become maladaptive in modern times and now leads to severe health complications [15].

The conceptual underpinnings of Neel's hypothesis are true to some extent, because we do know that genes determine body weight and fat distribution as well as the development of T2D (Figure 1.3 and 1.4) [22]. Although a clear genetic link between obesity and T2D has been difficult to establish, certain genes have been implicated in both obesity and diabetes. For example, those who are homozygous for the affected allele of the fat-mass and obesity-related (FTO) gene are at higher risk for

diabetes and also at risk to be 2-3 kg heavier than those homozygous for the variant allele (Figure 1.5) [23-27].

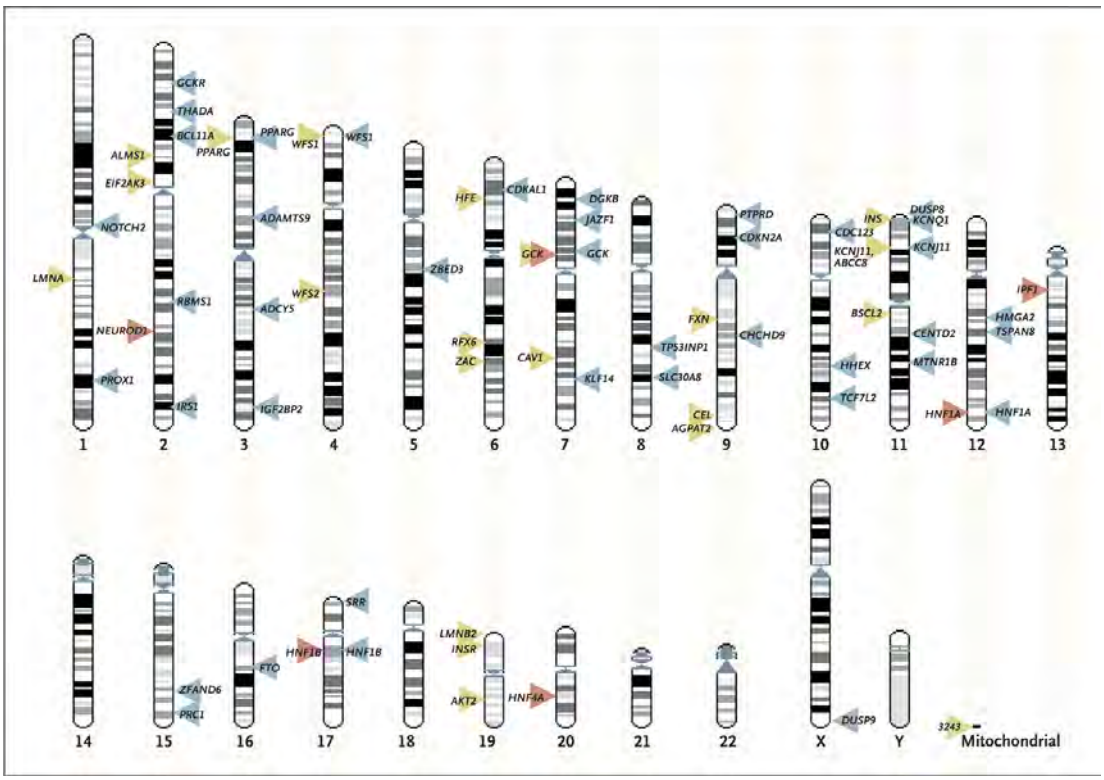


Figure 1.3. Genomic Locations of Proven Signals of Nonautoimmune Forms of Diabetes. Signals are shown according to their location on each chromosome. Genes causing monogenic and selected syndromic forms of diabetes are shown to the left: genes implicated in maturity-onset diabetes of the young (red triangles) and those representing loci causal for other monogenic and syndromic forms of diabetes (green triangles). Common variants that have significant genomewide associations with multifactorial forms of diabetes are shown to the right (blue triangles); for these variants, the genes named within the triangles are indicative of signal position, but in most instances, formal proof that these are the specific genes responsible for the association is lacking. Adapted from McCarthy, M.I. (2010). Genomics, type 2 diabetes, and obesity. N Engl J Med *363*, 2339-2350.

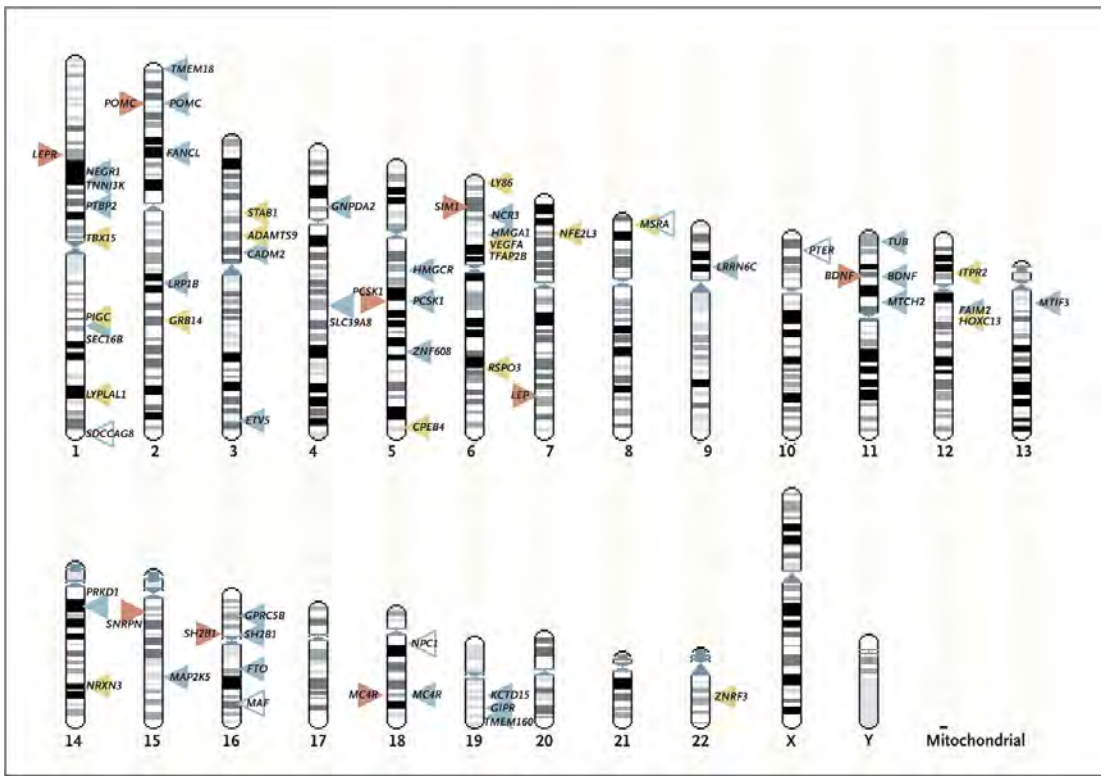


Figure 1.4. Genomic Locations of Proven Signals of Body-Mass Index (BMI), Obesity, and Related Phenotypes. Signals are shown according to their location on each chromosome. Genes causing monogenic and selected syndromic forms of obesity (red triangles) are shown to the left. Common variants that have significant genomewide associations with BMI or multifactorial obesity are shown to the right: loci implicated in BMI or weight variation at the population level (solid blue triangles), additional loci identified in case–control analyses of extreme obesity (open blue triangles), and variants identified primarily because of their association with waist circumference or waist-to-hip ratio (solid green triangles). For the variants shown to the right, the genes named within the triangles are indicative of signal position, but in most instances, formal proof that these are the specific genes responsible for the association is lacking. Adapted from McCarthy, M.I. (2010). Genomics, type 2 diabetes, and obesity. N Engl J Med *363*, 2339-2350.

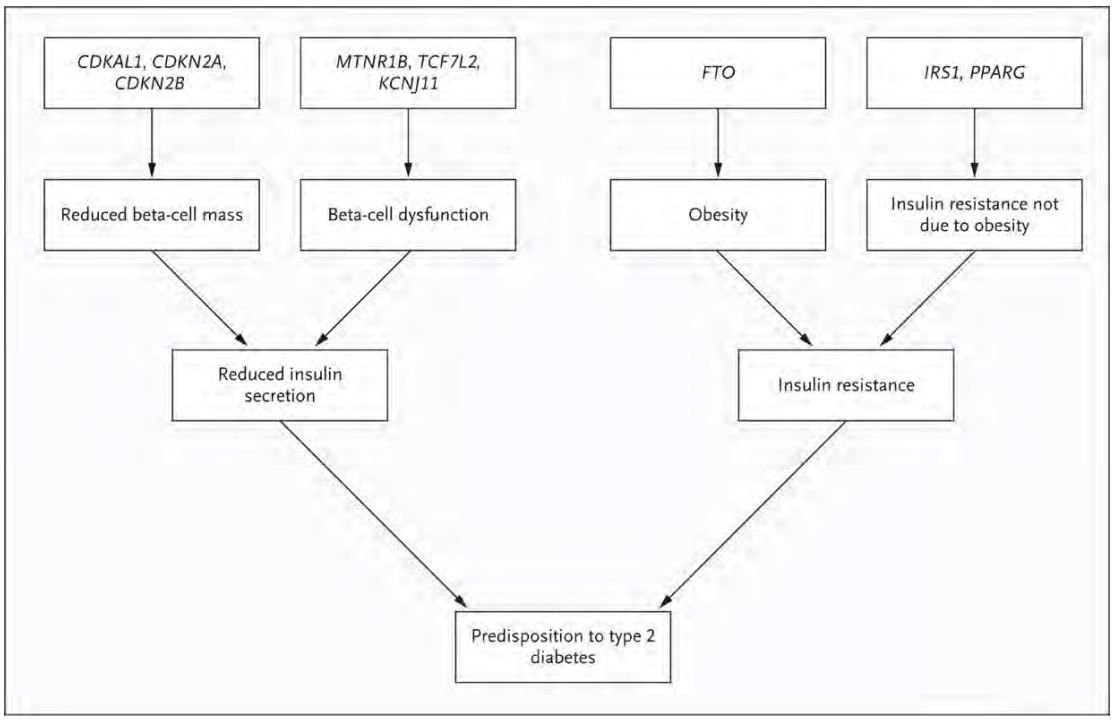


Figure 1.5. **Pathways to Type 2 Diabetes Implicated by Identified Common Variant Associations.** Type 2 diabetes results when pancreatic beta cells are unable to secrete sufficient insulin to maintain normoglycemia, typically in the context of increasing peripheral insulin resistance. The beta cell abnormalities fundamental to type 2 diabetes are thought to include both reduced beta cell mass and disruptions of beta-cell function. Insulin resistance can be the consequence of obesity or of obesity-independent abnormalities in the responses of muscle, fat, or liver to insulin. Examples of susceptibility variants that, given current evidence, are likely to influence predisposition to type 2 diabetes by means of each of these mechanisms are shown. Adapted from McCarthy, M.I. (2010). Genomics, type 2 diabetes, and obesity. N Engl J Med *363*, 2339-2350.

Thrifty Phenotype: An alternative to Neel's hypothesis is posed by David Barker, who argues that exposure to malnutrition *in utero* results in disturbances that lead to the impaired glucose tolerance and other metabolic syndromes [16, 28, 29]. Epidemiological data support the Barker hypothesis. In 1944-1945, World War II devastated Western parts of Netherlands and famine ensued. During the Dutch famine, food was rationed and dairy intake dropped below 1000 calories [30]. It was found that fetal exposure to malnutrition during the first half of gestation resulted in significantly higher obesity rates in men and decreased glucose tolerance in adults [31, 32]. Others have found that low birth-weights were associated with higher rates of diabetes [33-35]. Furthermore, studies in animal models involving nutrient restrictions and high-caloric feeding revealed that maternal and paternal health have profound and lasting effects on fetal endocrine function [36-38]. Several pieces of evidence suggest that fetal adaptation to uterine environment may negatively affect phenotype in adult life.

Predator Release: Although Neel and Barker suggest different hypotheses for the alarming increase in obesity, they both cite adaptive mechanisms. A non-adaptive hypothesis has been suggested recently by John Speakman [39]. Speakman states that adaptive hypothesis might be flawed because a large population is still normal-weight. If indeed nature favored those especially efficient at storing fat, we would see high occurrences of obesity during historical periods of "feast." His "predatory release" theory posits that the recent rise in obesity is instead due to genetic drift. In the 'hunter-and-gather' days, predation selected against obese people. In the absence of predation, random mutations and genetic drifts could explain the rapid emergence of obesity [17].

Adipose Tissue

The mechanism by which obesity has rapidly emerged is one of the most pressing public health issues of our era and is still hotly debated. However, the fact that it negatively impacts human health and happiness is undisputable. At the center of all discussions and of this body of work is the adipose tissue. There are two established types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT).

White Adipose Tissue: Excess caloric intake is stored in WAT, which is widely distributed in visceral and subcutaneous deposits [40]. Visceral fat depots are found in intra-abdominal, perigonadal and retroperitoneal regions. Subcutaneous fat depots are mostly found in the gluteal, inguinal and abdominal regions. Histologically, white adipocytes appear spherical with variable size, depending on the lipid droplet (unilocular) within them. Mitochondria present in white adipocytes are thin and elongated (Figure 1.6) [41].

WAT has two primary functions. First, it stores energy in the form of triglycerides in times of surplus and releases energy in the form of fatty acids in times of fasting. Second, it serves as the largest endocrine organ in the body, secreting adipokines such as leptin and adiponectin that are vital in regulating energy balance.

Brown Adipose Tissue: Adipose tissue is composed of not only white adipocytes, but also brown adipocytes, which are involved in energy expenditure rather than energy storage. BAT is present in the axillary, cervical, perirenal and periadrenal regions of newborns [42]. Although it was once thought that BAT becomes relatively insignificant in older people, Cypress *et al.* recently demonstrated through [^{18}F]-2-fluoro-D-2-deoxy-D-glucose (FDG) positron emission tomography

(PET) that metabolically active BAT is present in the cervical, supraclavicular, axillary and paravertebral regions of normal adults [43]. Others have since confirmed that BAT exists in human adults [19, 20, 42, 44, 45]. Histologically, brown adipocytes are polygonal and have many small lipid droplets (multilocular) within them. Their mitochondria are abundant and appear large, spherical and filled with laminar cristae (Figure 1.6) [41].

Brown adipocytes express uncoupling protein-1 (UCP-1), a 32 kDa protein that permits protons to leak through the inner mitochondrion membrane [46]. It uncouples ATP synthesis from the electron transport chain, allowing production of heat rather than ATP from the electrochemical gradient [47]. UCP-1 is specific to BAT and is entirely responsible for non-shivering thermogenesis [48]. In humans, BAT function is inversely correlated with obesity [21, 49], while mice genetically engineered to have reduced brown fat develop T2D and obesity [50, 51]. Because of its anti-obesity effect, induction of BAT in humans represents a potential tool in the prevention of obesity and its metabolic consequences

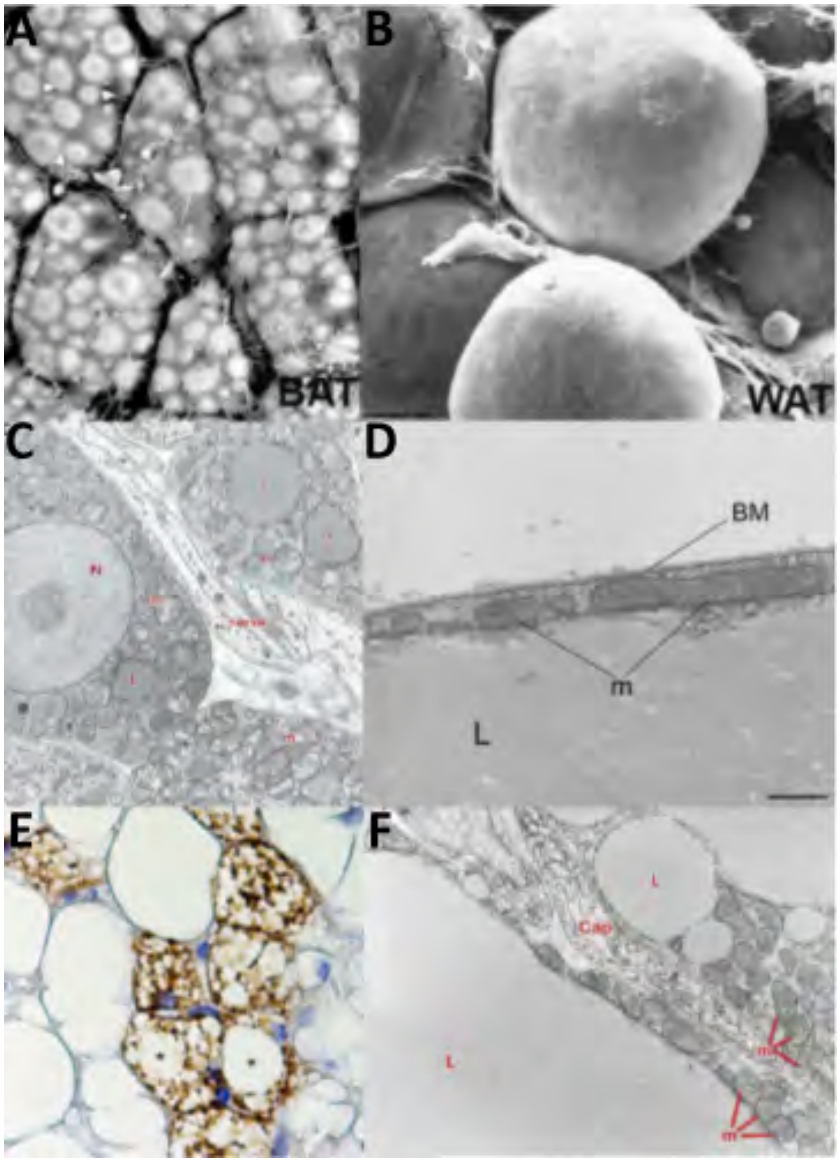


Figure 1.6. Histology of BAT and WAT. Brown adipocytes are multilocular with abundant spherical mitochondria (**A** and **C**) and white adipocytes have unilocular lipid droplets and flattened mitochondria (**B** and **D**). In some WAT, there is presence of brown adipocytes, indicated by expression of UCP-1, distinct multilocular appearance and spherical mitochondria (**E** and **F**). m: mitochondria, l: lipid droplet, m: mitochondria, n: nucleus, cap: capillary. Modified from Frontini, A. and S. Cinti, *Distribution and development of brown adipocytes in the murine and human adipose organ.* Cell Metab, 2010. **11**(4): p. 253-6 and Cinti, S., *Transdifferentiation properties of adipocytes in the adipose organ.* Am J Physiol Endocrinol Metab, 2009. **297**(5): p. E977-86.

Stromal Vascular Fraction of Adipose tissue: White adipocytes and brown adipocytes are supported by cells in the stromal vascular fraction (SVF) of the adipose tissue. The SVF loosely defines a population of cells isolated from adipose tissue via collagenase digestion. It is comprised of all non-adipocyte cells, including preadipocytes, autonomic nerves, vasculature, leukocytes and connective tissue. The SVF plays a crucial role in supporting the maintenance and expansion of the adipose tissue.

Obesity and diabetes at the molecular perspective:

At the molecular level, it is thought that adipose tissue dysfunction in obesity leads to insulin resistance. Although this hypothesis is still evolving, it is widely accepted that obesity-related insulin resistance is in part due to (1) a sequestration defect that leads to free fatty acid (FFA) circulation, deposition and subsequent disruption of insulin signaling and (2) changes in adipokine secretion that impair adipogenesis and decrease peripheral insulin sensitivity [52].

Lipotoxicity: The hypothesis that FFA circulation and deposition in peripheral organs cause insulin resistance is supported by studies demonstrating that short-term FFA infusion acutely induces insulin resistance. This is further corroborated by findings that intra-myocyte lipid content correlate positively with insulin resistance better than BMI measurements [53-55]. Consistent with this conclusion is the observation that patients who have congenital lipodystrophies have high levels of circulating triglycerides and are also insulin resistant [56]. Mouse lipodystrophic models also show glucose impairment in the absence of fat [57, 58]. Although it is widely accepted, Karpe and colleagues recently challenged the hypothesis that there is

higher plasma concentration of FFA in cases of obesity and diabetes. They found that when normalized for mass, adipose tissue FFA release is decreased [59]. It was also noted that women tend to have higher FFA than men, however they are more insulin sensitive.

Inflammation: Besides the "lipotoxic" effect, adipose tissue dysfunction can also lead to perturbation in adipokine secretion to cause insulin resistance. Patients who are obese have lower levels of plasma adiponectin, an insulin sensitizer [60, 61]. In the initial stages of obesity, adipocytes grow in size to accommodate excess nutrition. It has been found that hypertrophied adipocytes secrete higher level of chemo-attractants to recruit immune cells [52, 62]. Inflammation of the visceral adipose tissue has been correlated with insulin resistance [63]. In obese patients, macrophages can represent as much as 40% of cells [64]. Macrophages and adipocytes themselves in turn secrete inflammatory signals, such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β). TNF-α has been show to negatively affect insulin substrate receptor (IRS) Ser/Thr phosphorylation, a key mediator of insulin signaling [52, 65].

Beyond impairing insulin signaling, TNF-α also down-regulates peroxisome proliferator-activated receptor γ (PPARγ) at the transcriptional and post-translational levels. PPARγ is a transcription factor that is necessary and sufficient for adipogenesis [66]. It is required for the development and maintenance of adipose tissue and is important in many tissues as demonstrated by numerous knock-out studies (Table 1). Thus, not only does TNF-α disrupt insulin signaling, it impairs the formation of new healthy adipocytes to help regulate metabolism.

