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TRANSCRIPTIONAL REGULATION OF ADIPOCYTE FUNCTION

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

Kıvanç Birsoy

June 2009

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TRANSCRIPTIONAL REGULATION OF ADIPOCYTE FUNCTION

Kıvanç Birsoy, Ph.D.

The Rockefeller University 2009

The increased white adipose tissue mass associated with obesity is the result of both hyperplasia and hypertrophy of adipocytes. While adipocyte development and transcriptional processes are well studied *in vitro*, regulation of *in vivo* genes (such as leptin), the identity of the adipocyte progenitor cells and the development of the adipose organ have not been defined *in vivo*.

In this thesis, firstly KLF4 was discovered to be an essential early regulator of adipogenesis. KLF4 together with Krox20 cooperatively transactivates C/EBPβ, suggesting that KLF4 and Krox20 are part of an immediate early transcriptional network. This network is upregulated in a lipodystrophic animal model, encoding a dominant negative transgene against C/EBP factors, suggesting that these animals carry hyper-adipogenic residual fat pads, which provide a niche for transplantation experiments for identifying possible adipocyte progenitors. When injected into the residual fat pads of lipodystrophic mouse, indeed, a cell population, sorted from stromavascular fraction reconstitutes a functional white adipose tissue. Next, through a leptin-luciferase animal model, where luciferase is expressed only in adipocytes (see below), the location and timing of embryonic adipose development were

determined. Identification of the location and timing led the way to study gene regulation and morphology of the developing adipose tissue in embryos.

Leptin is an *in vivo* regulated adipocyte hormone, which is the afferent signal in a negative feedback loop controlling body weight and energy expenditure. Leptin is secreted in proportion to adipose tissue mass. This suggests the possibility that cellular lipid content is sensed and that a fuller understanding of the mechanisms of leptin production could lead to the delineation of a lipid sensing mechanism in fat cells and possibly other cell types. To discover this mechanism, we searched for the region/s in the leptin gene promoter that control the transcription of the leptin gene using a deletion series of BAC transgenic mouse lines that express luciferase under the control of overlapping leptin regulatory sequences. Cis elements that confer qualitative and quantitative control of the leptin gene are located between – 762B and +8kb relative to the transcription start site.

Since luciferase is driven by leptin regulatory sequences in leptin-luciferase animals and leptin levels are highly correlated with the amount of body fat, luciferase expression can be used as a surrogate for studying changes in the amount of adipose tissue. To study the responses of adipocytes to changes in weight, leptin-luciferase animals were used to show that weight loss induced by fasting or leptin treatment results in the retention of lipid-depleted adipocytes in adipose depots. This work led to the identification of a cellular program that controls the recovery of adipose fat mass after weight loss.

I dedicate this thesis to my family who gave me the love and support necessary to achieve my goals in every part of my life.

Aileme ve Yakinlarima…

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I also want to thank the members of my committee, Drs. Roeder, Darnell and Lazar for being very supportive and providing great guidance throughout my graduate career. I thank Susan Korres who has been delightful to work with and has been helpful with countless aspects of my work. Matt Rodeheffer, Zhu Chen and Alex Soukas have played a substantial role in some of the projects I have pursued here. Kristina Hedbacker and Zachary Knight have always been willing to listen and provide great input both scientific and non-scientific. Finally, Mats Ekstrand, Bobby Wysocki, Christian Perez, Giovanni Ceccarini, Scott Sternson, Lisa Pomeranz, Sarah Stanley, Allyn Mark, Zhiying Li, and Ana Domingos have all made the lab a friendly and fun place to work. For embryo injections, I would like to specially thank Gulya Fayzikhodjaeva.

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CHAPTER 1:

Introduction

Obesity as a Global Health Problem

The first law of thermodynamics basically states that a thermodynamic system can store or hold energy and this energy is conserved. The regulation of body weight also adheres this principle of thermodynamics. Body fat increases when energy intake is consistently greater than energy expenditure and decreases when energy intake is smaller than energy expenditure. Excess body fat and obesity are the result of sustained positive energy balance. In the last 20 years, medical and public health interest in obesity has skyrocketed due to the increased prevalence of obesity in United States. Today, the obesity epidemic has spread from the US to Europe and is now emerging in middle and even low income countries. In the United States, this trend affects all ages, races and socio-economic groups (Kuczmarski et al., 1994b).