Table 1. PPARγ knock-out mouse models*

Tissue	Study	Approach	Phenotype	Comments
Whole body	Barak *et al*., 1999 [67]	Insertion of *lacZ*-neomycin resistance cassette upstream of DNA-binding domain	Embryonic lethal due to impaired placental vascularization and trophoblast development	Shows that PPARγ is necessary for maintenance of adipocytes
	Imai *et al*. 2004 [68]	aP2-CreER^{T2}/ PPAR$^{L2/L2}$	Lipodystrophy, tamoxifen-induced necrosis of mature adipocytes and subsequent formation of new adipocytes	
	Duan *et al*, 2007 [57]	Rescued the embryonic lethality by breeding Mox2-Cre mice with floxed-PPARγ mice to delete PPARγ in the embryo proper	Severe lipodystrophy, insulin resistance and hypotension	
Adipose	He *et al*., 2003 [69]	aP2-Cre/PPARγ$^{f/f}$	Progressive lipodystrophy, decreased plasma leptin and adiponectin levels, increased plasma FFA and triglycerides levels. On high-fat diet (HFD), developed hepatic steatosis, hyperinsulinemia, and insulin resistance (IR) compared to controls.	TZD improved insulin-stimulated muscle glucose disposal rates, but did not lower FFAs as seen in controls.
	Jones *et al*., 2005 [70]	aP2-Cre/PPARγ$^{lox/lox}$	Lipodystrophy, decreased plasma leptin and adiponectin but did not developed IR or glucose intolerance on HFD	Suggested that discrepancies in phenotype with previous study are due to different transgenes used in the aP2-Cre mouse and that hepatic compensation prevents IR development on HFD

Endothelial cells	Nicol *et al.* 2005 [71]	Tie2Cre/ PPARγ$^{fl/fl}$	HFD-induced hypertension	TZD did not improve HFD-induced hypertension
	Kleinhenz *et al*, 2009 [72]	Tie2Cre/ PPARγ$^{fl/fl}$	Hypertensive. Impaired Ach-induced NO production and vasorelaxation	
	Kanda *et al.*, 2009 [73]	Tie2Cre/ PPARγ$^{fl/fl}$	Increased insulin sensitivity, fasting plasma FFA and TG levels, decreased TG accumulation in skeletal muscle as compared to WT mice. Fat mass reduced compared to WT on HF diet.	TZD failed to increase epididymal fat mass in knockouts as seen in controls. Also, did not lower plasma FFA and triglyceride levels, suggesting direct action on endothelial cells.

*Table displays a limited list of models that are most relevant to this study

Although the mechanisms by which obesity leads to diabetes are still debated on the genetic and molecular levels, it is clear that the healthy adipocytes are integral to metabolism. It would follow that generating healthy adipocytes would be beneficial in at least two ways, first by sequestering free fatty acid and decreasing lipotoxicity and second by normalizing adipokines secretion to maintain insulin sensitivity. This study aims to establish the origin of new adipocytes.

Adipocyte expansion:

Adipocytes expand either by multiplying in numbers (hyperplasia) or by increasing in size (hypertrophy) [74-76]. Analysis of subcutaneous fat and visceral fat has led many researchers to conclude that hyperplastic growth may have a protective effect on metabolic dysfunction [77, 78]. While increase in visceral fat seen in abdominal obesity is associated with metabolic disease, increase in subcutaneous fat seen in peripheral obesity is associated with metabolic health [79-83]. Rate of proliferation of precursors isolated from subcutaneous adipose tissue is higher than that of visceral adipose tissue, and thus it is indirectly concluded that hyperplastic growth is metabolically beneficial [77, 78, 84].

New adipocyte formation is part of physiological growth. Experiments using radioactive tracers revealed that new adipocytes form and die throughout life [85-87]. Through analyzing adipose tissue of people who were exposed to radiation during atomic testing, Spalding *et al.* estimated that the rate of adipocyte turnover is approximately 10% per year [88]. Mature adipocytes do not divide, thus precursors exist and are constantly making adipocytes and renewing themselves in the adipose tissue.

Adipocyte precursors reside in the adipose tissue vasculature [89-91]. Adipogenesis and angiogenesis are temporally and spatially coupled in development [92-95]. Studying postnatal epididymal adipose tissue, Han *et al.* found that angiogenesis precedes adipogenesis and that impairment of the VEGF/VERGFR2 signaling pathway disrupts adipogenesis [93]. This result is further corroborated by findings from Kolonin and colleagues demonstrating that targeting adipose tissue endothelial cells with apoptotic peptide inhibited weigh gain in mice and primates fed high fat diet [96, 97]. It is important to note that some of the anti-obesity effect associated with anti-angiogenesis agents may be due to a confounding anorexic affect, and not to a direct effect on adipocyte growth and development.

Although the mechanisms by which anti-angiogenic drugs decrease weight gain are still debated, it is clear that a close relationship exists between cells in the vasculature and adipocytes. There are several microscopy studies that illustrated the unique relationship between pericytes and adipocytes. Micrographs depict the juxtaposition between preadipocytes and vasculature, some suggesting that preadipocytes might be emerging from the vasculature [98-101]. In 1983, Hausman *et al.* showed that endothelial cells of adipose tissue in rats have many processions into the lumen and perivascular space. Some of these endothelial processes are contiguous with adipocytes [102]. These morphological evidences are suggestive of a pericyte-endothelial nature of preadipocytes and adipocytes [95].

Morphological studies set the stage for the recent genetic experiments that have shed light on the identity of the adipocyte precursors. Development of novel genetic tools has allowed for the tracing of cell lineages throughout a life of the mouse [103, 104]. Tang *et al.* generated a knock-in mouse that has the tetracycline

transactivator (tTA) under the control of PPARγ [91]. Then they crossed the tTA-PPARγ mouse to one that has Cre-recombinase under the control of the tetracycline response element (TRE). The resulting transgenic animal was then mated with two reporter strains, one which as an indelible LacZ signal and the other with mitotic-sensitive GFP signal. The reporter would be active in the absence of doxycline (Dox; Tet-off) but would be inactive in its presence. Using these two models, the authors discovered that preadipocyte are present before birth, and most WAT expansion occurs postnatal.

Cells FACS sorted from PPARγ-GFP mouse for the reporter signal also expressed Sca-1 and CD34. Using stromal vascular particulate (SVP) isolation techniques, the Graff laboratory found that the GFP+ cells are located near vessels and co-localize with cells expressing pericytes markers, NG2, SMA, and PDFGRβ [91]. Based on these findings, Tang *et al.* concluded that mural cells of the adipose tissue give rise to adipocytes. To test this hypothesis they made a PDFGRβ-Cre/R26R reporter mouse. It was found that adipocytes indeed expressed LacZ in these transgenic animals, further supporting a pericytic origin of new adipocytes. Tang *et al.* reported that cells expressing PDGFRβ in the vasculature of the adipose tissue have the capacity to undergo adipogenesis when transplanted into nude mice [91]. In culture, isolated PDGFRβ+ cells from WAT, but not from other tissues e.g. kidney, were able to undergo adipogenesis in the presence of a thiazolidinedione (TZD), a PPARγ agonist. Thus, the vasculature of the adipose organ not only supports the maintenance and expansion of adipose tissue, but also harbors cells that can differentiate into adipocytes.

An important aspect to realize is that these results do not exclude the possibility that endothelial cells may give rise to new adipocytes. It is reported that endothelial cells also express PPARγ [71-73]. PDGFRβ is also expressed in endothelial cells of microvasculature and is not restricted to pericytes [105-107]. Thus, although the Graff laboratory demonstrated that pericytes can give rise to adipocytes, questions remain whether cells of an endothelial lineage have the same capacity. The first goal of this study is to address this question using morphological and genetic approaches.

Around the same time, a seminal study by Rodeheffer *et al.* revealed the identity of the early adipocyte progenitors [108]. The Friedman group used fluorescence-activated cell sorting (FACS) to isolate cells expressing stem cell markers CD29, CD34, Sca-1 and CD24 ($CD24^{+}$) from the SVF. Within this population, they also depleted cells expressing known lineage markers: CD31, CD45 and Ter119 (Lin-:CD24+). Approximately 50,000 of Lin-:CD24+ cells from ubiquitin-GFP mice were transplanted into wild-type and A-Zip lipodystrophic mice [58]. Although no GFP+ adipocytes were found in wild-type mice, GFP+ adipocytes were present in newly-formed fat pads of the A-Zip mice 12 weeks after injection of the CD24+ progenitors. Furthermore, these newly-formed adipocytes were able to improve the insulin resistance associated with the A-Zip lipodystrophic model. These experiments not only revealed the identity of an early progenitor population, but they also demonstrated the importance of the microenvironment in adipogenesis.

With respect to diet-induced obesity, changes in the microenvironment appear to play an important role in regulation of adipogenesis. Rodeheffer *et al.* illustrated this with transplantation experiments involving CD24+ cells from leptin-luciferase

transgenic animals [108]. In this model, luciferase expression is restricted to the adipose tissue [109]. Two weeks after injection, wild-type mice were fed high fat diet for six weeks. Luciferase signal was detected in three of the eight animals injected with CD24+ cells and subsequently fed HFD. The development of new adipocytes from CD24+ precursors in A-Zip and HFD-fed animals indicates that adipose expansion from transplanted cells may only occur in a permissive environment.

Rodeheffer *et al.* demonstrated the importance of the microenvironment and its role in determining cell fate. Cells in the SVF are capable of differentiating into a number of cell types such as osteocytes, chondrocytes, adipocytes, myocytes, and endothelial cells *in vitro* [90, 91, 108, 110-116]. Furthermore, Planat-Benard and colleagues demonstrated that adipocytes can be stimulated to become endothelial cells in culture [115]. Cellular plasticity exists and cell fate is less rigid than thought. In this study, we examine whether endothelial cells arising from the aorta of mice have the capacity to become adipocyte, similar to cells in the adipose tissue.

Up to date, the seminal works on adipocyte progenitor cells have been focused on white adipocyte. This may be because only recently has BAT been discovered to be relevant to human adult health. BAT and WAT are both mesodermal and it was assumed that they share the same origin. However, two recent studies have shown that this may not be the case. The Conlon and the Spiegelman laboratories have found that a population of brown adipocytes is derived from a muscle-related En1 and Myf5 lineages [117, 118].

Study by Seale *et al.* also suggests that there are several origins of brown adipocytes. They noted that brown adipocytes stimulated by β-adrenergic agonists are

not derived from a Myf5+ lineage. The concept that multiple cell types can give rise to brown adipocytes is supported by experiments that show transdifferentiation of white and brown adipocytes under certain physiological conditions. For example, in the presence of cold or after treatment with β3-adrenoceptor agonist, it has been found that white adipocytes can transdifferentiate into brown adipocyte [119-122]. Under obesogenic conditions, brown adipocyte accumulate lipid and take on storage function like white adipocytes [123]. From an evolutionary perspective, multiple origins of brown adipocyte might be beneficial, as in the right physiological and pathophysiological conditions, pluripotent cells residing in specific organs can be programmed to take on function that would ensure optimal performance.

Recent Development: Since the work described herein began, there have been several important papers published by the Spiegelman's laboratory on this subject. Although these studies were completed too late to directly influence the design of this work, their findings support our hypothesis and will be discussed. In 2010, Gupta *et al.* reported that finger protein 423 (ZFP423) is enriched in fibroblast cell lines that have the capacity to undergo adipogenesis (Figure 1.7) [124]. Overexpression of ZFP423 in NIH3T3 cells, which do not undergo adipogenesis, causes them to differentiate into adipocytes under permissive conditions. The ZFP423 knock-out mouse has diminished and irregular brown and white adipocytes. It was concluded that ZFP423 was an adipocyte predetermination factor.

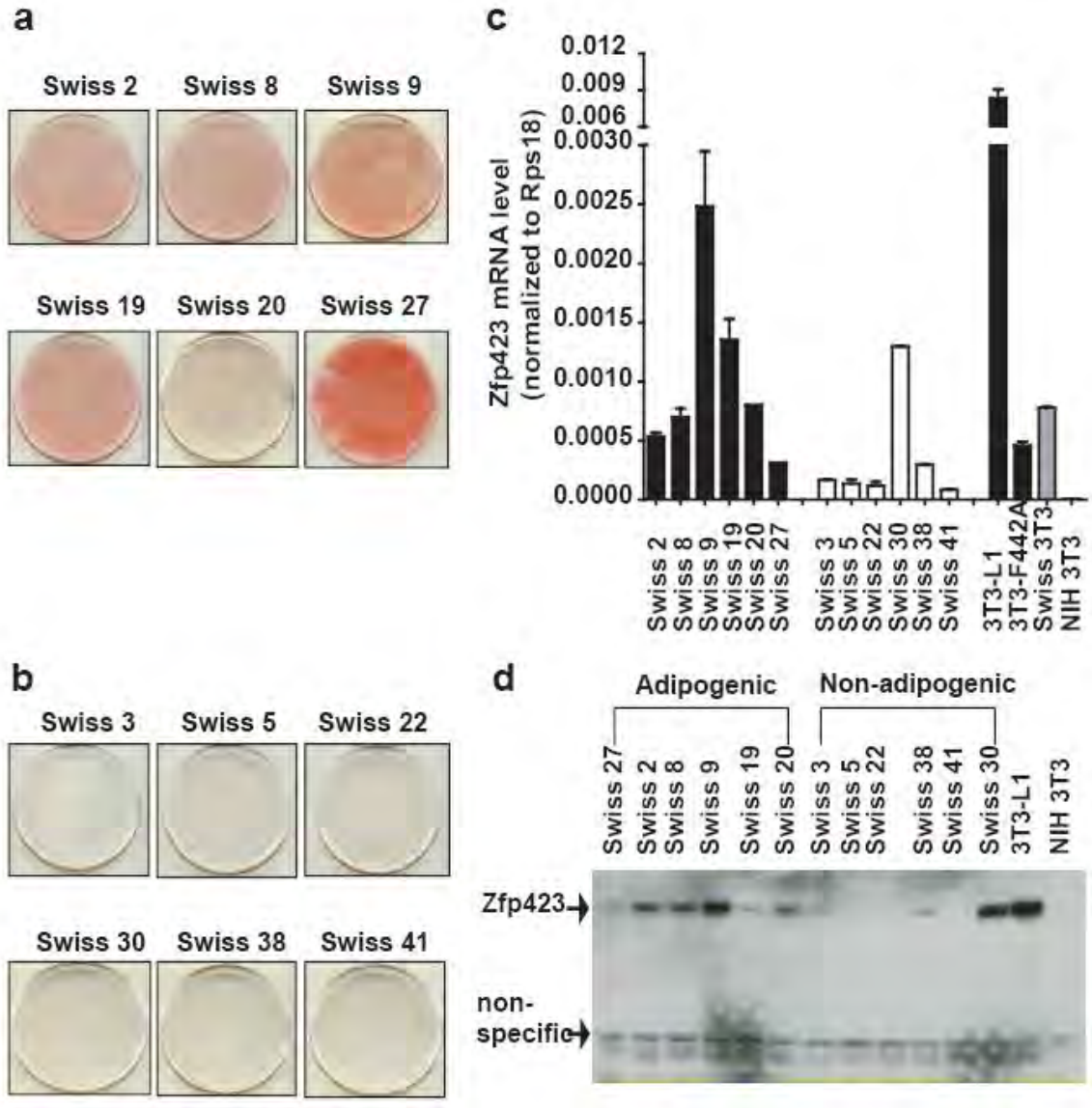


Figure 1.7. The C2H2 zinc finger protein Zfp423 is enriched in preadipocytes. Oil-red-O (ORO) staining of preadipocyte cell lines (a) and non-adipogenic cells lines (b) at six days following the induction of adipogenesis with DMI. (c) Zfp423 expression in sub-confluent Swiss 3T3 subclones and existing preadipocyte and fibroblast cell lines. (*n*=3 replicates per cell line). In this, and other figures, bars represent mean ± standard deviation from the mean. (d) Western Blot of endogenous Zfp423 protein levels in fibroblast cell lines grown under non-differentiating conditions. Adapted from: Gupta, R.K., et al., *Transcriptional control of preadipocyte determination by Zfp423*. Nature, 2010. **464**(7288): p. 619-23.

The Spiegelman laboratory then went on to make ZFP423^{GFP} reporter animal [89]. This animal expresses GFP under a non-functional ZFP423 transgene, allowing for the identification of precursor cells. In their study, Gupta *et al.* found localization of ZFP423 to pericytes and endothelial cells of the adipose tissue (Figure 1.8). This supports the results of our experiments. Interestingly, ZFP423 is only expressed in endothelial cells of the adipose tissue and not in other tissues, e.g. muscle. This may reinforce the concept that environment controls gene expression on the molecular and global levels.

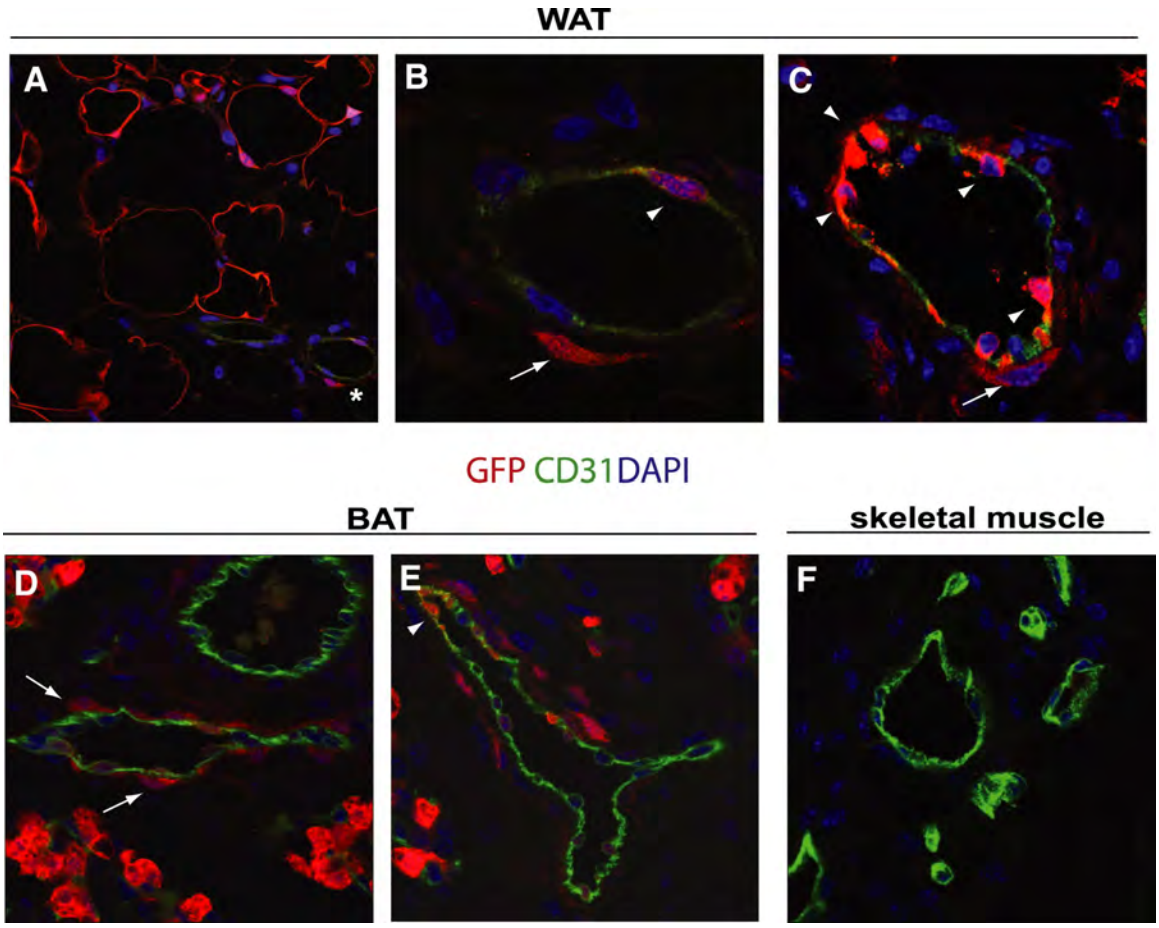


Figure 1.8. GFP+ Preadipocytes Reside in the Adipose Vasculature as a Subset of Both Pericytes and Capillary Endothelial Cells (A and B) Confocal images of adult inguinal WAT stained with antibodies recognizing GFP (red) and the endothelial cell protein CD31 (green), with nuclei counterstained with DAPI. In (A), note the expression of GFP in mature adipocytes and in some blood vessels (*). In (B), note the expression of GFP in a subset of perivascular cells (arrow) and in a subset of endothelial cells (arrowhead) of the blood vessel highlighted in (A).(C) Confocal image of developing inguinal WAT from postnatal day 4 mice stained with antibodies recognizing GFP (red) and the endothelial cell protein CD31 (green). Note the expression of GFP in a subset of perivascular cells (arrow) and in a strong subset of endothelial cells (arrowhead) even before the full development of mature adipocytes at this stage.(D and E) Confocal images of embryonic day 18.5 interscapular BAT stained with the same antibodies shown in (A)–(C). In (D), note the expression of GFP in mature adipocytes and in numerous perivascular cells (arrows). In (E), note the expression of GFP in a subset of endothelial cells (arrowhead).(F) Confocal image of skeletal muscle directly adjacent to the interscapular BAT shown in (D) and (E). Note the absence of GFP+ cells in the vasculature of this tissue. Adapted from Gupta, R.K., et al., *Zfp423 expression identifies committed preadipocytes and localizes to adipose endothelial and perivascular cells.* Cell Metab, 2012. **15**(2): p. 230-9

SPECIFIC AIM

The goal of this body of work is to further characterize the identity or identities of the adipocyte progenitors. Adipose tissue expansion requires the enlargement of existing adipocytes, differentiation of adipocyte progenitors, and the development of a supporting vascular network. Evidence suggests that adipocyte precursors reside in the vasculature of the adipose tissue. Recently, Tang *et al.* have shown that pericytes of adipose tissue vessels give rise to adipocytes. Pericytic origin of adipocytes has been corroborated by other studies. However, it is not completely clear if endothelial and adipocytes may also share the same origin.

It has also been established that microenvironment is crucial in adipogenesis. Pericytes from the kidneys are not able to undergo adipogenesis. Furthermore, adipocytes progenitors from the adipose tissue will not undergo adipogenesis when transplanted into host animals unless the environment of the recipient adipose tissue is conducive to the formation of new adipocytes. This brings into question whether progenitor cells of other organ systems can be stimulated to make new adipocytes if permissive conditions *in vitro* are established.

We hope to contribute to the current knowledge of adipocyte progenitors. The specific aims of this study are:

1. To determine whether cells of the endothelial lineage may give rise to new adipocytes during physiological growth.
2. To determine whether endothelial cells arising from the aorta have the capacity to undergo adipogenesis.

CHAPTER II: Origin of white and brown adipocyte form vascular endothelium

This chapter represents the manuscript:

Tran, K.-V.*, Gealekman, O.*, Frontini, A.*, Zingaretti, M. C., Morroni, M., Giordanom, A., Smorlesi, A., Perugini, J., De Matteis, R., Sbarbati, A., Corvera, S., Cinti, S. (2012) The vascular endothelium of the adipose tissue gives rise to both white and brown fat cells. Cell Metab. *15*, 222-229.

*These authors contributed equally

This work is a collaborative effort between the Corvera and Cinti laboratories. Animal experiments were performed by Khanh-Van Tran and Andrea Frontini. Real-time PCR and FACS analysis were performed by Khanh-Van Tran. Immunohistochemistry was performed by Olga Gealekman. All electron microscopy were performed by the Cinti laboratory.

SUMMARY

Adipose tissue expansion involves the enlargement of existing adipocytes, the formation of new cells from committed preadipocytes, and the coordinated development of the tissue vascular network. Here we find that murine endothelial cells (EC) of classic white and brown fat depots share ultrastructural characteristics with pericytes, which are pluripotent and can potentially give rise to preadipocytes. Lineage tracing experiments using the VE-cadherin promoter reveal localization of reporter genes in EC, and also in preadipocytes and adipocytes of white and brown fat depots. Furthermore, capillary sprouts from human adipose tissue, which have predominantly EC characteristics, are found to express Zfp423, a recently identified marker of preadipocyte determination. In response to PPARγ activation, endothelial characteristics of sprouting cells are progressively lost, and cells form structurally and biochemically defined adipocytes. Together these data support an endothelial origin of murine and human adipocytes, suggesting a model for how adipogenesis and angiogenesis are coordinated during adipose tissue expansion.

INTRODUCTION

The obesity epidemic associated with increased risk of type 2 diabetes have underscored the need to understand the relationship between excess caloric intake, white adipose tissue (WAT) development, and metabolic disease. Mammals, including humans, also have metabolically active brown adipose tissue (BAT) [20, 21, 43], and BAT precursors can be found in adipose organ of adult humans [125]. BAT has anti-obesity properties, therefore the mechanisms by which brown adipocytes emerge under different physiological and pharmacological conditions is under intensive investigation.

Adipose tissue growth is mediated by adipocyte hypertrophy, but in obesity adipose tissue may reach 60-70% of body weight, requiring hyperplasic growth [76, 126]. Therefore, defining the identity of adipocyte precursors is an area of intense interest. Markers of precursor cells giving rise to committed preadipocytes are being identified [124], and a population of early adipocyte progenitor cells expressing stem cell markers has been characterized in mouse WAT [108]. Furthermore, PPARγ lineage tracing studies indicate that the vascular wall of adipose tissue capillaries represents the niche of adipocyte precursors[91].

Adipose tissue growth requires concomitant expansion of its capillary network [127, 128], but how adipocyte formation and capillary expansion are coordinated is unclear. The stromal-vascular fraction (SVF) cells of adipose tissue differentiate *in-vitro* into either an adipogenic or a perivascular phenotype [114, 129-132], but the identity of these progenitor cells *in-vivo* is unknown. Since cells morphological

features are closely related to their function, a careful ultrastractural analysis during development could provide information on the origin and anatomical localization of adipose tissue precursors.

VE-cadherin is required for the formation of vasculature, and is expressed specifically in endothelial cells (EC) of fetal and adult mice. VE-cadherin-Cre-dependent LacZ and eGFP reporter strains show localization of the reporters in EC of many tissues [133-138]. However, VE-cadherin is also expressed in sub-populations of hematopoietic cells before E11.5, and thus their descendants are potentially labelled into adulthood [136]. To circumvent this problem, mice in which VE-cadherin-driven Cre is induced during adulthood have also been created, resulting in negligible excision (lower than 0.4%) in the hematopoietic lineage [134]. Lineage tracing with constitutive and inducible VE-cadherin driven Cre can provide information on the relationship between vascular and adipose cell development by allowing the identification of cells that express this EC gene at any point during embryonic and postnatal periods.

An additional tool to define the relationships between newly formed vasculature and the genesis of new adipocytes is the use of mouse and human adipose tissue explants cultured *ex-vivo* [139, 140]. In this manuscript, we have used these morphological, genetic and functional approaches, and obtained evidence that EC of capillaries in developing WAT and BAT depots can give rise to mature adipocytes. Our findings will enable further studies of the biochemical and physiological cues controlling adipose tissue growth.

RESULTS AND DISCUSSION

Ultrastructural identification of endothelial-pericytic cells as possible intermediate between EC and preadipocytes

Before birth rat (Figure 2.1A) and mouse (not illustrated) epididymal fat (eWAT) shows the typical features of a poorly differentiated mesenchymal tissue, consisting of a homogeneous population of fibroblast-like cells, often in mitosis (Figure 2.1A, m), in a loose connective matrix with small and sparse capillaries (Figure 2.1A, asterisks). In contrast, at postnatal day 6-8 (P6-8) (Figure 2.1B), well-circumscribed areas with majority of cells identifiable as adipocytes due to the predominantly unilocular cytoplasmic lipid droplets (Figure 1B, yellowish colour in semi-thin section), are observed. These areas are delimited by fibroblast-like cells, and contain numerous large capillaries (Figure 2.1B, asterisks). These are about 3-fold larger than capillaries found in eWAT of adult animals, suggesting they have functions additional to oxygen and nutrient exchange. Adipogenesis appears to be restricted into these vasculo-adipocytic islets. Electron microscopy of these islets reveals dense collagen fibrils in the interstitial matrix, and thick capillary walls due to the presence of abundant pericytes (Figure 2.1C and D). Most of the cells located in the peri-capillary position of the vasculo-adipocytic islets, correspond to the cells described above by EM, and show nuclear staining for transcription factors widely considered as markers of adipogenic conversion, such as PPARγ, C/EBPα and C/EBPβ (Figure 2.S1A-C). These results are consistent with studies suggesting that pericytes are precursors of preadipocytes [91, 101]. Some EC are also positive for C/EBPβ (Figure 2.S1D, arrowhead), which is located upstream of PPARγ and C/EBPα in transcriptional control of adipogenesis [141].

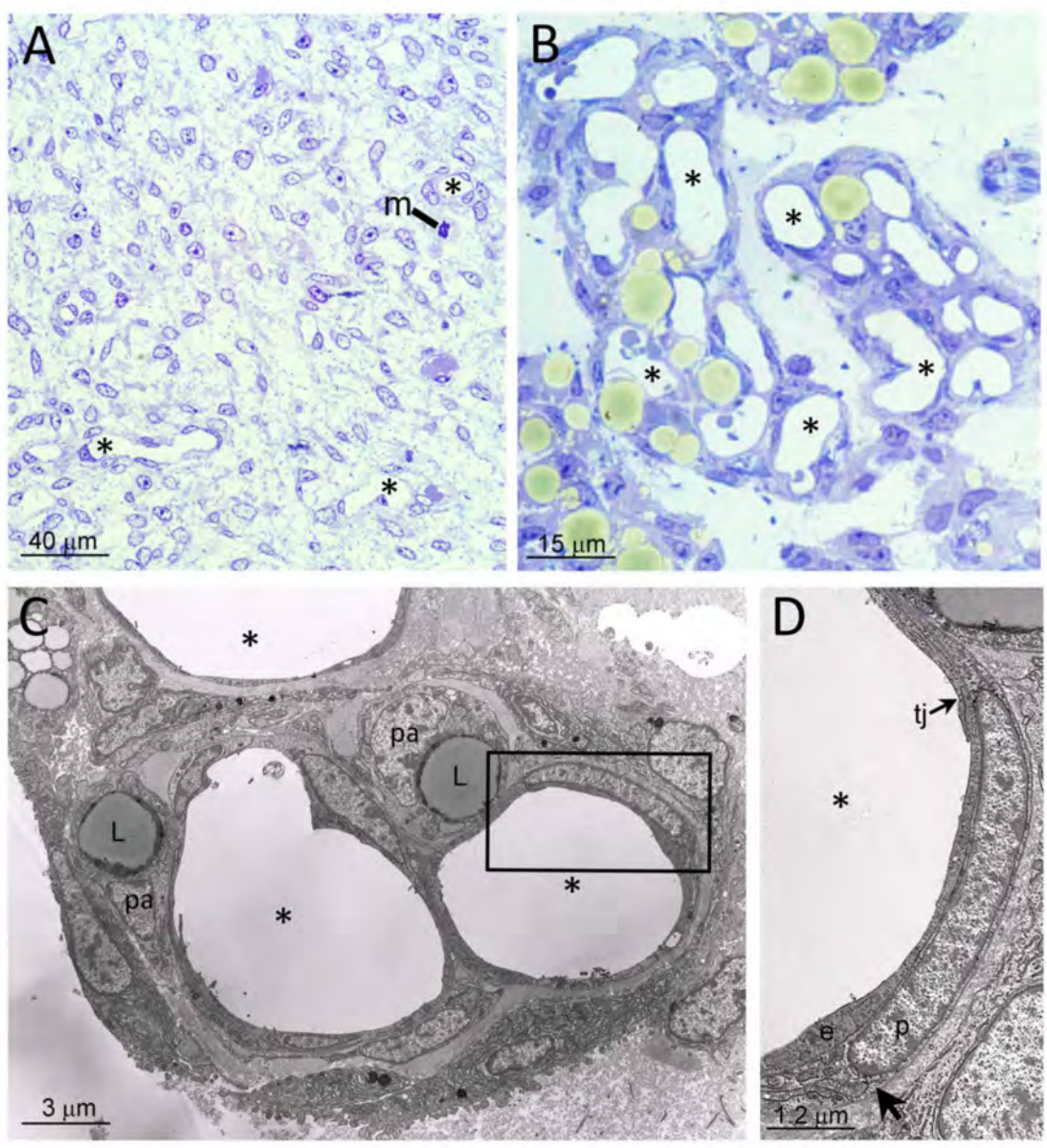


Figure 2.1: Murine embryonic and postnatal eWAT morphology. A. eWAT depot at E18 composed of poorly differentiated mesenchymal cells(m = mitosis; some capillaries indicated with asterisk). **B.** eWAT at P7, where adipocytes appear yellowish in areas with abundant large capillaries (asterisks). **C,D.** Electron Microscope micrograph of a vasculo-adipocytic islet showing EC (e; elongated cells), tight junctions (tj), pericytes (p; poorly differentiated cells with glycogengranules and surrounded by a distinct basal membrane),preadipocytes (pa; cells with small lipid droplets, (L)), andglycogen granules in pericyte (arrow).

In about 1-3% of vasculo-adipocytic islet capillaries, some EC exhibited novel, unusual features by being exposed to the capillary lumen, but also extended over a vicinal EC to adopt a pericytic position (Figure 2.2 A-C, endothelial-pericytic cells). Importantly, the junction of these cells with adjacent EC was composed of typical oblique tight junctions (Figure 2.2C, arrow), confirming the endothelial nature of these cells. EC and pericytes were sometimes joined by tight junctions between a protrusion of the EC crossing the basal membrane, and the complementary indentation in the pericyte (Figure 2.S1E). Some of the EC and endothelial-pericytic cells contain glycogen granules (Figure 2.2D-F, arrows), a characteristic feature of adipocyte progenitors [142]. Almost all pericytes (Figure 2.2D), some EC (Figure 2.2E, arrow), and cells that are partially associated with the capillary wall and also abut into the interstitial space (Figure 2.2F) also contain glycogen particles. These data reveal a complex relationship between cells in the vasculo-adipocytic islets where extensive adipogenesis is ongoing, and suggest that endothelial-pericytic cells represent an intermediate between endothelial and preadipocyte stages (drawing in Figure 2.S1F).

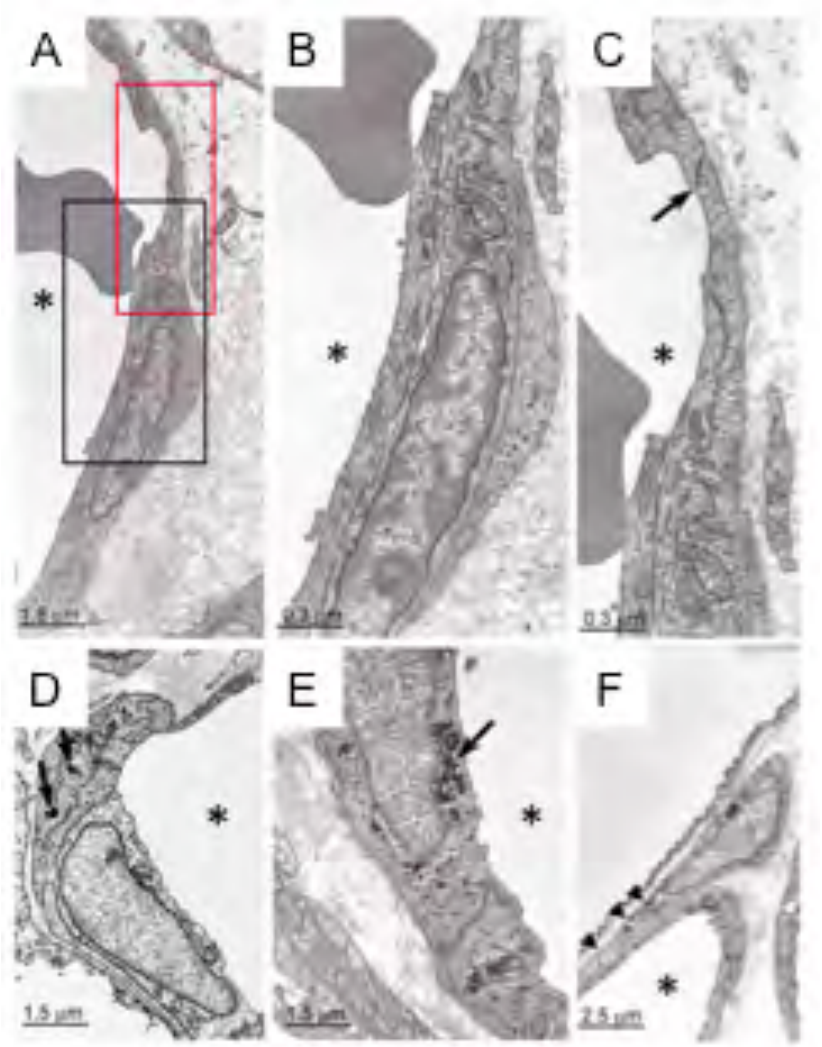


Figure 2.2 Endothelial cells in pericytic position A. EC and pericytes of a capillary wall (asterisk indicates the capillary lumen). **B**. Enlargement of the black squared area in E revealing EC in endothelial-pericytic position. **C**. Enlargement of the red squared area in E highlighting typical oblique tight junction (arrow) joining cell in endothelial-pericytic position to adjacent EC. **D.** Example of cell in endothelial-pericytic position containing abundant glycogen (arrows). **E.** Example of a "pure" EC containing abundant glycogen (arrow). **F**. Example of a cell partially associated with the capillary wall (arrowheads) and partially abutting into the interstitial space.

Lineage tracing studies show an endothelial origin of adipocytes

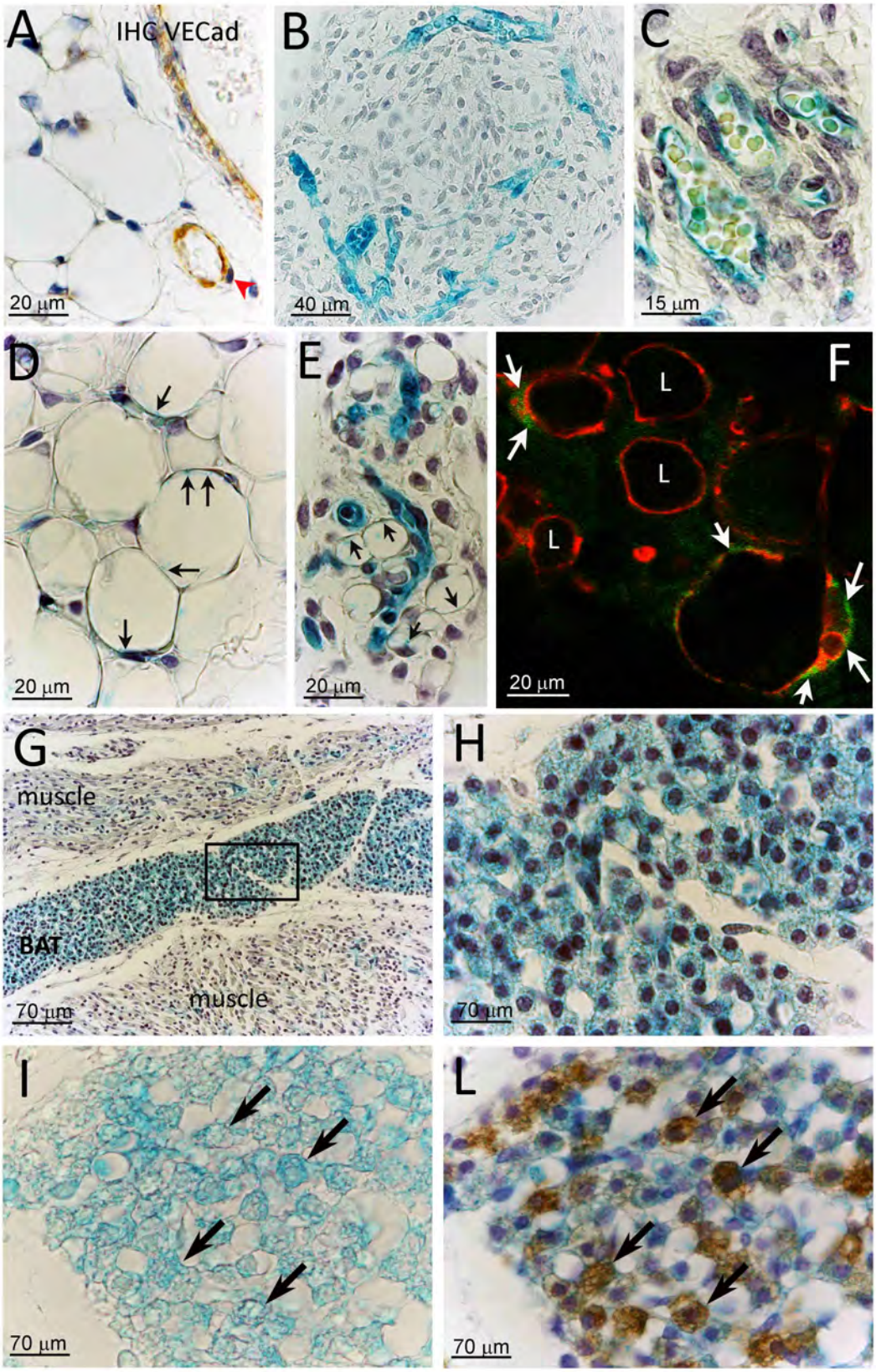
Data shown above suggest that EC may be amongst the cells that give rise to new adipocytes. To address this hypothesis directly we used VE-cadherin promoter-driven lineage tracing. VE-cadherin was expressed only in EC, and not in pericytes (red arrowhead) or adipocytes, of subcutaneous (scWAT) (Figure 2.3A) and eWAT (data not shown) of adult animals. Similarly, prior to adipocyte development in fetal and early postnatal eWAT (Figure 2.3B, C) and scWAT (not shown) of VE-cadherin-Cre/R26R-LacZ mice, only EC were X-gal positive. In contrast, from P6-8 (Figure 2.3D, E) to adult (Figure 2.S2A), adipocytes at different stages of lipid accumulation in eWAT and scWAT were also X-gal positive, indicating that VE-cadherin promoter was expressed at some point during adipocyte development. Control animals were always X-gal negative (Figure 2.S2B). To verify that the reporter was localized to the adipocyte cytoplasm, we used EM and observed a precise localisation of X-gal crystals in EC, pericytes, adipocytes (Figure 2.S2C), and preadipocytes (Figure 2.S2D). Similar results were found using eGFP as a reporter; in developing eWAT adipocytes of VE-cadherin-Cre/R26R-eGFP mice, perilipin and eGFP colocalized in adipocytes (Figure 2.3F). Internal positive control (vessels), and other eGFP positive adipocytes are shown in Figure 2.S2E. These results further support the hypothesis that adipocytes can develop *in vivo* from cells of endothelial origin.

These data prompted us to investigate whether brown adipocytes might also have an endothelial origin. Immunohistochemistry for VE-cadherin confirmed its presence in EC and its absence in BAT pericytes and adipocytes (not illustrated). However, the interscapular region of E17-19 VE-cadherin-Cre/R26R-LacZ mice

showed strong X-gal staining in both EC and adipocytes (Figure 2.3G, H), while in the surrounding muscles only EC were stained (Figure 2.3G). The X-gal staining colocalized with the classical brown adipocyte marker UCP-1 (Compare Figure 2.3I and 2.3L). Moreover, in the developing interscapular brown fat depot (Figure 2.S3A-B), some ECs display characteristic markers (Figure 2.S3C-E) and structural features (glycogen and mitochondria) of UCP-1- positive brown adipocyte precursors (Figure 2.S3F-I). Taken together, these data strongly suggest that EC of developing WAT and BAT capillaries are a source of adipocyte precursors. Further evidence supporting the possibility that certain EC populations can give rise to adipocytes is the finding by Gupta et al (B. Spiegelman and R. Gupta personal communication) that pericytes and some EC of mouse adipose tissue express GFP driven by the promoter for Zfp423, which marks cells determined to form preadipocytes [124]. Moreover, PPARγ excision with the use of the Tie-2 promoter driven Cre-recombinase, which is expressed in a mosaic pattern in capillary endothelium [143], results in a decrease in adiposity and adipocyte size in response to rosiglitazone treatment in a manner dependent on endothelial, but not bone-marrow, PPARγ expression [144]. These findings constitute further evidence supporting the possibility that certain EC populations can give rise to adipocytes

Figure 2.3: VE-Cadherin lineage tracing in WAT and BAT. A. Immunohistochemical analysis on scWAT from VE-cadherin-Cre/R26Rmice showing the specific expression of VE-cadherin only in EC (brown), note the negative pericytes indicated by red arrowhead. **B, C.** In early neonatal eWAT only vasculature is X-gal positive. **D, E.** scWAT (D) and eWAT (E) from P7 mice revealing X-gal staining (arrows) in developing and mature adipocytes. **F.** Confocal microscopy of eWAT from VE-cadherin-Cre/eGFPRmice showing a single optical plane of adipocytes (arrows), containing eGFP (green) and perilipin (red). Some eGFP-negative/perilipin-positive adipocytes are also visible (L). **G.** X-gal positive staining in developing BAT, and in muscle capillaries. **H.** Enlargement of the squared area in G. **I, L.** X-gal (I) and UCP-1 (L) co-localization to brown adipocytes (arrows).