Obesity is associated with many other health complications including diabetes mellitus, coronary heart disease, several forms of cancer, and sleep-breathing disorders (Kopelman, 2000a, b) (Friedman, 2000). Because of the health and economic costs of obesity, the immediate need to understand why humans are gaining weight has increased in recent years. The global epidemic of obesity possibly results from a combination factors such as genetic factors, increased availability of high-calorie foods and decreased requirement for physical activity due to sedentary lifestyle in modern society. Obesity became so

common within the world's population that it is beginning to replace common problems such as infectious diseases and under-nutrition as the most significant contributor to ill health (Kopelman, 2000a).

Definition of obesity

Obesity is defined as excessive accumulation of body fat. Body fat is most simply estimated by using a formula that combines weight and height. Invented by a Belgian polymath, body mass index (BMI) is defined as the individual's body weight divided by the square of his/her height, producing a unit measure of kg/m^2 (Dalton et al., 2003). The formula assumes that most variation in weight for people of the same height is due to fat mass. The assumption, for example, does not hold true for people with increased muscle size.

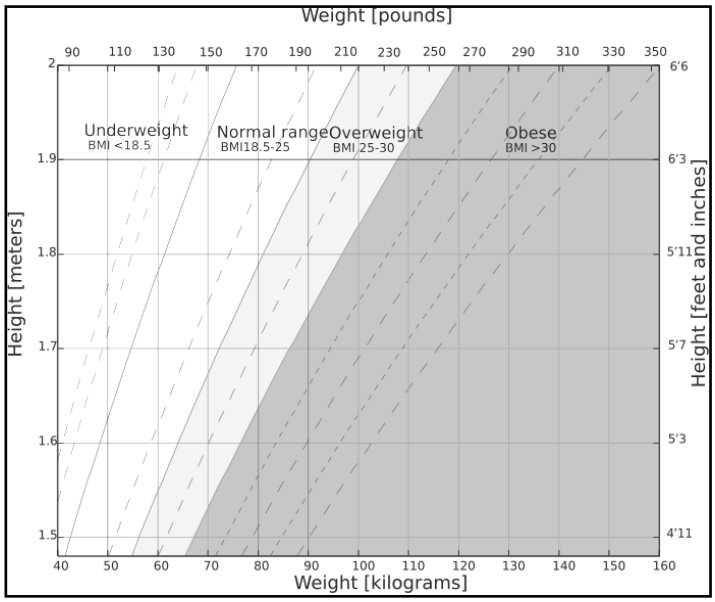

Figure 1 BMI and classification of obesity (adapted from W.H.O.)

World Health Organization classifies individuals with BMI more than 25 as overweight, more than 30 as obese and more than 40 as morbidly obese respectively (Figure 1) (Committee, 1995; WHO, 1997). It is important to note

that, owing to differences in body proportions, BMI may not correspond to the exact same degree of fatness among different people as indicated above. Despite the fact that it does not provide an accurate measure of percent body fat, BMI allows meaningful comparisons of weight status within and between populations and the identification of individuals at risk of morbidity and mortality. For this reason, BMI sets up a general index and is very useful for epidemiological studies. Consistent with this, increased BMI has been found to be associated with the incidence of type II diabetes, CHD and hypertension (Willett et al., 1999).

One other accepted measure for obesity is waist circumference which correlates with the risk for CHD such as hypertension or blood lipid levels (Dalton et al., 2003). The National, Heart, Lung and Blood Institute classifies a high risk of obesity if men have a waist circumference greater than 102 cm, and women have a waist circumference greater than 88 cm (Colman et al., 1995; Flegal, 2007). The choice of cut-off points on the waist circumference involves a trade-off between sensitivity and specificity similar to that for BMI.

Obesity as an emerging health concern

Obesity is becoming a major health concern in the United States and an increasing problem in the developing world. The most recent National Health and Nutrition Examination Survey (NHANES) indicates that 66% of adults in the US are either overweight or obese, (Ogden et al., 2006). Obese individuals made up 32.2% of the American adult population as of 2004, compared to 12.8% in 1962

(Kuczmarski et al., 1994a; Kuczmarski et al., 1994b). Interestingly, this increasing trend manifests itself even more among children and adolescents. The prevalence of overweight/obese children in the United States more than doubled since 1970s (Troiano et al., 1995; Yanovski and Yanovski, 2002).