Figure 2.3


A
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A hematopoietic origin of adipocytes has been suggested [145-147]. To exclude the possibility that X-gal positive adipocytes originate from hematopoietic lineage, we complemented our studies with a tamoxifen-inducible transgenic VE-cadherin-CreER^{T2} model in which cells derived from hematopoietic precursors are not labeled [134]. VE-cadherin-CreER^{T2}/R26R-LacZ mice at eight weeks of age were induced by tamoxifen injection for five consecutive days. Three weeks later X-gal staining was observed in capillaries of adipose tissue, but also in numerous adipocytes distributed among the eWAT (Figure 2.4B, C) inguinal (E, F) and brown (H, I) depots. Number of X-gal positive adipocytes was lower, as compared to the results described above, possibly due to a lower number of adipocytes being formed during this postnatal expansion period, but still clearly significantly above the background observed in negative controls (Figure 2.4A, D, G). PPARγ agonists affect murine adipose tissue by increasing multilocularization of existing adipocytes and by stimulating pre-adipocyte differentiation into mature adipocytes [122, 148]. Mice treated with rosiglitazone for three weeks following induction displayed numerous multilocular adipocytes, many of which were X-gal positive (Figure S2.3J-L). Thus, under both normal and stimulated adipogenesis, adipocytes arise from VE-cadherin expressing progenitors non-hematopoietic origin.

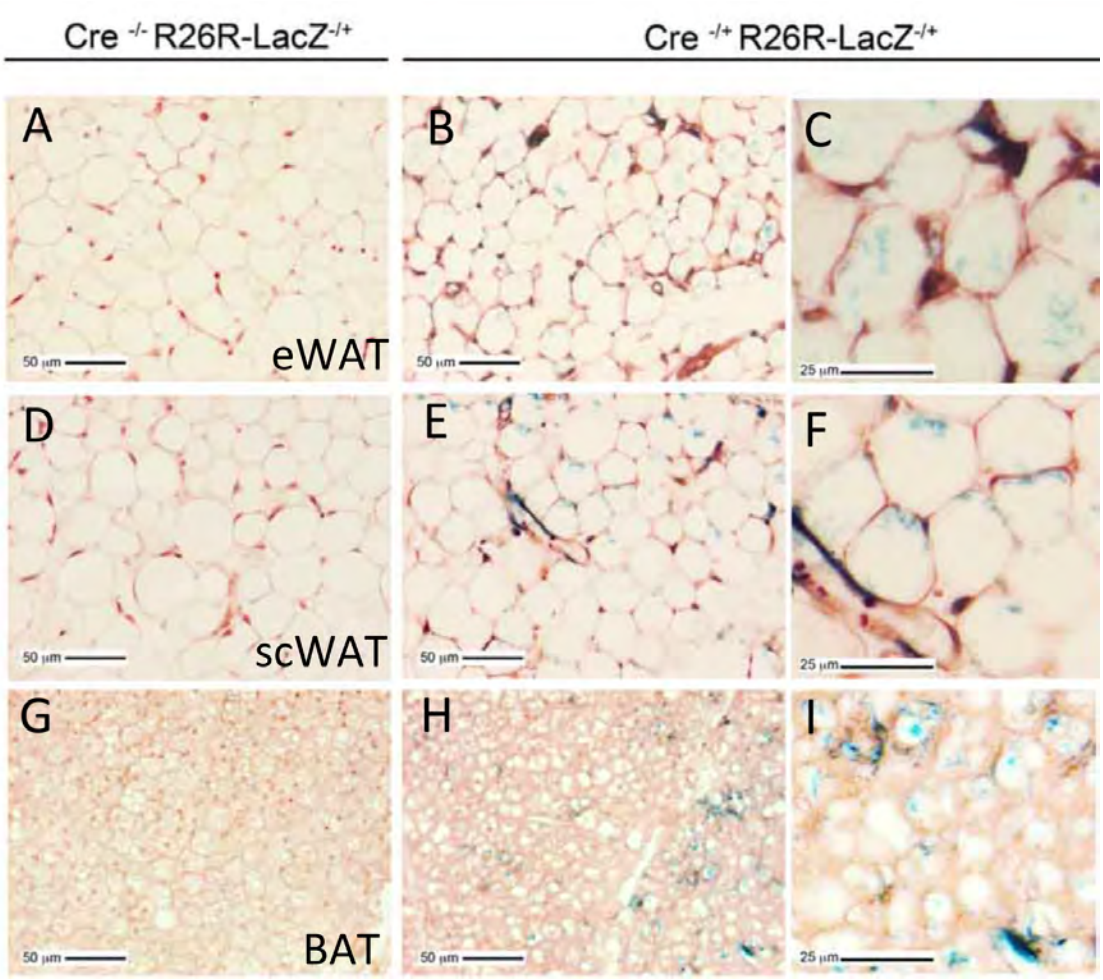


Figure 2.4. VE-Cadherin fate tracing in WAT and BAT. eWAT **(B, C),** scWAT **(E, F),** and BAT **(H, I)** adipose tissue from VE-cadherin-CreER$^{T2-/+}$/R26R$^{-/+}$ mice showing X-gal positive staining in adipocytes and EC. Adipose tissues fromVE-cadherin-CreER$^{T2-/-}$/R26R$^{-/+}$control mice **(A, D, G)**.

A vascular origin for white and brown adipocytes may seem to be in contradiction with the work of Seale et *al.,* in which a common origin of BAT and skeletal muscle cells [118] was proposed. In this work, the authors found expression of YFP in skeletal muscle and BAT from Myf5-Cre/R26R3-YFP mice, consistent with both these tissues being derived from a myogenic, Myf5 expressing progenitor. Interestingly, De Angelis et al. have shown the existence of progenitor cells from embryonic dorsal aorta that express both myogenic and endothelial markers including Myf-5 and VE-cadherin[149]. Thus, one explanation for the results of Seale *et al.* and ours is that BAT is derived from a population of VE-cadherin positive, Myf5 positive cells, while muscle can be derived from Myf5 only positive cells. Alternatively, as the Myf-5-Cre mouse model labels multiple cell lineages from somite origin [150], other somite-derived cell progenitors that subsequently express VE-cadherin could in theory give rise to BAT. If the brown adipocyte and muscle cell fates were determined before the expression of VE-cadherin, lineage tracing experiments using the VE-Cadherin-Cre mouse model would not label myocytes.

Human adipose tissue endothelial sprouts give rise to adipocytes

The finding that murine cells with endothelial characteristics can give rise to adipocytes prompted us to investigate whether human adipocytes share this origin. Fragments of human adipose tissue give rise to capillary sprouts when embedded in Matrigel, and cultured in pro-angiogenic media [139]. Classical EC ultrastructural features, including tight junctions, irregular nuclei and pinocytic vesicles were seen in cells comprising these sprouts (Figure 2.5A-D). In regions more proximal to the tissue explant, capillary lumens could be observed (Figure 2.5E-G), indicating that

angiogenic development is recapitulated *ex vivo*. Upon exposure to the PPARγ agonist rosiglitazone, many cells within the emerging capillary sprouts produced lipid droplets (Figure 2.5H-J). These cells displayed canonical ultrastructural features of white adipocytes, including a large lipid droplet, displaced nucleus, small and elongated mitochondria and glycogen particles distributed in the cytoplasm (Figure 2.5K). Interestingly, these adipocytes showed tight junctions identical to those found in sprouting EC, and between EC and pericytes *in vivo* (Figure 2.5L, compare with Figure 2.S1). These results suggest that the processes of capillary expansion and adipocyte formation seen in explants *ex vivo* reflect those occurring in the intact organism.

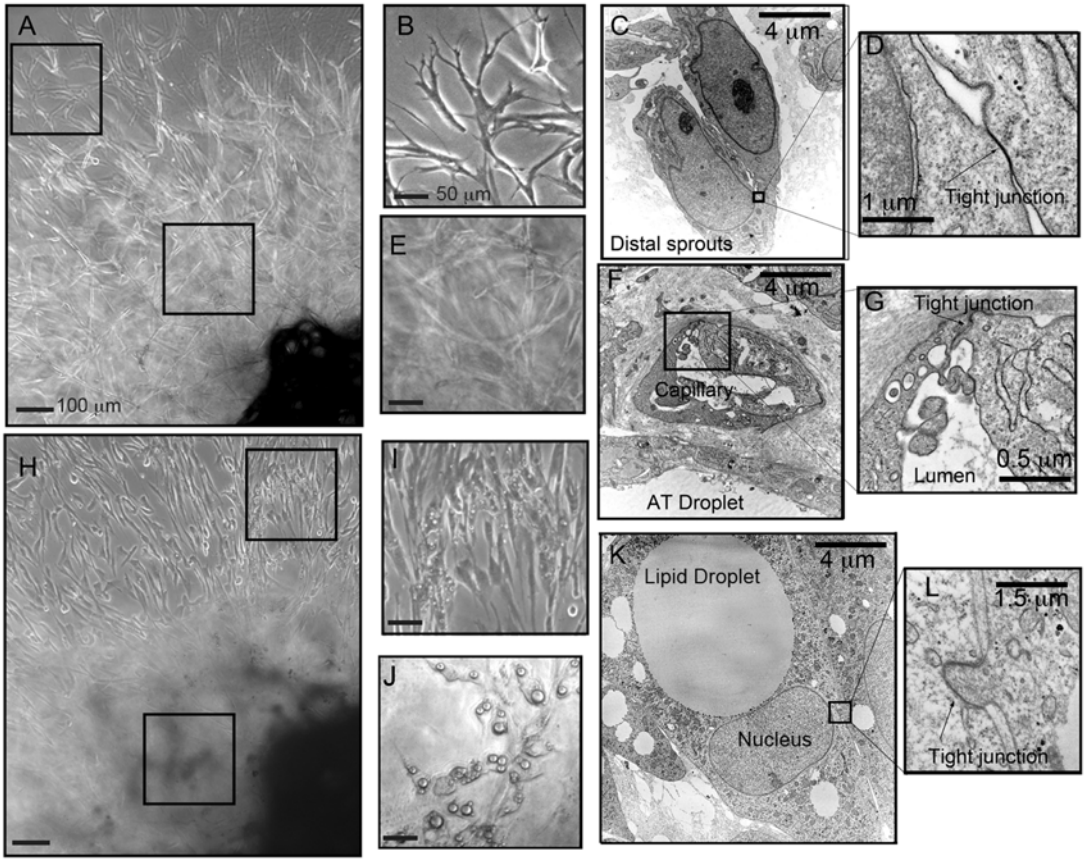


Figure 2.5: Effect of rosiglitazone on angiogenic sprouts originating from human adipose tissue. A. Capillary outgrown after 15 days of culture in the absence of rosiglitazone, indicating areas distal and proximal to the embedded explants. **B**. Enlargement of area distal to the explants. **C**. Electron microscopy of area similar to that shown in B, where tight junctions can be seen to connect cells. **D**. Enlargement of tight junction found between two EC. **E**. Enlargement of area proximal to the explant. **F**. Electron microscopy of area similar to that shown in E, revealing lumenized capillaries formed by EC joined by tight junctions. **G**. Enlargement of area shown in F. **H**. Capillary outgrowth after 15 days in the presence of rosiglitazone. **I**. Enlargement of area distal to the explant, revealing lipid droplets in cells interspersed among the capillary sprouts. **J.** Area proximal to the explant containing cells harboring larger lipid droplets. **K**. Electron microscopy of lipid-laden cells revealing features of classical white adipocytes, and of EC such as the tight junction in the squared area. **L**. Enlargement of area outlined in K.

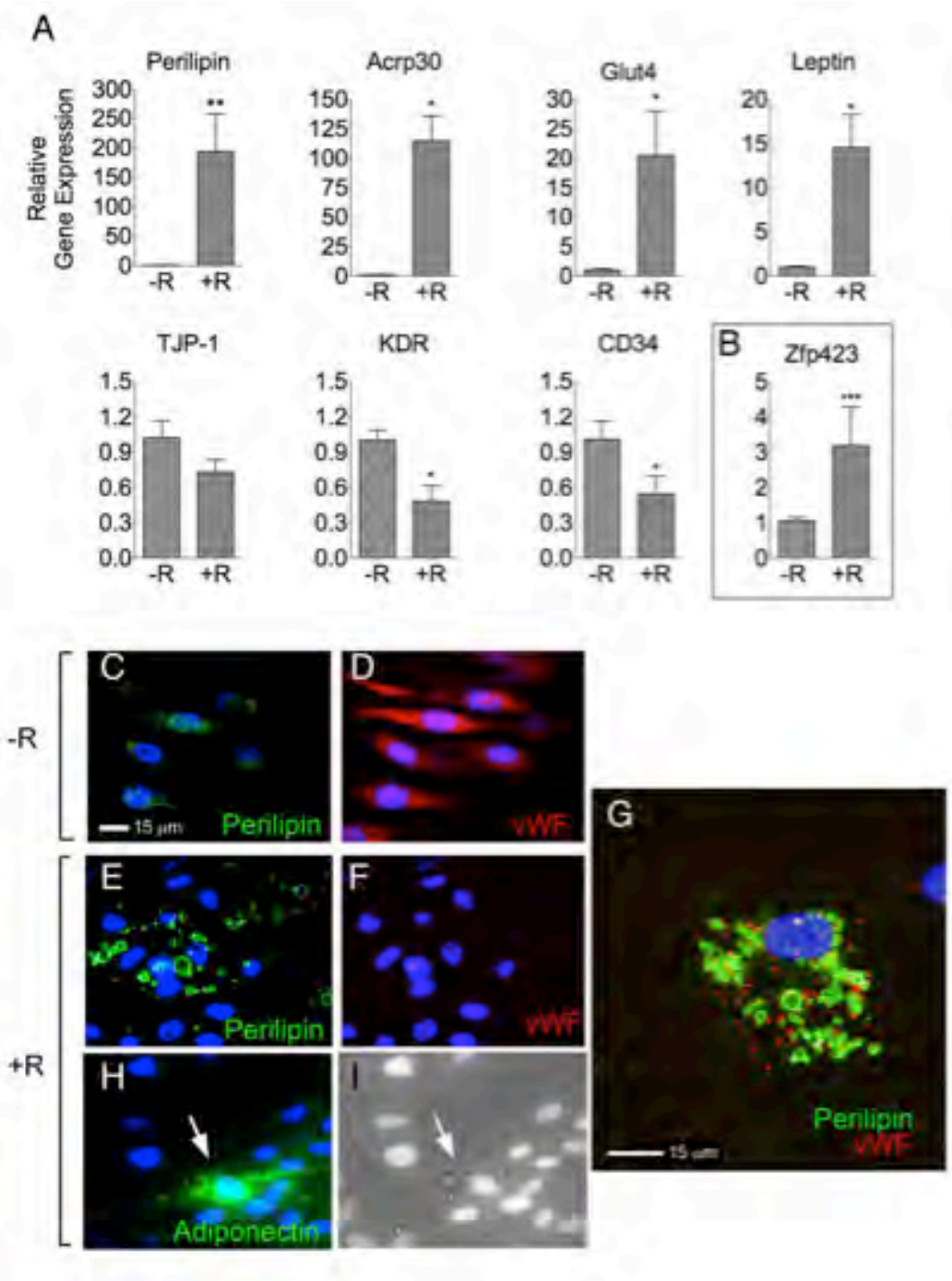
We then asked whether these morphologically defined adipocytes express genes that confer adipocyte function. Induction of mRNAs for Glut4, adiponectin, and leptin, and an >100-fold increase in the expression of perilipin mRNA were observed (Figure 2.6A, top row) in parallel with decreased expression of EC markers; tight junction protein TJP-1, CD34, and KDR (Figure 2.6A, bottom row). In addition, the transcription factor Zfp423, considered a marker of adipocyte lineage pre-determination[124], was detected and increased significantly in response to rosiglitazone (Figure 2.6B). The presence of this pre-determination marker, and the strong induction of genes that define adipocyte function, is consistent with the possibility that cells with endothelial characteristics can give rise to adipocytes in human adipose tissue.

To further examine the relationship between endothelial and adipocyte markers at a single cell level, we performed immunofluorescence analysis. In the absence of rosiglitazone, most cells contained low or undetectable levels of perilipin (Figure 2.6C), and high levels of von-Willebrand Factor (vWF) (Figure 2.6D). In the presence of rosiglitazone, majority of cells became vWF-negative (Figure 2.6F), but exhibited typical morphological feature of adipocytes, a perilipin-coated lipid droplets (Figure 2.6E). Nevertheless, approximately 5% of cells containing perilipin-coated lipid droplets also contained vWF (Figure 2.6G). Similar cells containing lipid droplets were also found to express adiponectin (Figure 2.6H and I), another specific feature of mature adipocytes. These results are consistent with the hypothesis that adipocytes arise from EC, or precursors expressing EC features. In line with these observations, recent findings indicate that ECs can be converted into mesenchymal stem cells,

which can differentiate into adipocytes, chondrocytes and osteoblasts [151]. Furthermore, others have described that adipocytes have the potential to rapidly acquire an endothelial phenotype in vitro (Planat-Benard et al., 2004), raising the possibility that adipocytes and ECs are plastic enough to undergo inter-conversion to maintain a homeostatic equilibrium during adipose tissue expansion and reduction.

Figure 2.6: Co-expression of endothelial and adipose cell genes in human and mouse systems. A. Relative mRNA levels of canonical adipocyte (top row) or EC (bottom row) genes in capillary outgrowth from explants grown for 14 days in the absence (-R) or presence (+R) of rosiglitazone. **B.** Relative mRNA levels of the adipocyte pre-determination marker Zfp423. Plotted are the means and SEM of 6-8 independent experiments. Statistical significance was assessed by non-paired, two tailed student t-test *=p<0.05; **=p< 0.001; **C, D.** In absence of rosiglitazone cells growing from human adipose explants are mostly perilipin-negative (green) and vWF-positive (red). E-**G.** In the presence of rosiglitazone, perilipin coating around lipid droplets is present (E), with majority of cells becoming vWF-negative (F), and about 5% of cells co-expressing perilipin (green) and vWF (red) (G).**H, I.** Cells growing from adipose explants, that accumulate lipid droplets in the presence of rosiglitazone(I) are also adiponectin-positive (H).

Figure 2.6


A
Perilipin
Acrp30
Glut4
Leptin
Relative Gene Expression
-R
+R
TJP-1
KDR
CD34
B
Zfp423
C
D
E
F
G
H
I
-R
+R
15 μm
Perilipin
vWF
Adiponectin

Expression of VE-cadherin in adipocyte progenitors characterized by stem-cell markers.

To further examine the possibility that adipocytes and EC derived from each other or share a common precursor, we asked whether cells expressing adipocyte stem cell markers [108], were also positive for VE-cadherin (CD144). CD45- cells from the SVF were successively gated for the presence of CD29, CD24 and Sca1 (Figure 2.7A). Approximately 5% and 10% of the cells in the SVF of eWAT and BAT, respectively, were CD45-CD29+CDC24+, and this difference was statistically significant (Figure 2.7B, upper panel). Approximately 17% and 52% of the CD45-CD29+CDC24+Sca-1+ population in eWAT and BAT, respectively, were also positive for VE-cadherin (CD144) (Figure 2.7A), comprising 0.5% and 4% of the cells in the SVF of eWAT and BAT, respectively (Figure 2.7B, lower panel). The percentages of adipogenic stem cells expressing VE-cadherin is in line with the amount of endothelial-pericytic cells found in WAT by EM studies *in vivo* and the much larger proportion of VE-cadherin positive cells in BAT is consistent with its denser vascular network. These data support the hypothesis of an endothelial origin of adipocyte populations in these depots.

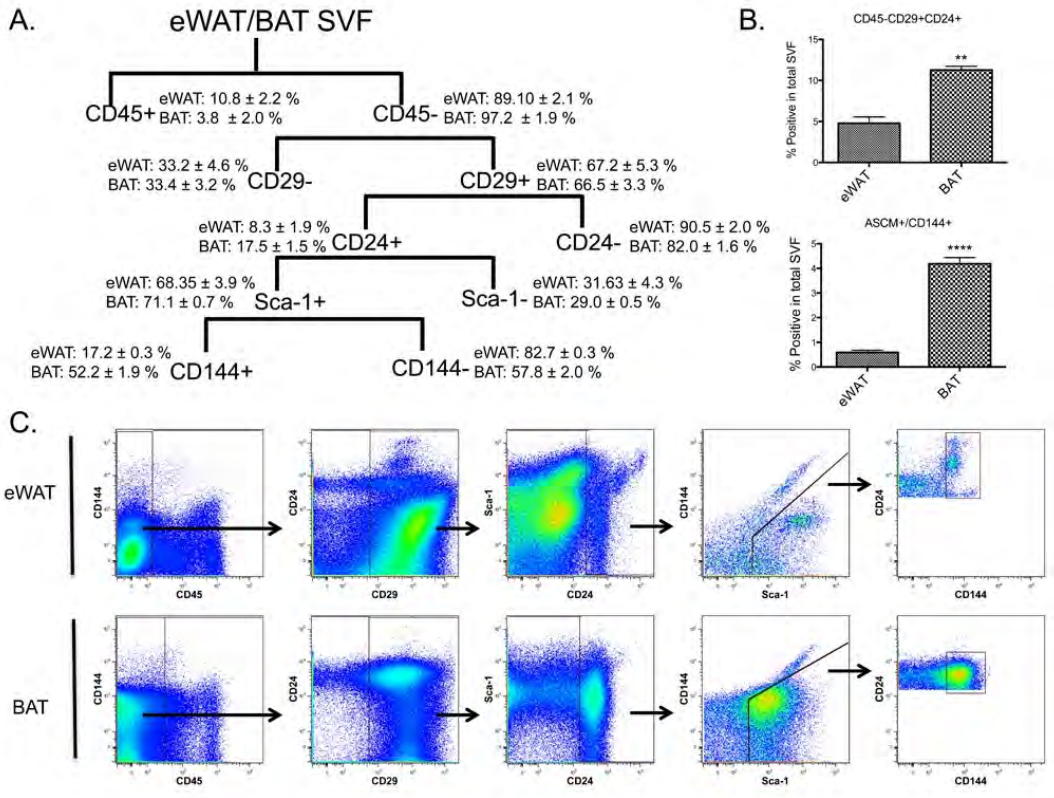


Figure 2.7. Adipocyte progenitors express VE-Cadherin (CD144) A. FACS analysis scheme of cells from SVF of eWAT and BAT. **B.** Comparison of CD45-CD29+CD24+Sca1+ population and CD45-CD29+CD24+Sca1+CD144+ (ASCM+) between depots. **C.** Gating strategy for experiments. **p< 0.005 and *p < 0.00005 and derived from two-tailed student t-tests, n = 3-4. Fluorescence minus one antibody controls are in Figure 2.S4.