Obesity is not only a problem of United States, but a common condition in almost every continent. In Europe, a very comprehensive study (MONICA) showed that on average, 15% of men and 22% of women were obese. More than half the adult population between 35 and 65 years of age in Europe were either overweight or obese (Silventoinen et al., 2004).

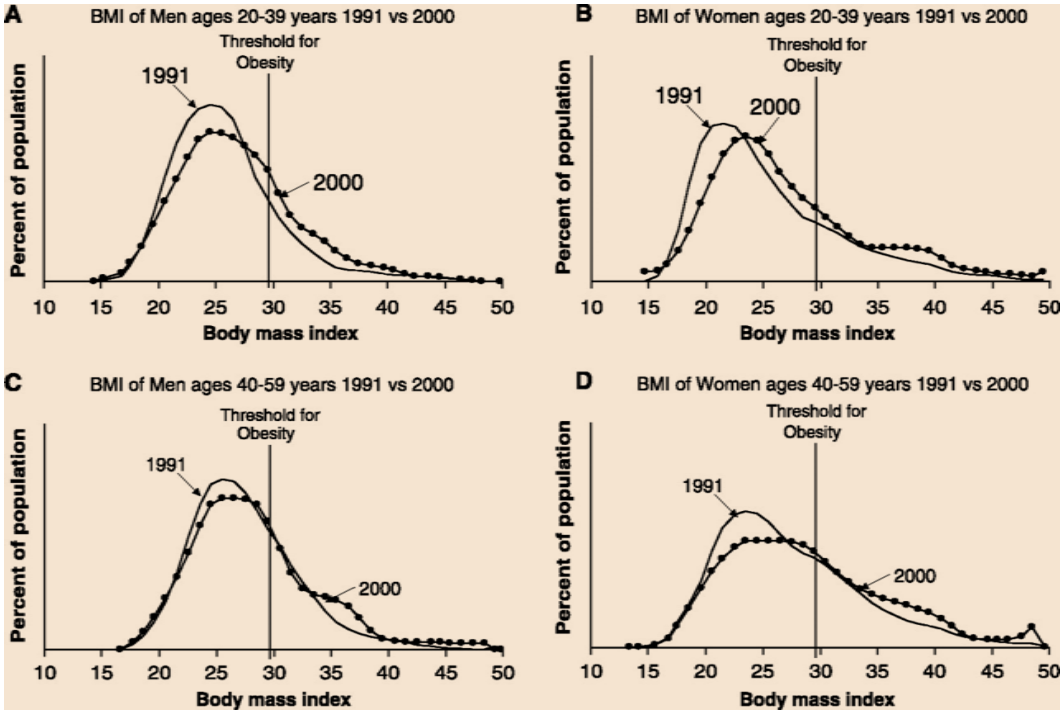

Figure 2 Distribution of BMI in different age and sex categories (Adapted from Friedman, 2000)

Even though obesity is often viewed as a problem of rich and developed countries due to food availability, it is actually rapidly increasing even in the developing world (Taubes, 1998). In fact, it is estimated that the number of obese

people in the world is equivalent to those suffering from hunger. For instance, in Turkey, the overall prevalence rate of overweight was 25.0% and of obesity was 19.4% as of 2003. The prevalence of overweight among women was 24.3% and obesity 24.6%; 25.9% of men were overweight, and 14.4% were obese (Hatemi et al., 2003). Incidence of obesity has also plummeted in countries such as Japan, Korea, China, and Thailand , where being overweight has traditionally not been a concern due to the traditional lifestyle and food habits (Popkin, 1994). Obesity rates amongst Pacific Islanders are astonishingly high, with a prevalence of over 60% in certain populations, which has developed a lot of interest for researchers to identify possible genetic factors (Bonnen et al., 2006; Hodge et al., 1995; Shmulewitz et al., 2001).

Obesity as a disorder is defined as a threshold. In this regard; small changes in average weight might have a different effect on its incidence. Incidence of obesity has increased from 23.3% to 30.9% in recent years (Figure 2). On the other hand, the average weight of a typical American increased only 7 to 10 pounds (Friedman, 2003). This suggests that a small increase in average BMI of a population such as US population can lead to marked increase in the number of obese individuals. It is thus important to consider obesity as a threshold when interpreting the statistics for prevalence of obesity epidemic (Friedman, 2003) (Figure 2).