In summary, we present morphological and genetic evidence that adipocytes in white and brown depots originate from cells that display endothelial characteristics. Further research to identify the physiological signals that determine adipocyte and/or EC fates will lead to a better understanding of the mechanisms responsible for coordinating the formation of new adipocytes with angiogenic expansion during adipose tissue growth.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague Dawley rats and B6 Mice (Harlan, Udine, Italy) were studied at different developmental stages. At least 5 animals per group were used for morphological studies performed by light, confocal, electron microscopy and immunohistochemistry. Homozygous VE-cadherin (VE-cadherin-Cre-recombinase) transgenic mice were crossed to homozygous ROSA26R (R26R) reporter and ROSA-eGFPR lines (Jackson Labs., Bar Harbor, ME; stock n. 6137, 3474 and 4077 respectively) to reveal the activity of the Cre-recombinase by detection of LacZ and eGFP reporter genes. The VE-cadherin-R26R and VE-cadherin-eGFPR mice were studied at developmental stage E18 (± 12h) and at P6-8. Heterozygous VE-cadherin tamoxifen-inducible Cre-recombinase transgenic mice (VE-cadherin-CreER^{T2}) were crossed to homozygous R26R reporter animals. VE-cad-CreER^{T2}/R26R mice (8 weeks old) were IP injected with tamoxifen (2mg/day) for five consecutive days [134]. After injections, mice were fed normal diet with or without rosiglitazone (10mg/kg/day) for three weeks. Mice were then sacrificed, and brown and white adipose tissue from epididymal and inguinal depots was harvested for X-gal staining.

Animal care and handling were in accordance with Italian Institutional Guidelines and the Animal Care and Use Committee at UMass Medical School.

Light microscopy and immunohistochemistry

Mouse and rat embryos were collected after mother perfusion with 4% paraformaldehyde in 0.1M PB pH 7.4 and fixed by overnight immersion in the same solution. Each embryo was then dehydrated, paraffin embedded and oriented to be cross-sectioned through the interscapular region (head-neck). Newborn rats and mice were perfused intracardially using the same fixative; bilateral testis with peri-epididymal fat were dissected and embedded in paraffin. Light microscopy, immunohistochemistry and confocal microscopy were performed using standard methods described in the Supplementary Methods.

Electron microscopy and immuno-gold staining

After perfusion, small fragment of iBAT of fetal and postnatal eWAT were fixed in 2% glutaraldehyde – 2% paraformaldehyde in 0.1M PB pH 7.4 for at least 4 h, post-fixed in a solution of 1% osmium-tetroxide and - 1% potassium hexacyanoferrate (II), dehydrated in acetone and finally epoxy-resin embedded. Sectioning, mounting and examination are further described in the Supplementary Methods.

X-gal staining for β-galactosidase tissue localization

Male E18-19 and P6-8 and adult VE-cadherin-Cre/R26R mice were fixed in 2% paraformaldehyde, 0.25% glutaraldehyde in PBS pH 7.3 for 1 h and washed in PBS. Thick (500μm) cross-sections through the interscapular region (head-neck) of

iBAT and Ewat were stained for β-galactosidase with the chromogenic substrate X-gal, sectioned, counterstained and imaged as described in the Supplementary Methods.

Human adipose tissue explants

Human scWAT was obtained from discarded tissue of patients undergoing panniculectomy at UMASS Memorial Hospital. Pieces of $1mm^3$ were prepared and embedded in Growth Factor Reduced Matrigel (BD Biosciences)on 35 mm glass-bottom culture dishes (MatTek Corporation) as described previously (Gealekman et al., 2011), in the absence or presence of 1 μM rosiglitazone maleate. After 14 days in culture immunofluorescence was performed as described previously (Gealekman et al., 2011). Primary antibodies used are stated in the Supplementary Methods. For RT-qPCR, the adipose tissue fragment was mechanically excised and the endothelial sprouts remaining in the Matrigel were isolated using Dispase II (Roche, 2.4 U/mL), centrifuged, and RNA extracted from the pellet using an Ambion RNA extraction kit. Probes used are specified in the Supplementary Methods.

Fluorescence Activated Cell Sorting

SVF from eWAT or inter-scapular brown fat pads from 8 week old mice were isolated as described [152], and stained with blue stain (Invitrogen) at 4°C for 20 min. Cells were incubated with anti-mouse CD16/CD32 (BD Biosciences) for 15 min and then with respective FACS antibody for 2h at room temperature. Antibodies used are stated in the Supplementary Methods.

Acknowledgements

The research described in this study was supported by grants from Università Politecnica delle Marcheand Cariverona Foundation to Saverio Cinti and NIH grant DK089101to Silvia Corvera. The FACS analysis was funded in part by the NIDDK Diabetes and Endocrinology Research Center DK52530.

Figure 1.S1: Morphological analysis of eWAT at P7. **A-D.** Nuclear staining of poorly differentiated cells in pericytic position (arrows), and adipocytes (L; lipid droplet), for transcription factors indicated in each panel. In D the nucleus of EC (indicated by arrowhead) also positive for C/EBPβ. **E.**EC joined to adjacent pericyte with a tight junction between a protrusion of its cytoplasm that crosses the basal membrane and the complementary indentation in the pericyte. The inset is an enlargement of the squared area. **F.** Hypothesis based on data shown in Figure 2 that describe developmental stages from endothelium to preadipocytes.

Figure 1.S1

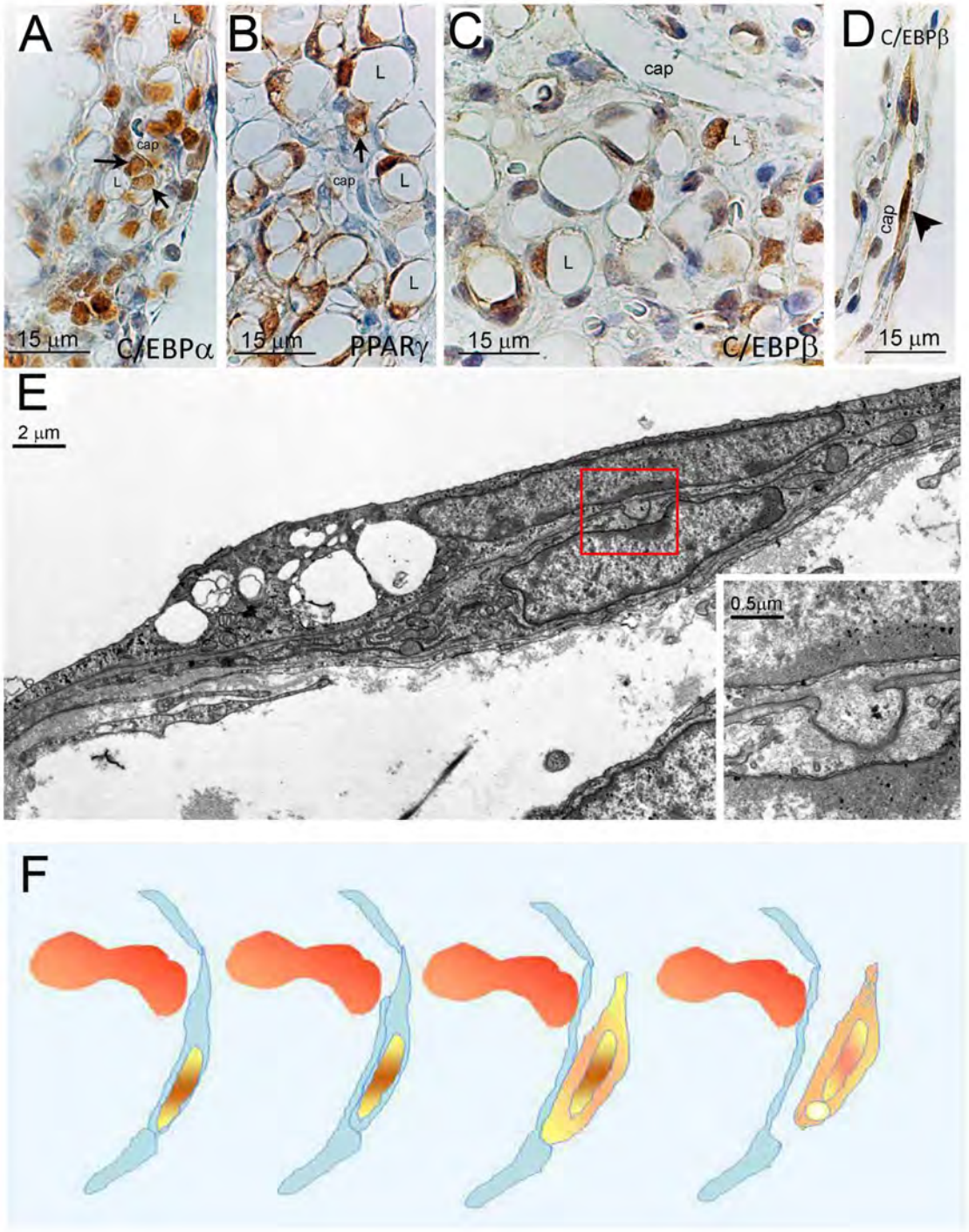

A
L
cap
L
15 µm
C/EBPα
B
L
cap
L
L
15 µm
PPARγ
C
cap
L
L
15 µm
C/EBPβ
D C/EBPβ
cap
15 µm
E
2 µm
0.5µm
F

Figure 2.S2: VE-Cadherin lineage tracing in WAT and BAT. Histological appearance of eWAT obtained from VE-cadherin-Cre/R23R (A) or VE-cadherin-Cre(B)adult mice subjected to X-gal staining. **C.** X-gal crystals in the cytoplasm of EC (e), pericytes (p) and adipocytes (a) found in eWAT of 4 weeks old VE-cadherin-Cre/R26R mice (arrows). **D**. Positive X-gal staining of eWAT preadipocyte (multiple small lipid droplets in the cytoplasm). **E.** eGFP localized in the vessels (V in inset), and adipocytes (asterisk)of VE-cadherin-Cre/eGFPR mice. n: nucleus.

Figure 2.S2

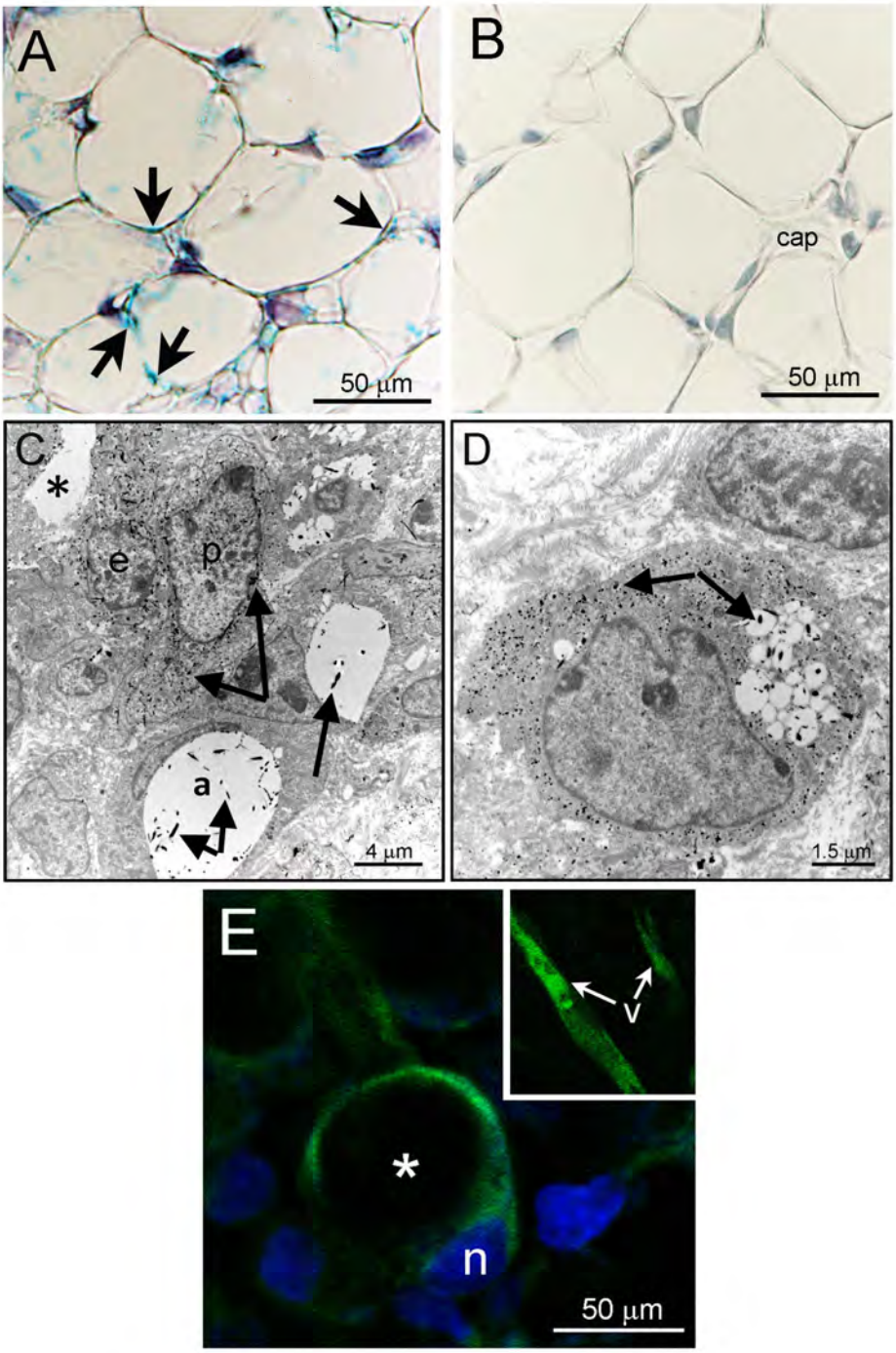
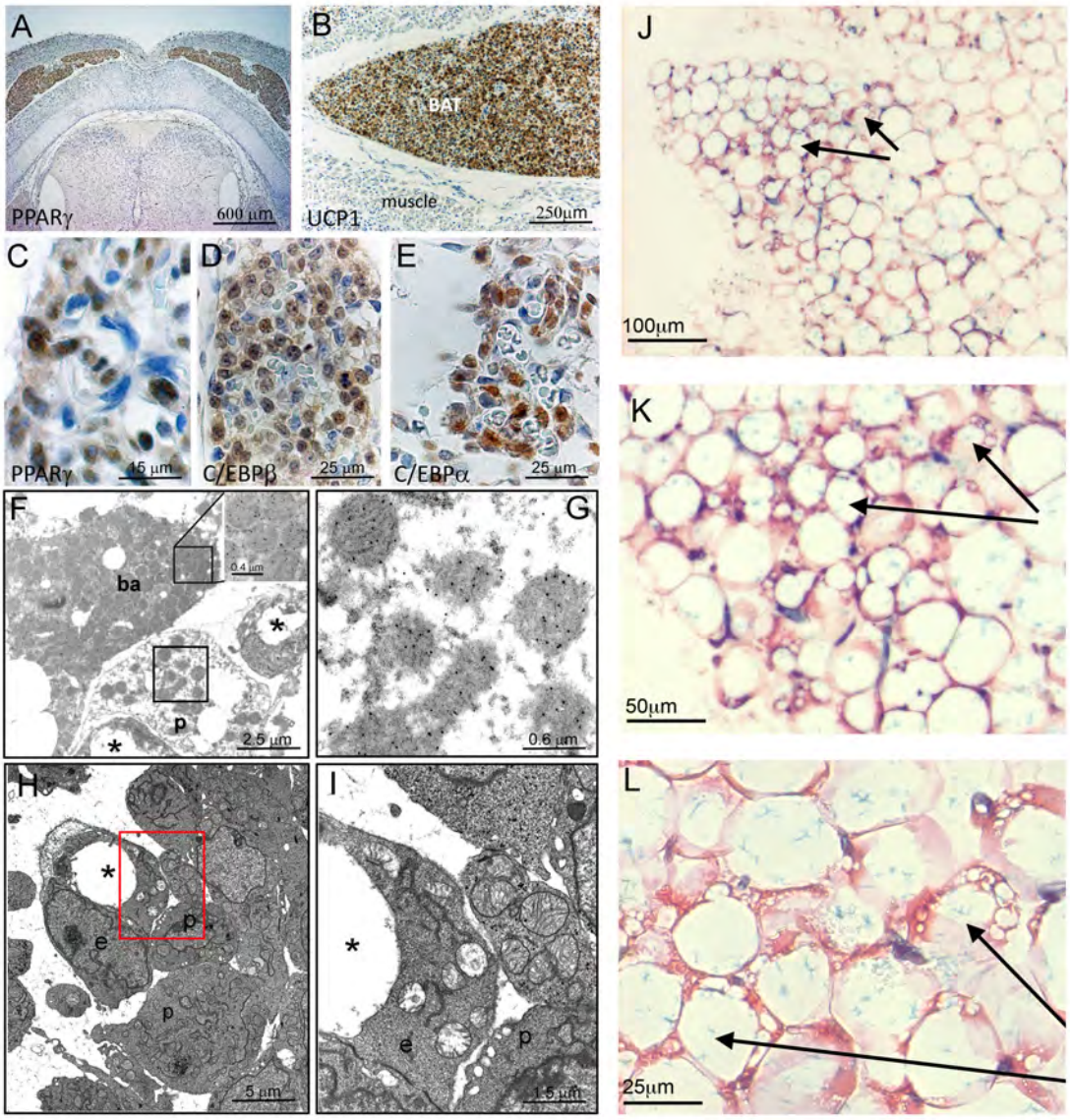


Figure 2.S3: Structural characteristic and lineage tracing support common origin of developing endothelial and fat cells. A. PPARγ positive staining (dark brown) of murine E18 brown adipocytes in interscapular BAT.**B.** UCP-1 immunohistochemistry of a serial section showed in A, revealing BAT staining and surrounding muscle being negative. **C-E.** Higher magnification images highlighting the positive staining for transcription factors involved in BAT development (PPARγ; C/EBPβ; C/EBPα) all in brown adipocyte nuclei. **F-G.** UCP-1-immunogold staining (black dots) showing positive mitochondria in brown adipocytes (ba, magnification in the inset), as well as in poorly differentiated cells (p) in pericytic position (G is an enlargement of the squared area in F). **H**. Some endothelial cells (e) showing mitochondria similar to those of poorly differentiated preadipocytes, close to the capillary wall. **I**. Enlargement of the red squared area in H outlining this similarity(asterisk indicating the lumen of capillaries).**J-L.** scWAT showing the appearance of X-gal labeled multilocular adipocytes (arrows) in rosiglitazone-treated VE-cadherin-CreER$^{T2-/+}$/R26R$^{-/+}$ mice.

Figure 2.S3


A
PPARγ
600 µm
B
BAT
muscle
UCP1
250µm
J
100µm
C
PPARγ
15 µm
D
C/EBPβ
25 µm
E
C/EBPα
25 µm
K
50µm
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ba
0.4 µm
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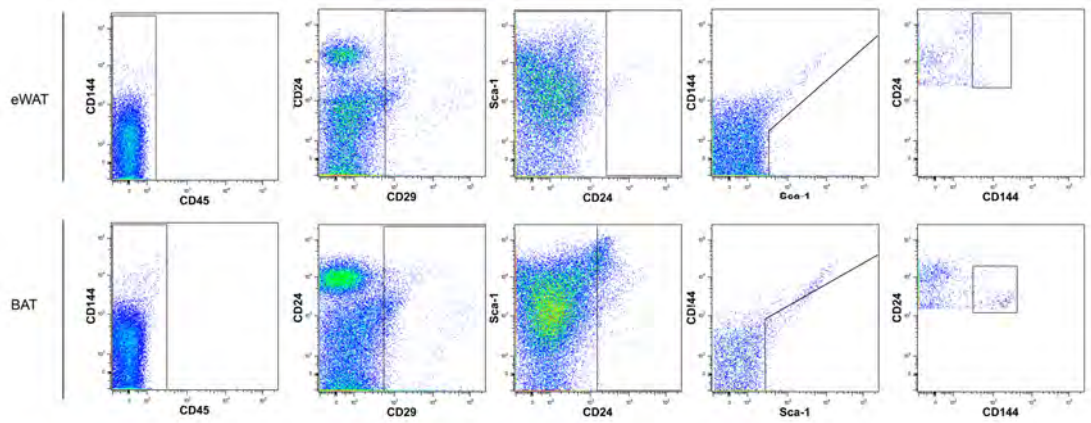
25µm



Supplementary Figure 4: FACS gating strategy. Respective fluorescence-minus-one plots used to define gates in FACS experiments, relating to Figure 4.

CHAPTER III: The microenvironment and adipogenesis

Olga Gealekman performed the microscopy work described in this chapter. Khanh-Van Tran performed the real-time PCR experiments. The study of regional differences between thoracic and abdominal aorta was a collaboration between the Corvera and Czech laboratories. Experiments were in parts designed by Timothy Fibzgibbons, Olga Gealekman and Khanh-Van Tran.

Summary:

Previously, we have demonstrated that cells from an endothelial lineage of adipose tissue can give rise to adipocytes. Here we investigate whether endothelial cells originating from aortic rings of C57Bl/6J mice have the capacity to undergo adipogenesis. We find that in the presence of the PPARγ agonist, rosiglitazone, cells with endothelial characteristics accumulate lipid droplets, express canonical adipocyte markers, and secrete adipokines. Furthermore, we investigated whether cells from thoracic and abdominal regions of the aorta have genotypic and phenotypic differences. We found that progenitors from different regions of the aorta express different levels of developmental gene Hoxc9. Cells from these distinct areas have different proliferative dynamics and give rise to distinct adipocyte types. Taken together, these results imply that progenitor cells from a non-adipose organ may have capacity to undergo adipogenesis and that microenvironment is crucial in determining genotype and phenotype of cells.

Introduction:

We recently found that progenitors of an endothelial cell lineage residing in the adipose tissue vasculature have the ability to give rise to a population of adipocytes [153]. There are reports of multiple stem cell niches in different organ systems [154-157]. In this chapter, we address the hypothesis that endothelial cells from aortas of mice are able to give rise to new adipocytes.

In 1990, Nicosia and Ottinetti designed aortic ring assay to study angiogenesis [158]. This development provides a semi-isolated model of angiogenesis while preserving some of the microenvironment that is representative of the physiological process. Cells arising from aortic rings form tubular structures and lumenize. Furthermore, the time course of this process mimics *in vivo* process [159]. Since its first introduction, this method has been widely used to study the development of new vessels, as it bridges the gap between *in vivo* animal work and *in vitro* cell culture [159-162].