Causes of Obesity

Recent evidence in clinical and basic research shed light on what could be causing the obesity epidemic. As for other diseases, it is plausible to think that

obesity is determined by combination of factors, both environmental and genetic. From a population genetics point of view, it is almost impossible that the genetic make-up has changed in the last century, which suggests that environmental alterations be the major determining factor. Consistent with this, obesity epidemic is occurring predominantly among humans and house pets (Kopelman, 2000a; Mason, 1970; Overgaauw and Kwant, 2008; Stuart, 2008). This observation itself implies that the shift of environment is the major cause of the recent epidemic. As will be discussed, one major theory suggests that increased food availability and shift to sedentary life styles with the development of technology can explain the increase in the incidence of obesity (Campbell, 2008; Levitan et al., 2006; Neel, 1999a).

Genetic factors are the major determinants of an individual's reaction to his/her environment. As much as 80% of variability in BMI can be attributed to genetic factors (Stunkard et al., 1990). Furthermore, twin and adoption studies suggest that body weight is among the most genetically controlled traits (Maes et al., 1997). Correlation between monozygotic twins reared apart/together does not differ significantly (Maes et al., 1997). In parallel, people sharing the same childhood do not necessarily show the same body weight. All these studies imply that genetic influences on BMI/body weight are substantial. Next sections will discuss the factors that affect obesity and possible evolutionary theories that explain the obese gene selection.

Genetic contribution to Obesity

The fact that body weight in adults tend to stay stable suggests that body weight is maintained by an inherent homeostatic circuit. When the food intake is restricted to maintain a lesser body weight, energy expenditure decreases to balance this alteration or when a person overfeeds himself/herself, energy expenditure increases for the same reason (Cohn and Joseph, 1962). This, itself suggests that body weight is regulated by inherent cues of the organism.

Studies in humans using both adoption and twins have shown that body weight is highly heritable. A large study of more than 100.000 individuals found a BMI correlation of 0.70 in monozygotic twins, with a heritability of %50-90 (Maes et al., 1997). This heritability is comparable to traits regarded as highly heritable such as height and even greater than traits which were accepted to have genetic roots. Furthermore, children can be predicted to be obese less than %10 chance if both parents have a normal body weight, %50 if one parent is obese and 70% if both parents are obese (Stunkard et al., 1980a; Stunkard et al., 1980b). In parallel, mouse studies show that genetically different mouse strains gain different weights upon high fat feeding. For example, exposure to high fat diet results weight gain but the degree of weight gain varies depending on the strain background. Among the mouse strains, C57B6/J and AKR/J become obese on high fat diet, on the contrary to SWR/J and FVBN/J (Astrup et al., 1994; Prpic et al., 2002; West et al., 1994).

In order to compromise with the environmental and genetic factors, many researchers propose that susceptibility to obesity is under genetic control and environment/behavior determine the phenotypic expressivity of the susceptible genotype.

Figure 3: Depiction of thrifty gene hypothesis

Rare Mendelian single gene disorders also occur in obesity, which presents another indication of the genetic basis of obesity. An example of this type of abnormality is ob and db mutations. Recessive mutations in the ob and db genes cause a syndrome of extreme obesity and diabetes (Coleman, 1978). Mice having mutations at either of these loci weigh 2 to 3 times more the wild type littermates. An early parabiosis experiment by Coleman and colleagues found that ob/ob partners of db/db mice experienced rapid weight loss, ate little food, became hypoglycemic, and died within 26 days of parabiosis surgery (Coleman, 1978). Similar responses were reported for normal, lean mice parabiosed to db/db mice, and it was concluded that both lean and ob/ob mice had normal satiety centers that were responsive to a satiety factor produced by db/db mice. The similarity of the obesity syndromes observed in the ob/ob and db/db genotypes also led to the conclusion that the two different genes influenced the same satiety mechanism. These observations led to the fact that ob/ob mice

had a normal satiety center but were unable to produce the humoral satiety signal, whereas db/db mice produced the signal but had a defective satiety center. This factor was later identified by our lab in 1994, which will be mentioned in the following sections of this thesis (Zhang et al., 1994).