In this study, we used the aortic ring assay to study progenitor cell plasticity. When cultured in EGM-2, vast majority of cells (over 90%) arising from the aortic ring express endothelial cell markers. There is also a small subset of cells (around 5%) that express pericyte marker, SMA. In this study, we used PPARγ agonist to stimulate adipogenesis in cells emerging from aortic explant. In the presence of rosiglitazone, we observed that cells expressing endothelial markers begin to take on adipocyte morphology. To confirm that these cells are adipocytes, we checked for expression of adipocyte genes at the transcriptional and translational levels. We found that rosiglitazone decreases the expression of endothelial cell makers and increases

the expression of adipocyte markers. Furthermore, rosiglitazone-induced adipocytes secrete adiponectin, a hallmark adipokine. Taken together, we show that cells arising from the aorta of C57/Bl6 mice can give rise to functional adipocytes.

There have been several reports that adipocytes have different characteristics depending where they are located in the body [83, 163-165]. Tchoukalova *et al.* recently demonstrated that subcutaneous femoral fat and abdominal fat have different proliferative dynamics. Fat accumulating in the femoral area tends to undergo hyperplasia, whereas fat in the upper abdominal area tends to undergo hypertrophy in the presence of overfeeding [84]. They attributed this observation to differences in the progenitor cell population.

In this study, we aim to study preadipocyte populations between the thoracic and abdominal regions of the aorta. We attempt to measure the proliferative capacity of the precursor cells by quantifying the endothelial sprouts arising from the aortic rings. We also examined the expression levels of classical adipocyte genes in the rosiglitazone-stimulated fat cells from different regions of the aorta. Results reported here are very preliminary. However, our experiments show that progenitor cells from the thoracic and abdominal aorta express different levels of the developmental gene, Hoxc9. Furthermore, the adipocytes they give rise to may be distinct.

Experimental Procedures:

Aortic Ring Assay: C57Bl/6J mice were sacrificed and thoracic and abdominal aorta fragments were removed. The two fragments of aorta were then cleaned under dissection microscope to ensure that all associated fat and connective tissue were removed. The aortic fragments were washed with EGM-2 media and cut into 1 mm

ring segments. Each aortic ring segment was embedded in a singular well of a 96-well plate containing 40 μL of Matrigel (BD Discovery Labware). After aorta was embedded in the 96-well plate, the plate was allowed to stand for 30 min in the 37° C so that the Matrigel would solidify. The cells were fed with 200 μL of EGM-2 media, which was changed every other day. At day 7 and day 14, number of capillary branches forming around the periphery of the aortic ring was quantified at x100 magnification by two independent investigators.

Staining and Analysis: Pieces of aortic rings were embedded in Growth Factor Reduced Matrigel (BD *Biosciences*) in 35 mm glass-bottom culture dishes (MatTek Corporation), and cultured in EGM-2 media for 14 days. After 14 days in culture, explants were fixed in 4% Formaldehyde (Ted Pella, Inc.) in PBS for 15 min and permeabilized in 0.5% TX-100 in PBS for 30 min. The primary antibodies used to characterize the origin of cells growing from the explants were: monoclonal mouse eNOS (BD Pharmingen, 1:200), mouse CD31 (1:50), mouse CD34 (1:50), mouse monoclonal Smooth Muscle Actin (1:100), polyclonal rabbit Von Willebrand Factor (Dako, 1:100), monoclonal mouse Adiponectin (Peirce, 1:200), and polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 1:200). Secondary antibodies were species matched Alexa Fluor 594, and Alexa Fluor 488 (Millipore; Molecular probes, 1:500). Negative controls treated with irrelevant mouse IgG instead of primary antibody were processed simultaneously. All sections were counterstained with DAPI (Millipore, Molecular Probes).

*Real-Time PCR***:** The mouse aorta fragments were mechanically excised and the endothelial sprouts remaining in the Matrigel were isolated using Dispase II (Roche, 2.4 U/mL), centrifuged, and RNA extracted from the pellet using an Ambion RNA extraction kit. Probes used are specified in Table 2.

Table 2. Sequences of probes use for RT-PCR

PROBES	SEQUENCES
VE-Cadherinf	CACTGCTTTGGGAGCCTTC
VE-Cadherinr	GGGGCAGCGATTCATTTTTCT
CD34f	AAG GCT GGG TGA AGA CCC TTA
CD34r	TGA ATG GCC GTT TCT GGA AGT
VEGFR2f	TTTGGCAAATACAACCCTTCAGA
VEGFR2r	GCAGAAGATACTGTCACCACC
Acrp30f	TGT TCC TCT TAA TCC TGC CCA
Acrp30r	CCAACCTGCACAAGTTCCCTT
Perilipinf	GGGACCTGTGAGTGCTTCC
Perilipinr	GTATTGAAGAGCCGGGATCTTTT
GLUT4f	GTG ACT GGA ACA ATG GTC CTA
GLUT4r	CCAGCCACGTTGCATTGTAG
Cideaf	TGACATTCATGGGATTGCAGAC
Cidear	GGCCAGTTGTGATGACTAAGAC
UCP-1f	AGGCTTCCAGTACCATTAGGT
UCP-1r	CTGAGTGAGGCAAAGCTGATTT
HoxC9f	ACT CGC TCA TCT CTC ACG ACA
HoxC9r	AGGACGGAAAATCGCTACAGT
Sca-1f	AGG AGG CAG CAG TTA TTG TGG
Sca-1r	CGT TGA CCT TAG TAC CCA GGA
CD24f	GTT GCA CCG TTT CCC GGT AA
CD24r	CCC CTC TGG TGG TAG CGT TA
m36B4f	TCCAGGCTTTGGGCATCA
m36B4r	CTTTATCAGCTGCACATCACTCAGA

Western Blotting: The mouse aorta fragments were mechanically excised and the sprouts remaining in the Matrigel were isolated using Dispase II (Roche, 2.4 U/mL). Cell lysates were prepared using the Complete Lysis-M kit (Roche). Protein concentration was measured using the Beckman DU-640B Spectrophotometer. Equal amounts of protein (20 µg), and conditioned media (40 µl) were loaded, and analyzed by Western blot in accordance with standard procedure. Primary antibody used was rabbit polyclonal anti-Adiponectin (Peirce, 1:10000). Secondary was goat anti-rabbit (Promega).

RESULTS:

We found that in the presence of rosiglitazone, cells arising from the aorta can accumulate lipid droplets (Figure 3.1). The morphology of cells is no longer spindle-like, rather they resemble multilocular fat cells. Oil-Red-O staining clearly identified lipid droplets in cells coming from the aorta (Figure 3.2)

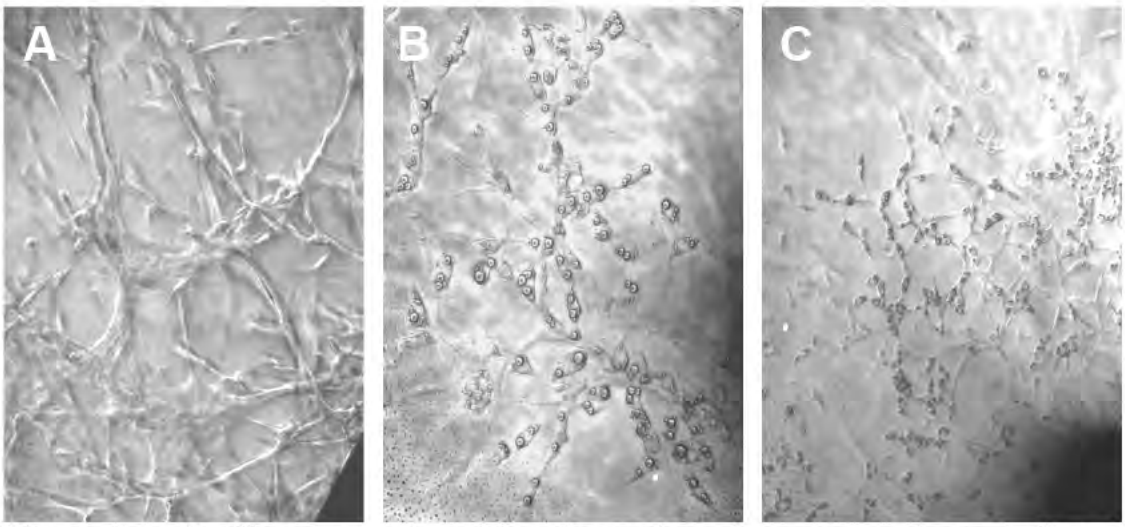


Figure 3.1. Capillary sprouts arising from thoracic aorta explants undergo adipogenesis in the presence of rosiglitazone. Representative photomicrographs of capillary sprouts growing from thoracic aorta explants under control conditions (**A**), and in the presence of Rosiglitazone (**B** and **C**) after 12 days in culture. Rosiglitazone induced dramatic alteration in morphological appearance of capillary sprouts characterized by accumulation of lipid droplet - like structures. Original magnification was x20 for A and B, and x10 for C.

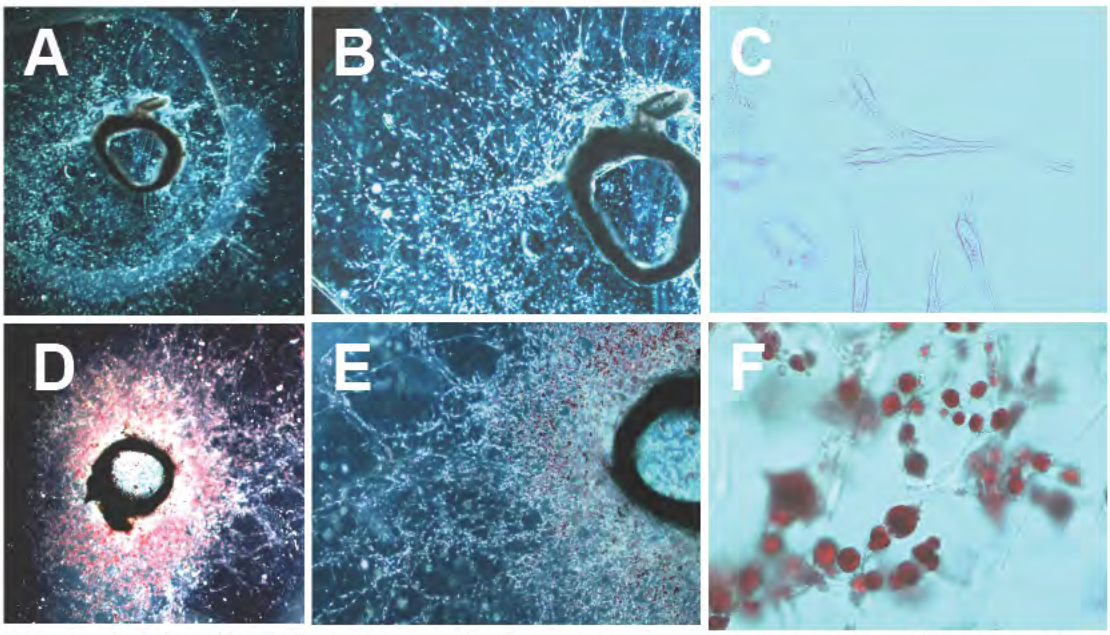
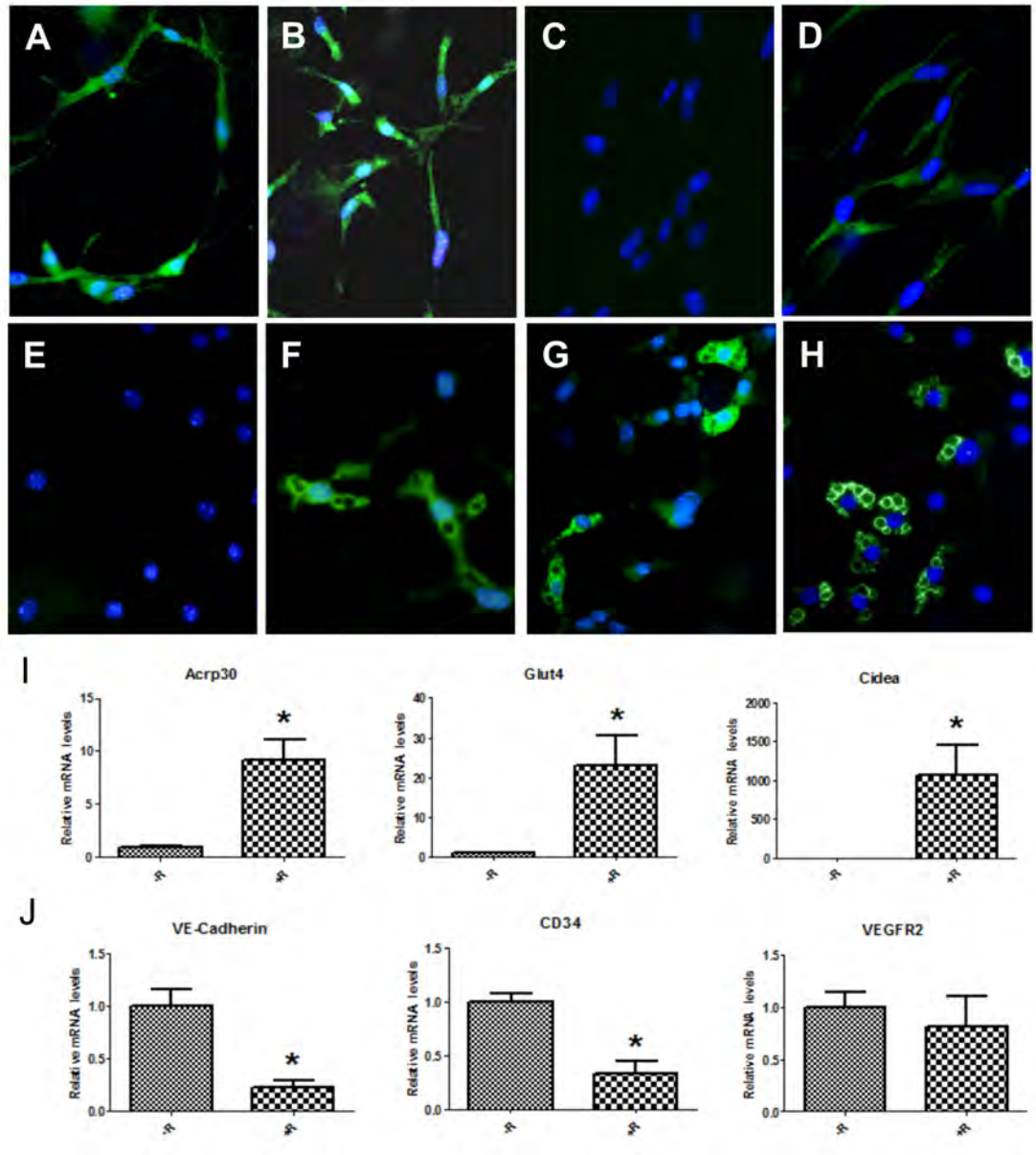


Figure 3.2. Oil-Red-O staining of cells arising from the aorta in the presence of rosiglitazone. Representative photomicrographs of capillary sprouts growing from thoracic aorta explants under control conditions (**A**, **B**, and **C**), and in the presence of Rosiglitazone (**D, E** and **F**) stained with Oil-Red-O for identification of lipid **droplets.** Positive Oil-Red-O staining is observed only in the presence of Rosiglitazone. Original magnification is 100x for **A**, and **D**, x200 for **B**, and **E**, and x400 for **C**, and **F**.

To confirm that these cells are adipocytes and not endothelial cells accumulating lipid droplets, we used immunoflourescence and real-time PCR to check for the expression of several adipocytes and endothelial markers. We found that in the presence of rosiglitazone, the expression of CD31 is decreased and the expression pattern of CD34 is distinctly altered (Figure 3.3). Conversely, the expressions of adiponectin and perilipin are increased. In particular, perilipin expression pattern in these cells is consistent with that of an adipocyte, i.e. surrounding a lipid droplet. The reciprocal change in endothelial cell and adipocyte markers observed using immunoflourescence is consistent with real-time PCR results demonstrating a decrease in endothelial cell markers, VE-Cadherin, CD34 and VEGFR2 and an increase in adipocyte markers, Acrp30, Glut4 ,and Cidea (Figure 3.3). We further tested cell lysates and condition media of sprouts growing from aorta explants for the expression and secretion of adiponectin. We confirmed that these rosiglitazone-induced fat cells are secreting adiponectin, a hallmark adipokine (Figure 3.4).

Figure 3.3. Reciprocal changes in endothelial cell and adipocyte markers. In the absence of rosiglitazone, cells arising from aortic rings are positive for CD31 (**A**) and CD34 (**B**), but are negative for adiponectin (**C**) and perilipin (**D**). In the presence of rosiglitazone, expression of CD31, and CD 34 (**E** and **F**) is altered, and there is an increase in the expression of adiponectin and perilipin (**G** and **H**). Furthermore, mRNA levels of cells arising from aortic rings at day 14 in culture in the absence (**I**) and presence of rosiglitazone (**J**) were assessed. There is a statistically significant increase in adipocyte markers, Acrp30, Glut4, and Cidea, and a reciprocal decrease in endothelial cell markers VE-Cadherin, CD34, and VEGFR2. n=3-6, data were analyzed using student t-test, *$p < 0.05$.

Figure 3.3


A
B
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Acrp30
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Relative mRNA levels
-R
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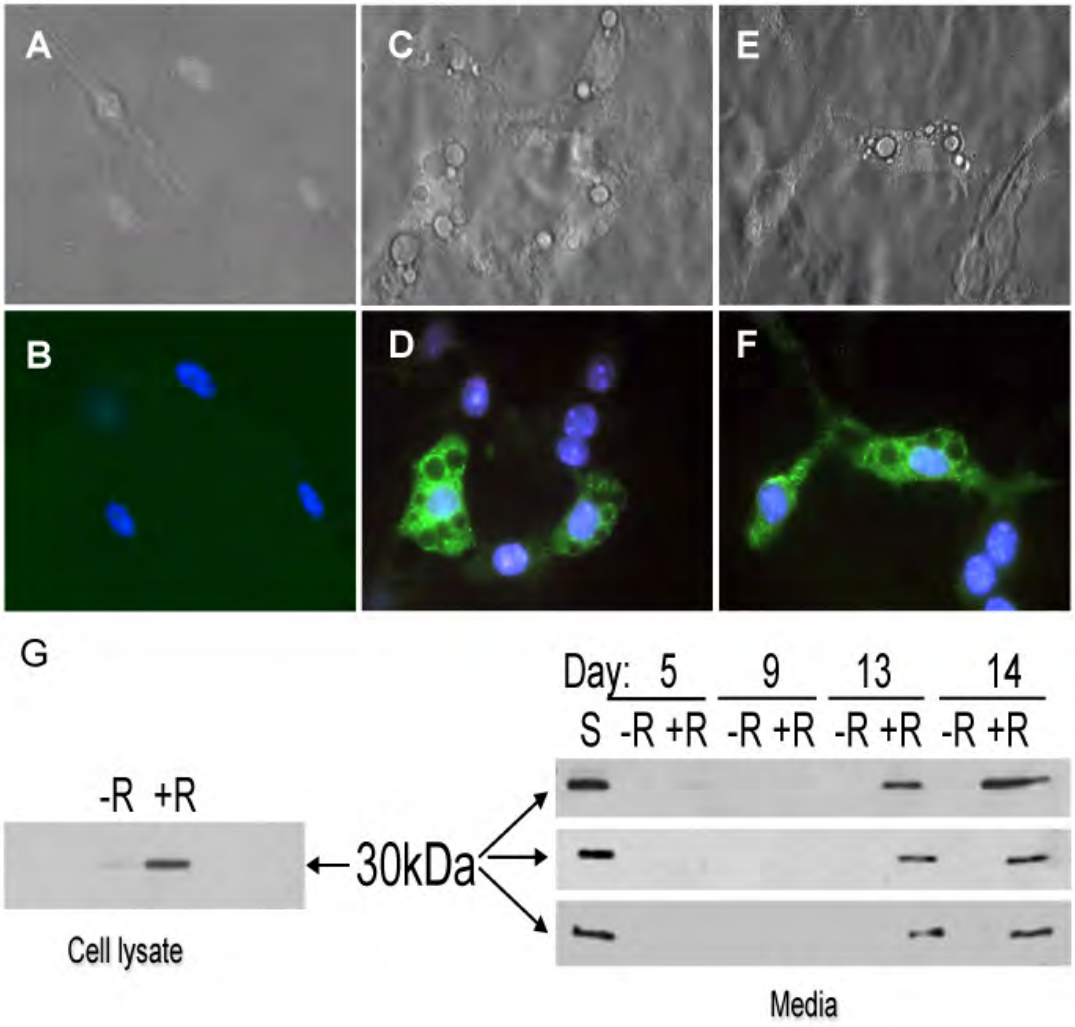

Figure 3.4. Rosiglitazone-induced adipocytes express and secrete adiponectin. Immunofluorescent analysis of Adiponectin expression in capillary sprouts forming from aorta explants under control conditions (**A**, and **B**), and in the presence of Rosiglitazone (**C** - **F**). Positive Adiponectin specific immunostaining was observed only in Rosiglitazone treated cells. A, C, and E are bright light microscopy images. B, D, and F are merged from the images taken using UV and green spectrum filters. Original magnification was x630. **G**. Western blot analysis of adipocyte marker Adiponectin in capillary sprouts growing from aorta explants under control conditions (-R) and in the presence of Rosiglitazone (+R). Rosiglitazone induced expression of Adiponectin in the cell lysates, as well, as secretion of Adiponectin into the media. Normal mice serum (S) was used as a positive control for Adiponectin detection.

We then asked if adipocyte precursors in different regions of the aorta are unique (Figure 3.5). Interestingly, we found that abdominal aorta has higher mRNA levels of Hoxc9, a homeobox gene expressed higher in WAT than BAT [163, 166]. Although, we found no differences between expression of Sca-1, and CD24 in these populations, it is clear that rosiglitazone decreases the expression of CD24 (Figure 3.6).