An evolutionary perspective of obesity: Environment vs Genetics

Body weight is determined by environment and behavior. As discussed above, body weight is kept in limits through homeostatic mechanisms. In this regard, then comes the question why this balance mechanism did not work at least for humans during the obesity epidemic. Relevant observations from recent studies show that every other species on earth, fat metabolism is well regulated and that most wild animals are in fact very lean and that they remain lean even when adequate food is supplied unless there is an infectious disease causing obesity (Dhurandhar, 2001). The thrifty gene hypothesis proposed by geneticist James Neel gives an explanation to this fundamental problem (Figure 3) (Neel, 1962) (Neel, 1999a, b). Obesity is quite common, and it is evident that it likely had a strong genetic basis. Neel proposed that obesity/diabetes genes historically were advantageous in ancient times where food was scarce, but they became detrimental in the modern world. These obesity genes, named as thrifty genes by Neel, are genes which enable individuals to efficiently collect and process food to deposit lipid during periods of food abundance in order to use in times of food scarcity which was possibly frequently happening in ancient times (Neel, 1962, 1999a, b) (Figure 3).

According to the hypothesis, the 'thrifty' genotype was advantageous for hunter-gatherer populations, especially for women, because it would allow them to fatten more quickly during times of abundance (Neel, 1962). Fatter individuals carrying the thrifty genes would thus better survive times of food-scarcity. However, in modern societies with a constant abundance of food, this genotype prepares individuals for a famine that never comes. When you combine the current environment with the sedentary lifestyle of today, the result is widespread chronic obesity and related health problems like diabetes (Neel, 1999a, b).

The central principle of the thrifty gene hypothesis that famines were common and severe enough to select for thrifty genes in ancient times has been challenged by several researchers. It was argued that many of the populations that later developed high rates of obesity/diabetes seemed to have no history of famine (Speakman, 2008). A common example is the Pacific Islanders whose tropical islands had plentiful vegetation and fish. One other significant problem for the 'thrifty gene' idea is that it predicts that modern hunter gatherers should get fat in the periods between famines, which was not the case in African/Australian hunter-gatherers (Speakman, 2008).

Energy Homeostasis and Body Mass Regulation

Body weight is relatively stable over decades for many adults, implying that it is under strict control through life. In fact, one study shows that males, on average gains only 0.3-1kg per year, indicating that approximately 99.8% of the calories are spent (Weigle, 1994). This suggests that calorie intake is strictly

balanced with the energy expenditure, keeping the weight within a small range. Consistent with this notion, perturbations to the animal's normal body weight cause a counter reaction by altering food intake and energy expenditure. Rodents who have gained weight through overfeeding, once ad libitum, decrease the food intake and increase energy expenditure until the weight is normalized (Cohn and Joseph, 1962). Conversely, mice that are food restricted, once ad libitum, increase the food intake until the weight is normalized (Rothwell et al., 1982; Rothwell and Stock, 1982). Similar observations have also been made in both lean and obese humans. Obese individuals on strict diets, regain the lost weight in the long run (Drenick et al., 1977; Johnson and Drenick, 1977). All these studies suggest that there is a regulatory system that keeps body weight stable at a "set point" upon changes in food intake/energy expenditure.

The system that changes food intake acts through hypothalamus based on many studies. Firstly, many hypothalamic tumors are associated with obesity or cachexia (Bauer, 1954). Furthermore, bilateral lesions of VMH region of hypothalamus are associated with increases in fat tissue. These active lesions also cause hyperphagia, causing obesity (Powley and Keesey, 1970). These evidences suggest that body weight and energy balance in mammals is controlled by a negative feedback loop including central nervous system and peripheral organs. Lipostasis theory, first proposed by Kennedy and colleagues, states that metabolites/signals/humoral factors from adipose tissue inform the brain about the energy status of the body and any divergence from the hypothetical "set point" causes changes in calorie intake/expenditure, keeping

the body weight in limits (Kennedy, 1953). The possibility of a secreted factor was later shown to be true through parabiosis experiments as explained below.

A circulating factor for Lipostasis

ob (*obese*) and db (*diabetes*) animals have identical phenotypes on the same genetic background. They are hyperphagic, show decreased energy expenditure and develop early obesity accompanied with hyperinsulinemia, sterility and thermoregulatory defects. Since the metabolic profiles were identical, these two putative genes were suggested by Coleman to be genetically linked and postulated to be part of the same pathway (Coleman, 1978).

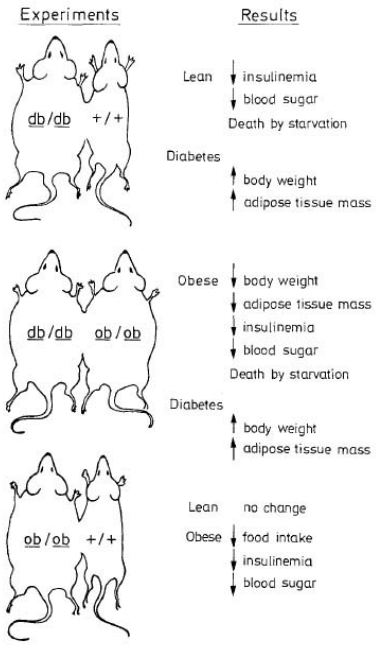

Figure 4: Parabiosis experiments performed by Coleman

Parabiosis experiments performed by Coleman led to the discovery of leptin gene. (Coleman, 1978)

The evidence to the link between db and ob gene came from parabiosis experiments (Figure 4). Parabiosis is a surgical procedure where two animals are joined through peritoneum and scapula creating a capillary graft between animals, hence allowing the direct exchange of blood. Interestingly, when Coleman performed the parabiosis between an ob animal and a wild type, ob animal gained much less weight compared to ob animal parabiosed to another ob animal. This suggested the existence of a blood

–borne factor that is absent in ob animals and present in wild types. When db animals are parabiosed to wt animals, Coleman did not see any change in db animals (Coleman, 1978). Conversely, wild type animals died of hypophagia/starvation in 50 days. Similarly, ob animals parabiosed to db animals became hypophagic, lost adipose stores and died of starvation as was the case for wild type animals.

Based on the abovementioned experiments, Coleman postulated that ob animals were lacking a factor that was present in wild type animals; further db animals were resistant to this secreted factor. Coleman reasoned that db gene product was the receptor for this humoral/secreted factor, which was negatively regulating body weight and missing in ob animals.

Additional evidence came from studies where VMH-lesioned obese rats are parabiosed to normal rats. In this setting, normal rats became hyperphagic and lost weight (Hervey, 1959). All together the evidence suggests that there is a blood-borne humoral factor and a receptor which possibly is expressed in hypothalamus.

Cloning of leptin and leptin receptor

The circulating factor that was missing in ob animals was successfully cloned using a positional cloning strategy by Jeffrey Friedman and his colleagues. Ob gene was found to be expressed predominantly in white adipose tissue and to encode a 167 aa protein (Zhang et al., 1994). Friedman and colleagues found that C57BL/6J ob/ob carries a nonsense mutation at codon

105. In ob/ob mouse, a transposon is inserted into the first intron and prevents mature ob RNA to be expressed.

In 1995, the leptin receptor was identified biochemically and later shown to be a cytokine family receptor with broad expression by Tartaglia et al. (Tartaglia et al., 1995). It was later shown that leptin receptor RNA was alternatively spliced and that only one of the splice variants, referred to as ObRb , was mutant in C57Bl/Ks *db*/*db* mice (Lee et al., 1996). Among the leptin receptors ObRb was highly enriched in the hypothalamus in precisely those nuclei that alter body weight when lesioned (See above) (Fei et al., 1997; Lee et al., 1996). This genetic evidence proved the Coleman hypothesis, that leptin acted on the hypothalamus to control food intake and body weight. In parallel with the evidence, injections or infusions of leptin reduce food intake and body weight of wild-type and *ob* mice but have no effect on *db* mice (Campfield et al., 1995; Halaas et al., 1997) (Figure 5).

Leptin action

Leptin primarily acts on a set of neural pathways in hypothalamus. Basically, it activates pathways that inhibit food intake and inhibits pathways that activate food intake (Figure 5). A significant part of leptin's actions seem to result from inhibition of neuropeptide Y/Agouti gene-related peptide (NPY/AGRP)–expressing neurons in the arcuate nucleus, a region in hypothalamus. These neurons stimulate appetite. On the other hand, leptin was also shown to activate pro-opiomelanocortin (POMC) neurons that reduce appetite by activating the

MC4 receptor in other parts of the brain (Friedman, 1997). It is very likely that there are other neural pathways that account for the full effect of leptin treatment. The identification of these extra circuits would provide extra understanding for delineating the molecular mechanisms. It has recently become apparent that leptin signaling has important modulator effects on dopaminergic neurons. These neurons are possibly a part of reward pathways that provide inputs to the nucleus accumbens (Hommel et al., 2006).