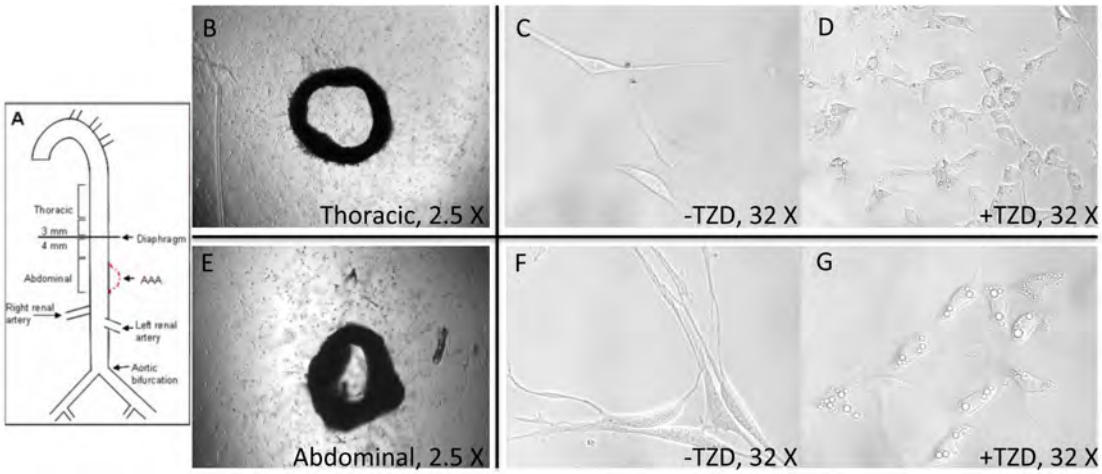

Figure 3.5. Capillary sprouts arise from both abdominal and thoracic aorta fragments. Cells arising from thoracic (**B**) and abdominal (**E**) rings were analyzed in the absence (**C** and **F**), and in the presence of rosiglitazone (**D** and **G**). Rosiglitazone induced the formation of multilocular adipocytes from both segments of aorta.

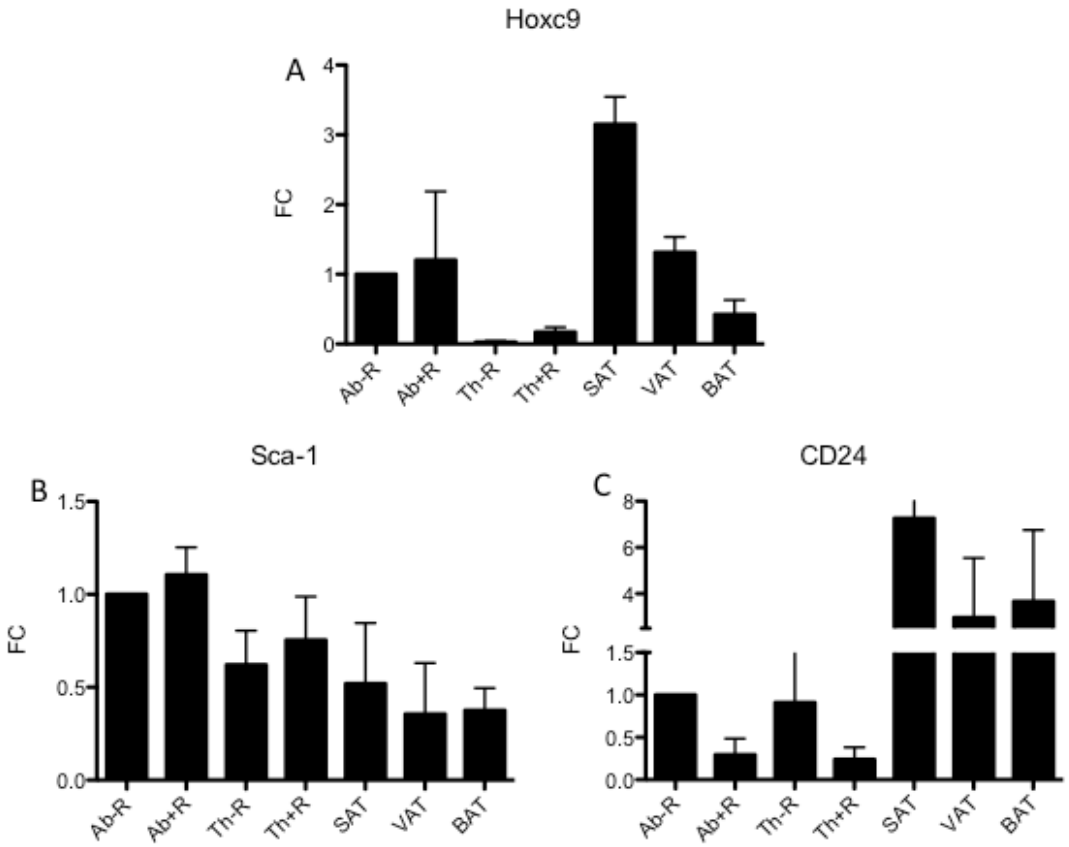

Figure 3.6. mRNA levels of developmental and precursor genes. Thoracic and abdominal regions of the aorta had different levels of expression of Hoxc9 (**A**). Although no difference in Sca-1 or CD 24 expression (**B** and **C**), we found that the expression of CD24 was decreased in the presence of rosiglitazone (**C)**.

We also examined the growth of capillary sprouts from the thoracic and abdominal regions of aorta. We found that significantly lower number of capillary sprouts grew from the abdominal aortic rings of the mice, as compared to the thoracic (Figure 3.7). We further tested to see if the adipocytes stimulated to form by rosiglitazone in different areas of the aorta were genotypically different. We found that rosiglitazone increased the expression of adiponectin, Glut4, and perilipin in cells arising from the abdominal and thoracic aortic rings. This same trend was observed with Cidea and UCP-1, canonical brown adipocyte genes. However, we observed that adipocytes arising from the thoracic aorta had much higher expression of both Cidea and UCP-1 (Figure 3.8).

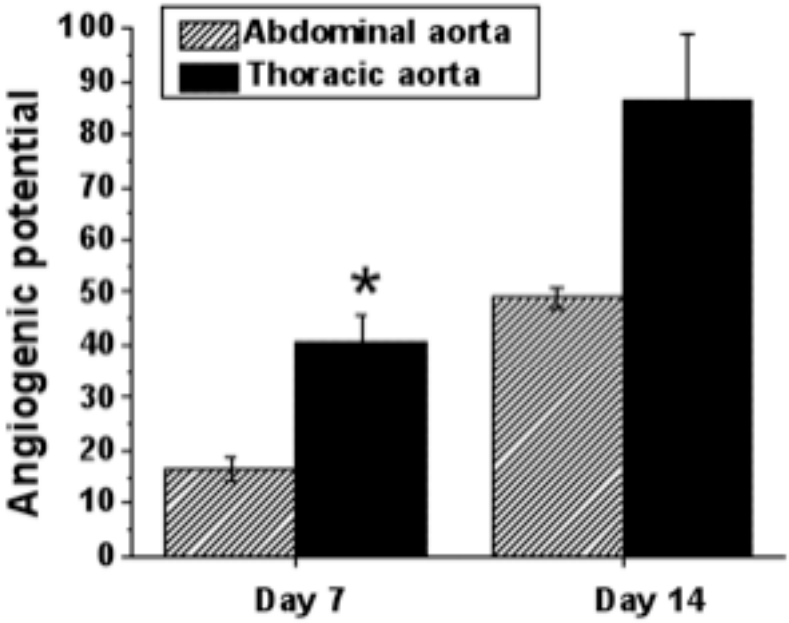

Figure 3.7. Abdominal aortic explants have less angiogenic potential. Quantitative analysis of angiogenic potential of abdominal and thoracic aorta explants from mice cultured for 14 days. Abdominal and thoracic aortas were surgically removed from 13 weeks old C57Bl/6J mice, embedded into Matrigel, and cultured for two weeks. After 7 and 14 days in culture number of capillary sprouts growing around perimeter of the aorta rings was counted by two independent investigators. Data was analyzed using student t-test, *$p<0.05$ and **$p<0.001$

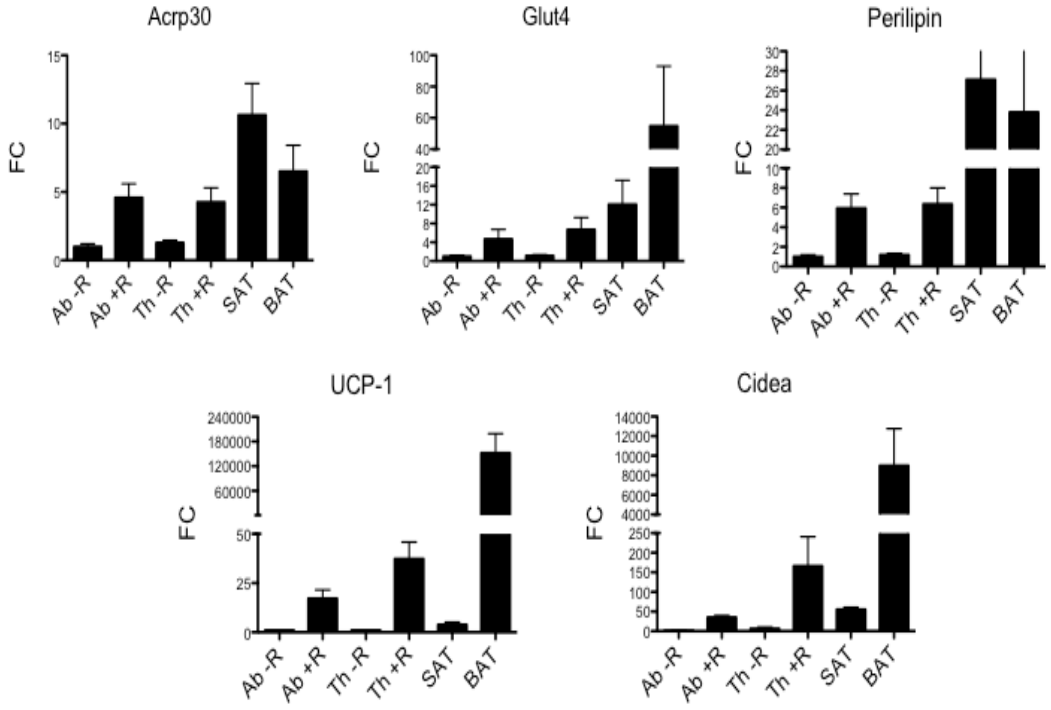

Figure 3.8. **Differential gene expression in rosiglitazone-induced adipocytes from abdominal and thoracic aortic explants.** Rosiglitazone increased the expression of perilipin, adiponectin, Glut4, UCP-1, and Cidea in cells arising from the aorta (**A** and **B**). However, the up-regulation of UCP-1 and Cidea in cells from the thoracic aortic region was much higher than that observed in cells from the abdominal aortic region.

Discussion:

Using a traditional model of angiogenesis, we found that endothelial cell progenitors residing in aorta can also undergo adipogenesis. This study demonstrates the plasticity of the progenitor population. These results are consistent with those of Joe *et al.* who have found that stem cells from muscle can give rise adipocytes [167]. It is worth noting that Tang *et al.* found that PDGFRβ+ cells from the kidneys are not able to undergo adipogenesis [91]. They concluded that only mural cells from the adipose tissue have adipogenic capacity. Perhaps the reason for the potential discrepancies between our results and that of the Graff lab is the difference in the microenvironment in which our cells were cultured. In our aortic ring assay, the cells have an *ex vivo* surrounding, in which some of the paracrine signaling existing between cells of the aorta are still preserved.

One obvious limitation stemming from the *ex vivo* model is that we are not completely sure about the identity of the cells that give rise to newly-formed adipocytes. Our conclusion that cells of endothelial origin from the aorta can give rise to adipocytes rests on the assumption that the spindly-like cells that express endothelial markers in the absence of rosiglitazone are the same cells that accumulate lipid droplets and express adipocyte markers in its presence. It is possible that in the presence of rosiglitazone we activated a population of precursors distinct from the one that gives rise to endothelial cell. Furthermore, there is a physiological fat pad around the aorta. Although we have tried to remove all fat around the aorta using micro-dissection, there is some possibility that some were left behind. Thus, what we are seeing might be adipocyte precursors from a non-endothelial cell lineage undergoing adipogenesis.

Although there are two clear limitations in our study, our results of the VE-cadherin fate tracing experiment support the hypothesis that endothelial cells can give rise to adipocytes. In the future, we hope to use aortas from the VE-Cadherin/R26R-eGPF mice to address this limitation.

Preliminary evidences from our study of thoracic and abdominal aorta show that precursor cells from different regions of the aorta have different genotypes and proliferative dynamics. Cells from the abdominal region of the aorta express higher levels of Hoxc9, a developmental gene that is expressed higher in WAT than BAT [163, 166]. In the presence of rosiglitazone, the progenitors also give rise to adipocytes that are unique. Adipocytes arising from the thoracic aorta have higher expression of UCP-1 and Cidea. This data indicate that the cells surrounding the thoracic aorta may have more of a “brite cell” phenotype, as opposed to a white adipocyte phenotype seen in fat cells surrounding the abdominal aorta [166]. Police *et al.* have reported that there are differences in aortic fat pads that may have a causative role in abdominal aortic aneurysm [168]. Our preliminary results support the notion that the adipocytes in different regions of the aorta have distinct characteristics. It was not our intention to study the pathogenesis of abdominal aortic aneurysm, however we do think that this *ex vivo* aortic ring model can give valuable insight to the role that adipocytes play in different regions of the aorta.

Our preliminary results also show that rosiglitazone decreases the expression of CD24 in the cells arising from the aorta. This is consistent with the findings of Tang *et al.* that chronic rosiglitazone treatment lowers CD29, Sca-1, CD44 and CD24 expression in progenitors cells by either depleting the precursor population or changing precursor dynamics [148].

In brief, our preliminary evidence suggests that precursor cells from an endothelial lineage arising from the aorta can give rise to adipocytes. The genotypes and proliferative dynamics of the progenitor cells from different regions of the aorta are distinct. Furthermore, the adipocytes that they give rise to are also unique and may have important physiological function.

CHAPTER IV: Summary and Final Thoughts

"For animals, belonging to two most distinct lines of descent, may have become adapted to similar conditions, and thus have assumed a close external resemblance; but such resemblances will not reveal—will rather tend to conceal their blood-relationship."

Darwin, On the Origin of Species, 1st ed., p. 427

Summary, limitations and future experiments:

Chapter II: In obesity, adipose tissue growth necessitates the development of new adipocytes and associated supporting vasculature. Here we find that endothelial cells of adipose tissue display similar morphological features to pericytes, which has been shown to give rise to adipocytes. Fate tracing using VE-Cadherin-Cre-dependent reporters revealed expression of LacZ and eGFP in endothelial cells, preadipocytes and adipocytes of BAT and WAT.

To examine whether our findings in mice adipose tissue translate to human adipose tissue, we analyzed capillary sprouts arising from human adipose explants. We found that capillary sprouts arising from human subcutaneous fat express Zfp423, a preadipocyte determination factor. In response to PPARγ activation, endothelial characteristics of sprouting cells are progressively lost, and cells form structurally and biochemically defined adipocytes. Taken together, these data reveal an endothelial origin of murine and human adipocytes. The ability of the vascular endothelium to give rise to adipocytes may explain how angiogenesis and adipogenesis can be temporally and spatially coordinated.

Our results are supported by a recent study from the Spiegelman laboratory which demonstrated that a subset of pericytes and endothelial cells express Zfp423, a marker of adipocyte pre-determination [89]. Moreover, PPARγ deletion using Tie-2-driven Cre-recombinase results in decreased adiposity and adipocyte size in response to HFD-feeding and rosiglitazone treatment [144]. It is important to note that Tie-2 is expressed in a mosaic pattern in capillary endothelium [143]. Preferential expression of Tie-2 in the arteriolar endothelium may explain how some adipocytes in the adipose capillaries may escape PPARγ deletion to give rise to normal adipocytes.

However, the presence of normal adipocytes in the knockout animals may also be due to the presence of precursors from a non-endothelial cell lineage.

There are reports of several distinct types of adipocytes—white, brown and brite. Within these categories, there are different subsets of cells that have variable physiological significance. Our results show that both white and brown adipocytes can originate from cells of endothelial origin. However, it is also clear from our FACS analysis that not all precursor cells express VE-Cadherin. Of the CD45-CD29+Sca1+CD24+ progenitor population, only 17% and 52% express VE-Cadherin in WAT and BAT, respectively. This analysis illustrates two important points: 1) there are adipocyte precursors that do not originate from a VE-Cadherin+ lineage and 2) the WAT and BAT have variable adipocyte precursor populations. Differences in composition and types of adipocyte progenitors may explain the difference in the adipocytes phenotypes that we observe in BAT and WAT.

Of note, we adopted a similar but altered FACS sorting scheme than that of the Graff laboratory to isolate adipocyte progenitors. We were not able to analyze the CD34 expressing cells due to technical challenges. We also felt that because CD34 can be expressed on both endothelial cell and adipocyte precursors, it was not essential for addressing the question asked in this work.

For the purpose of our study, it was important to rule out that our observations are not due to adipocytes arising from a hematopoietic cell lineage, and thus was our rationale for negatively selecting CD45 expressing cells. However, we did not analyze expression of CD31 or Ter119 in the SVF population. Consequently, we are unable to determine what percentage of the SVF population we analyzed is made up of Ter119 and CD31 expressing cells, which have low adipogenic potential. Interestingly, Gupta

et al. have found that CD31 positive cells of the adipose vasculature also express Zfp423, raising questions about the adipogenic potential of CD31+ endothelial cells.

Although this study supports an endothelial origin of a population of adipocytes, it does not exclude a hematopoietic origin of adipocytes. VE-Cadherin is expressed in endothelial cells and a sub-populations of hematopoietic cells before E11.5, and thus descendants from both lines are potentially labelled in our study [136]. To decrease the tracing of hematopoietic cells, an inducible VE-Cadherin-CreER^{T2} mouse model was used. Induction of Cre activity by Tamoxifen in adulthood results in negligible excision (lower than 0.4%) in the hematopoietic lineage [134]. However, this does not completely eliminate hematopoietic tracing. Furthermore, even if it was possible to trace only endothelial cells, our results cannot exclude the possibility that adipocytes can arise from a different lineage.

A limitation of this study is that it does not conclusively identify the point at which either a precursor cell or an endothelial cell is programmed to differentiate into an adipocyte (Figure 4.1). For example, do endothelial cells and adipocyte arise from a common progenitor cell that expresses VE-Cadherin? Alternative, are newly formed adipocytes created from endothelial cells that have divided and migrated into the tissue? Although, endothelial cell division and migration into extravascular space to be reprogrammed into a new adipocyte is plausible scenario in the case of obesity. The reverse situation in which an adipocyte is stimulated to become an endothelial cell seems unlikely. Our FACS analysis revealed that cells expressing CD29, CD34, Sca-1 and CD24 also express VE-Cadherin, suggesting that there is an existence of an early adipocyte progenitor from an endothelial lineage. However, the mechanism of new adipocyte formation is not yet clear.

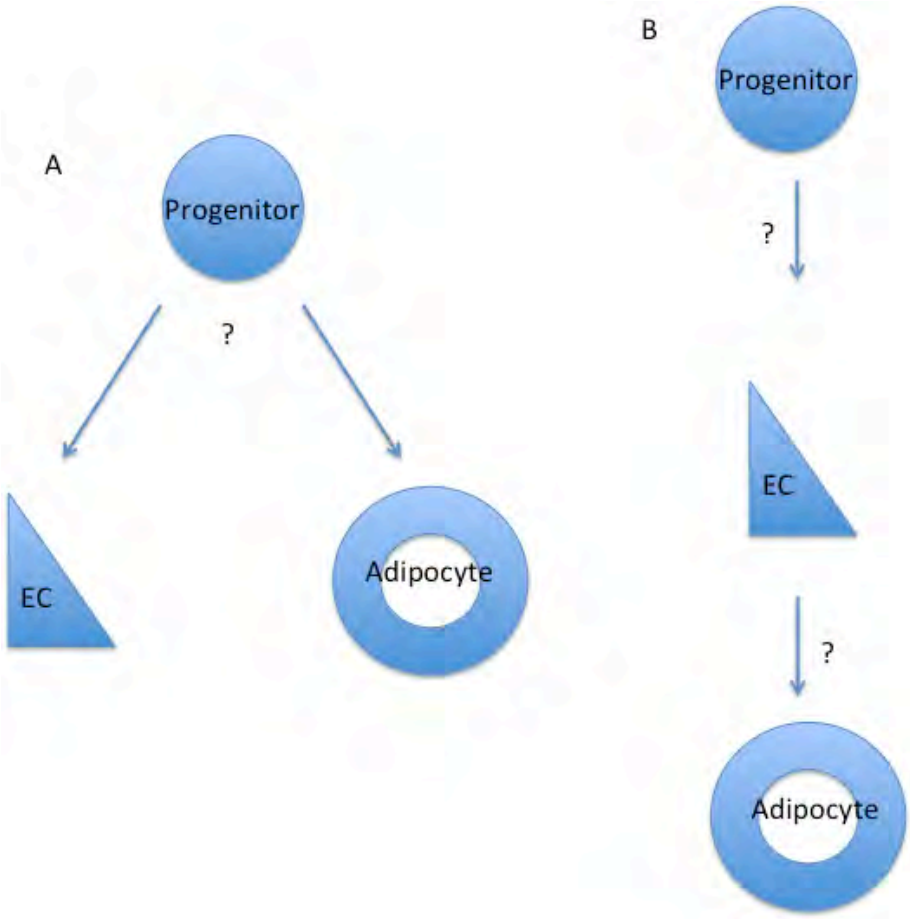

Figure 4.1. Possible sequences of new adipocyte formation. A VE-Cadherin+ precursor may give rise to both endothelial cells and adipocytes (**A**), or adipocytes can arise from an endothelial cell that is re-programmed to become a fat cell (**B**).

A natural progression of this project would be to isolate and characterize VE-Cadherin+ adipocyte progenitors. These cells can be sorted by FACS using several potential models (Figure 4.2). If indeed endothelial cells and adipocytes share a common progenitor, then we should observe VE-Cadherin+ cells undergo robust adipogenesis. Furthermore, we can use FACS to isolate distinct VE-Cadherin+ populations, e.g. populations also expressing Tie-2, to determine molecular signature of the adipocyte precursor arising from the VE-Cadherin+ lineage. It would also be beneficial to measure insulin sensitivity, mitochondrial content, oxygen consumption, and other metabolic parameters of these VE-Cadherin-derived adipocytes, as compared to other adipocyte populations. Finally, we should engraft VE-Cadherin+ adipogenic precursor cells into lipodystrophic or diet-induced obese animals to assess their physiological importance.

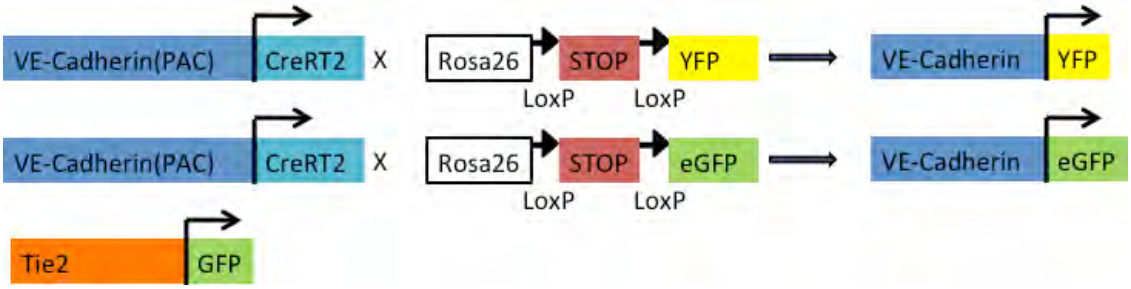

Figure 4.2. **Reporter models for isolation of cells from an endothelial cells lineage.** Either VE-Cadherin reporter models (**A** and **B**) or the Tie-2-GFP mouse model can be used to FACS sort endothelial cells and adipocyte progenitors

Chapter III: We find that rosiglitazone can stimulate cells with endothelial characteristics originating from the aorta to undergo adipogenesis, resembling the phenomenon we observed in human adipose tissue explants. Rosiglitazone-induced adipocytes from the thoracic and the abdominal regions of the aorta display different characteristics; cells from the thoracic aorta express higher levels of UCP-1 and Cidea, traditional brown adipocyte genes. The genotypic differences observed in adipocytes surrounding the aorta may have important physiological implications. For example, inflammation of adipose tissue surrounding the abdominal aortic region may be involved in the pathogenesis of abdominal aortic aneurysms [168]. The aortic ring assay may be a valuable tool to study the role that adipocytes play in pathologies relating to the abdominal or thoracic aorta.

Despite the fact that the aortic ring assay is a classical assay extensively used by numerous investigators to study angiogenesis ex-vivo, to the best of our knowledge, lineage tracing experiments to prove the endothelial origin of cells forming the capillary sprouts were never performed. Therefore, in order to obtain stronger evidence that cells of endothelial origin capable to undergo adipogenesis are present in other organs, aortic ring assays using VE-Cadherin-CreER^{T2}/YFP or eGFP reporter mice should be performed (Figure 4.2).

In addition, although results obtained using the *ex vivo* model are valuable, they represent an artificial process. Cells arising from the aorta or any other organ systems *in vivo* will never be subjected to the experimental conditions that we imposed; thus our results may not be reflective of normal physiology. However, these models allow us to study the differentiation potential of cells outside of the *in-vivo*

environment and to interrogate the paracrine signaling involved that would be challenging to study in a live animals.

On multiple origins of adipocytes: Although our study clearly shows that a population of adipocytes is derived from an endothelial cell lineage, it does not exclude alternate origins of adipocyte. As many others have shown and as supported by our FACS analysis, adipocytes can come from other cell lineages. Although it has been mentioned in Chapter II, we will briefly discuss the results of Seale *et al.* The Spiegelman laboratory showed that muscle cells and brown adipocytes are Myf5+, and that PRDM16 controls the switch between myocytes and fat cells phenotype [118]. They report that white adipocytes are not Myf5+. Thus, it is somewhat surprising that in our study VE-Cadherin reporters traced into white and brown adipocytes, but not into myocytes. One possible explanation for this is that VE-Cadherin is expressed later in development than Myf5. In that case the brown adipocyte lineage and the myocyte lineage have already diverged, and thus our model would not have labeled myocytes. There is little evidence in the literature to determine the expression pattern of these two genes in relation to each other.

It is also important to note that Myf5+ is not strictly a myocyte marker, but is expressed in several cell types [169]. Expression of Myf5+ has been found in neurons and in a population of cells from the dorsal aorta that also expresses VE-Cadherin [149, 170, 171]. Reports also indicate that not all muscle cells expressed Myf5 [172]. In this study, we detected a low level of Myf5 mRMA expression in capillary sprouts originating from human subcutaneous fat and mouse aorta. However, because

presence of other cells types in addition to endothelial cannot be completely excluded, question regarding Myf5 expression in endothelial lineage cells remains open.

Additional explanation that would bridge our study and that of the Spiegelman's group would be that brown adipocytes arise from multiple cell lineages, some of which are Myf5+, and some are Myf5-. Seale *et al.* supports this hypothesis by demonstrating that β-adrenergic-stimulated mutilocular adipocytes are not Myf5+. Reports of white fat cells taking on brown fat cell phenotype upon cold exposure illustrates brown fat formation from a Myf5- cell, indicating that there could be multiple origins of brown adipocytes.

Others have shown that cells of a hematopoietic lineage can give rise to adipocytes [118, 145, 173]. Most significantly, the Majka *et al.* have performed fate tracing experiments using the LysM-Cre/R26R mouse to show a population of adipocytes originating from a myeloid lineage. To further support this, Sera *et al.* have shown that eGFP+ hematopoietic stem cells (HSC) give rise to adipocytes when engrafted into recipient mice. It is also important to note that Zovein *et al.* have demonstrated that endothelial cells and hematopoietic cells arise from the same origin [135]. Thus, evidence supporting the origins of adipocytes from HSC and endothelial cells may not be mutually exclusive.

Recently, Billon *et al.* described a population of adipocytes originating from the neuroectoderm. *Sox10* is highly expressed in migratory neural crest cells; however it is not expressed in somatic and cephalic mesoderm. Billon *et al.* used Sox10-Cre/YFP reporter mice to show that adipocytes from the jaw are derived from cells of the neural crest [174, 175]. Because of the great heterogeneity within and between fat depots [84, 123], it makes biological sense that there are multiple

phenotypically and genotypically different progenitors. Figure 4.3 maps conclusions from some of the most recent studies on adipocyte precursor cells.

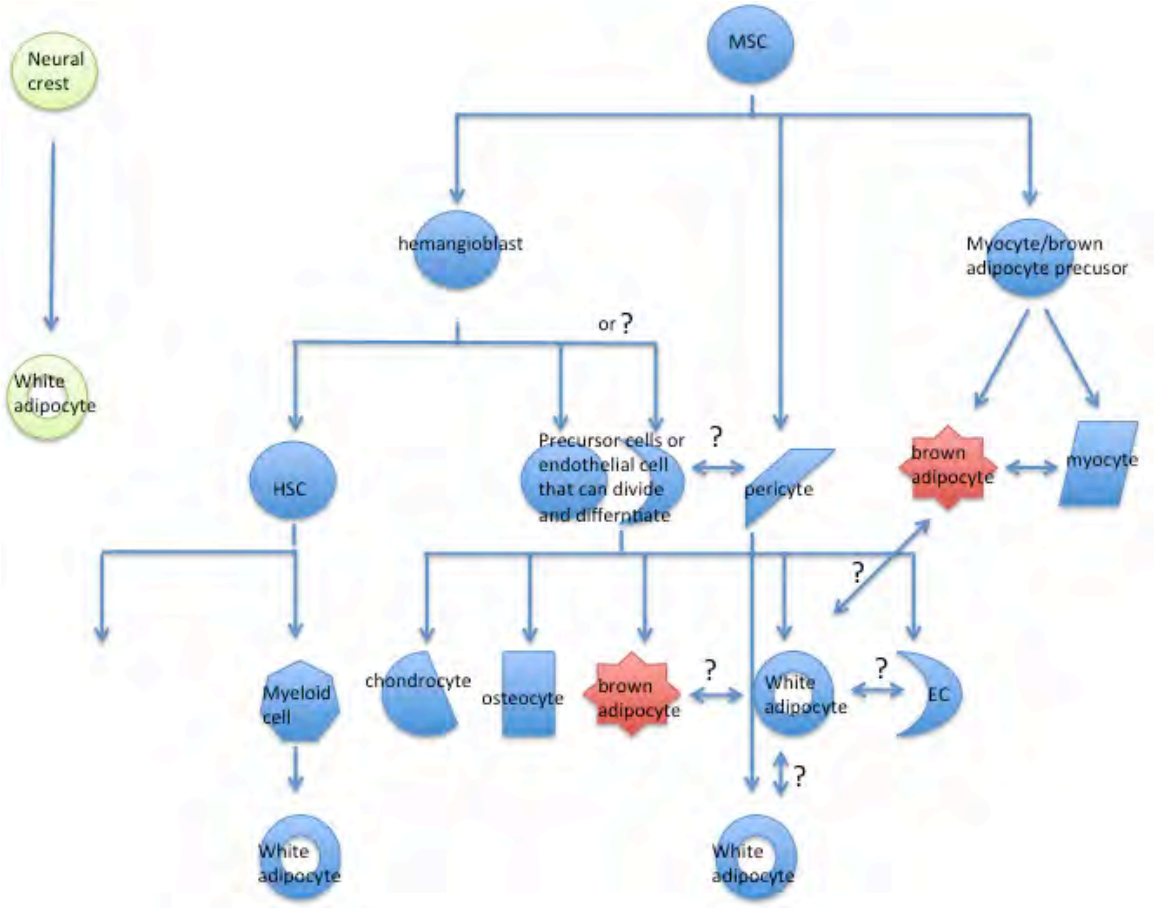

Figure 4.3. Model for multiple origins of adipocytes. Neural crest cells (Sox10+) can give rise to white adipocytes (**A**). Most adipocytes are considered to be of mesodermal origin (B). It is reported that hematopoietic cells and endothelial cell have common precursor, the hemangioblast. After diverging into the hematopoietic lineage and endothelial lineages, cells from both lineages were found to have the capacity to undergo adipogenesis. Progenitor cells that are My5+ can give rise to a population of muscle cell and brown adipocytes, but not white adipocytes. Questions remain about the sequence of events by which new adipocytes are formed, and whether there is enough plasticity for inter-conversion in between phenotypes.

The current body of evidence on adipocyte progenitors suggests multiple origins of adipocytes. This may reflect the vital role of storing energy in the body. All organ systems, regardless of their function, must store nutrients to survive. The most efficient way to store energy is through fat. There are reports of fat storage in multiple cell types in the animal kingdom. For example, *C. elegans* stores fat in the intestinal epithelium and sharks store fat in the liver [40]. Perhaps it is not too surprising that we find multiple cell types able to take on the adipocyte phenotype in mammals.

The concept that several cells types can be reprogrammed to take on a different phenotype for the benefit of an organ has been previously demonstrated. For example, Morroni *et al.* has shown that there is reversible trandifferentiation of adipocyte and secretory epithelial cells of the mammary gland [176]. During pregnancy, adipocytes of the mammary glands can transdifferentiate into secretory epithelial cells. Post-lactation, these cells revert back to their initial adipocyte phenotype. A less drastic example of transdifferentiation is between brown and white adipocytes. In case of cold exposure, white adipocytes of the adipose tissue will take on a “brown” phenotype and express UCP-1 [119]. Pathological reprogramming is illustrated by the heterotrophic ossification in patients with fibrodysplasia osssificans progressiva. Cells that make up ectopic tissue are found to be of vascular endothelial origin carrying a mutation in the activin-like kinase-2 gene [151]. Although, the mechanisms of cellular reprogramming are mostly undiscovered, it is clear that physiological and pathological conditions influence cell fate.

In obesity, the environment drives each organ to store fat. Although, the phenomenon of adipocytes forming in different organs from multiple cell lineages is not an example of convergent evolution, it may operate on the same principle within a

shorter time frame and on a microcosm. In order to adapt to an excess of nutrients, each system makes its own fat cells. This work does not conclude that every organ has a distinct fat cell progenitor, but rather postulates that in order to adapt to the overfed state, the body makes new fat cells, and that the cell lineage of the precursor is likely to be secondary to the immediate needs of the organ.

Future directions and significance: In moving forward, we should characterize the VE-Cadherin+-derived adipocytes, and determine the molecular signals that drive them towards differentiation. Although, the developmental aspects of this study are engaging, the prospect of identifying the progenitor stem cells and using those stem cells to treat patients who are lipodystrophic or have other obesity-related complications is most exciting. As Rodeheffer *et al.* have shown, injection of a small population of progenitor cells can reconstitute fat pad of a lipodystrophic animal and correct their metabolic impairment. Identifying the population of stem cells that gives rise to healthy white adipocytes can have profound implication for patients suffering from congenital or drug-acquired lipodystrophies.

Brown adipose tissue has been correlated with lower BMIs and greater metabolic health. Generating brown fat in people may be an effective anti-obesity treatment. Here, we find a common precursor to both brown and white adipocytes. Elucidating the pathway in which white to brown conversion occurs may provide a strategy for combating the obesity epidemic affecting our society.

Appendix I: Preliminary study of adipose tissue of NSG and huNSG mice

Introduction:

Hematopoietic origin of adipocytes has been proposed by several different groups [145, 173, 177]. We decided to examine this hypothesis using a mouse model that has severe disruption of hematopoietic cell development. The Schultz and Greiner laboratories have developed a NOD-Scid-Gamma (NSG) animal model by generating an additional mutation in the interleukin-2 receptor γ-chain locus of a NOD-scid mouse [178]. These NSG animals do not have mature T-cells, B-cells, and natural killer cells. A great advantage of this model is that the NSG mice can support hematopoietic stem cell engraftment derived from human cord blood (huNSG). This allows for the presence of human HSC, and immune cells that theoretically could be traced with human anti-nuclear antibodies [179-182]. In this work, we asked whether the NSG mice and the huNSG could gain weight on high fat diet (HFD). We show very preliminary data that the fat pads of NSG mice increase in weight on HFD. No conclusions were reached regarding the huNSG mice due to technical challenges.

Results and discussion:

After one month of HFD feeding, we found that fat pads of NSG HFD-fed animals were increased in weight compared to the normal diet (ND) fed animals (Figure A1.1). This increase is a trend and not statistically significant. The same trend was not observed in the huNSG groups partially because some animals did not survive long enough for HFD effect to be analyzed. Unfortunately, we were unable to find the right conditions for the human anti-nuclear antibody to detect human cells. No significant trend was noticed in the weight of the animals (Figure A1.2). This may also be due to their ill health and the short time period for which they were on HFD. These results

are very preliminary and very little conclusions can be made. However, we would like to note that mice with severe impairment in hematopoietic cell lines have morphologically normal adipocytes and that their fat pads seems to increase in weight on HFD (A1.3).

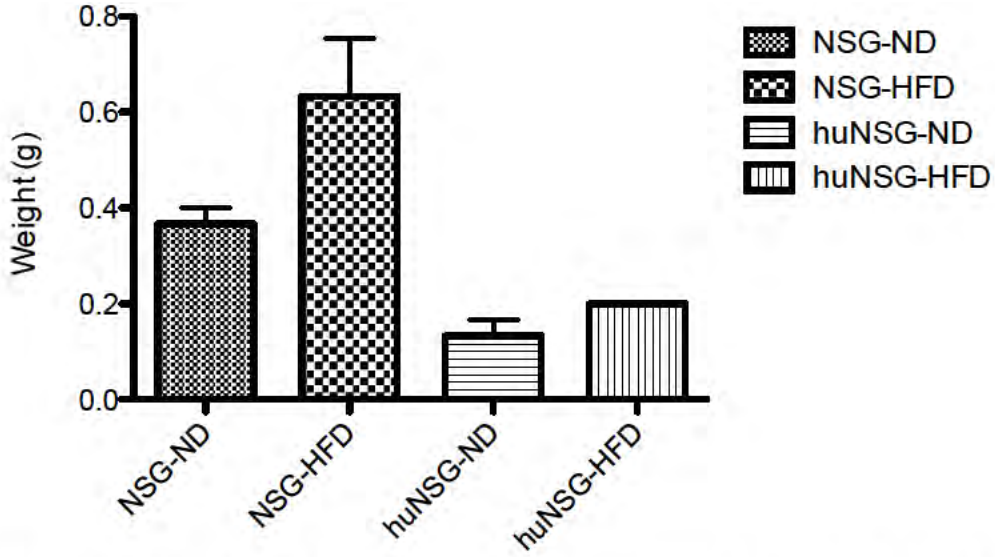

Figure A1.1. **Epididymal fat pads of NSG mice increased in weight on high fat diet feeding**. NSG and huNSG mice were placed on ND and HFD and their epididymal fat pads were measured after 31 days

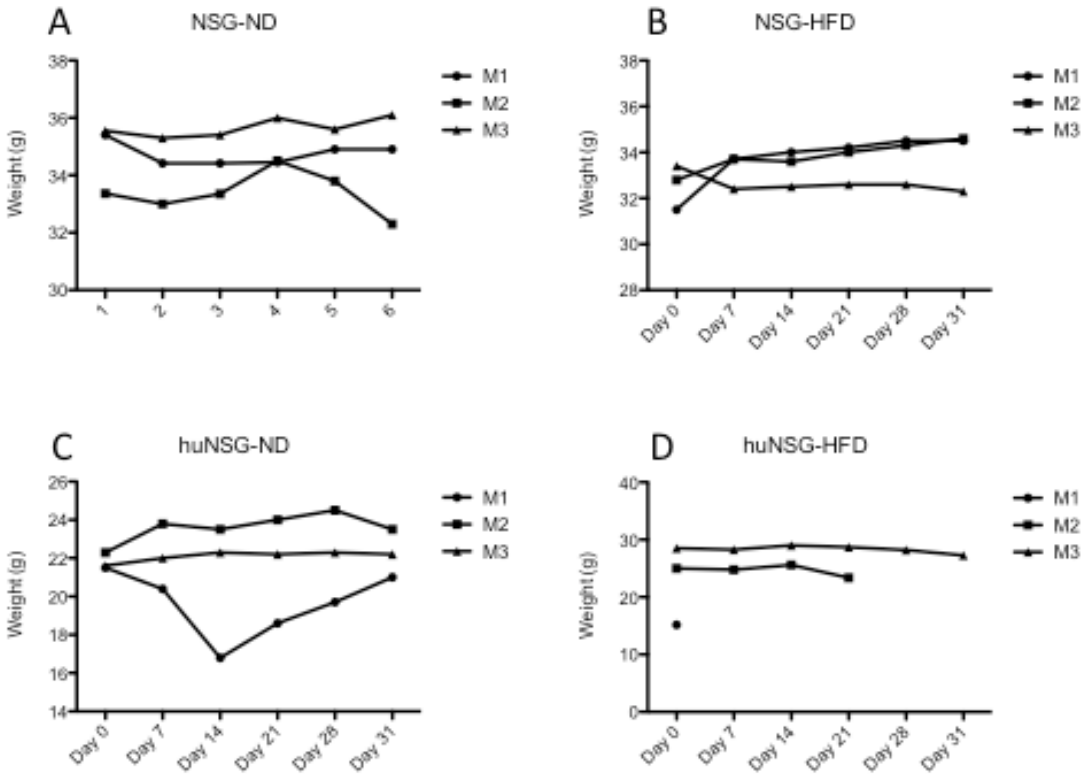

Figure A1.2. NSG and huNSG mice on HFD. No trend was observed in weights of NSG and huNSG mice on normal chow diet (**A** and **C**) and high fat diet (**B** and **D**).

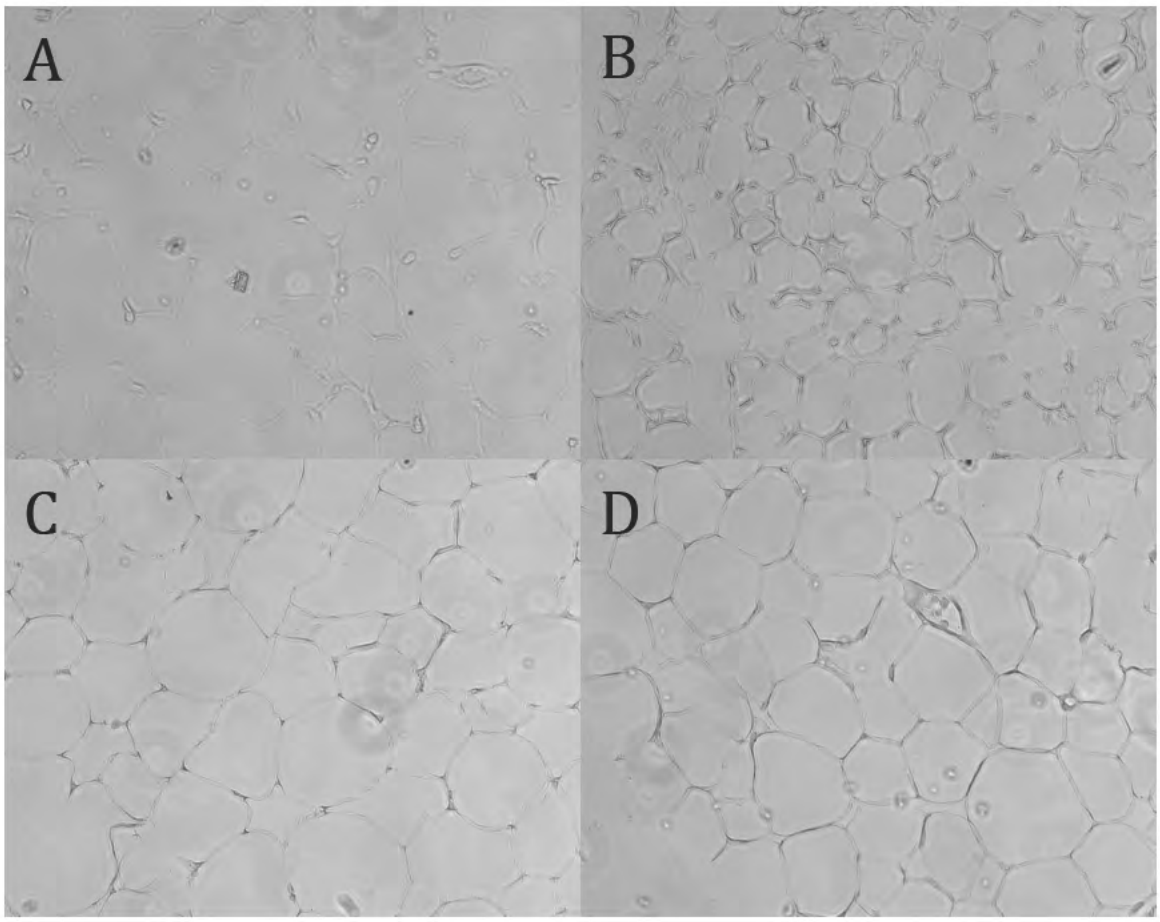

Figure A1.3. Histology of adipocytes of NSG and huNSG mice. Adipocytes from NSG mice on normal chow (**A**) and high fat diet (**C**) and adipocytes from huNSG mice on normal chow (**B**) and high fat diet (**D**) have normal morphology.

Experimental Procedures:

NSG and huNSG mice were kindly donated by the Greiner laboratory. At 15 weeks, they were placed on HFD for 31 days, during which time their body weight were monitored. After a month on HFD, the mice were sacrificed and their fat pads were measured.

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