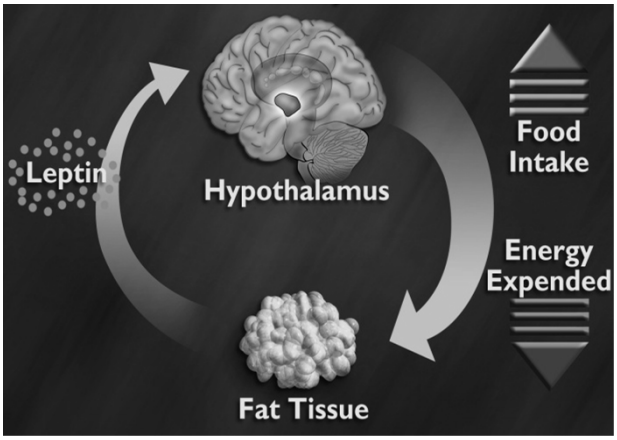

Figure 5: Leptin and Lipostasis

Leptin is secreted by adipose tissue relative to the amount of lipid stored. It acts on hypothalamus and changes food intake and energy expenditure to stabilize the body weight. (Courtesy of Jeffrey Friedman)

Leptin also acts on metabolism independently of its effect on body weight regulation. Leptin decreases glucose/ insulin levels in *ob/ob* mice independent of the weight loss as assessed by using very small doses (Pelleymounter et al., 1995). Some of the peripheral effects of leptin are still unknown if they are mediated directly on peripheral targets or through CNS. Leptin also increases sympathetic nerve activity in several peripheral tissues (Haynes et al., 1997).

Regulation of leptin gene

The leptin gene is expressed predominantly in the fat tissue (Birsoy et al., 2008; Maffei et al., 1995a). Furthermore the expression is very highly correlated

with the amount of fat mass as shown in many independent studies (Guo et al., 2004; Lonnqvist et al., 1997; Maffei et al., 1995b; Skurk et al., 2007). Obese mice such as ob/ob and high fat-fed models have elevated leptin mRNA levels (Maffei et al., 1995a). Conversely, lipodystrophic/fasted animals have lower leptin mRNA levels (Reitman and Gavrilova, 2000). Of importance, differences in leptin mRNA levels, as assessed using in situ hybridization, show marked differences in the levels of leptin RNA/cell as fat cells get larger or smaller (Maffei et al., 1995a). This correlation holds true in comparisons of adipocytes in obese vs. lean adipose depots, adipose tissue in fed vs. fasted in animals, comparisons among different adipose depots that vary with respect to adipocyte cell size and finally comparisons of leptin expression in fat cells *in vitro* vs. *in vivo.* The strong correlation between leptin gene expression and adipocyte size supports leptin's physiological role as a humoral signal of fat mass (Skurk et al., 2007).

Leptin is regulated by many other metabolic cues. Glucocorticoids directly stimulate leptin synthesis in adipocytes (Murakami et al., 1995). Leptin synthesis is also increased by infection/ endotoxins and cytokines such as TNF and and interleukin 1 (IL-1) (Bornstein et al., 1998; Sarraf et al., 1997). Conversely, administration of thyroid hormone decreases leptin levels in rodents (Escobar-Morreale et al., 1997).

Previously, changes in membrane cholesterol concentration related to adipocyte size have been proposed as regulators of gene expression in adipocytes. However, manipulation of cholesterol concentrations in 3T3-L1 adipocytes does not alter leptin gene expression but does change expression

patterns of several other genes, including TNF-alpha (Le Lay et al., 2001). Thus, the mechanism underlying the strong correlation between leptin gene expression and adipocyte volume are unknown and identification of the transcriptional mechanisms that regulate leptin could shed light on these questions.

While several prior reports have identified leptin promoter elements that respond to secretagogues such as insulin, glucocorticoids, endotoxins and thiazolidinediones,(Kolaczynski et al., 1996; Nolan et al., 1996; Zhang et al., 1996) the factors that confer fat specific expression of the leptin gene and that regulate its expression in parallel with changes in fat mass are not known. Specifically, it is not known which trans-acting factors are responsible for the induction or repression of leptin gene expression. Studies of the mouse, rat and human leptin promoter are limited, but have identified C/EBP alpha, Sp1, and Lp1 as playing roles in the regulation of leptin (de la Brousse et al., 1996; He et al., 1995; Hwang et al., 1996; Isse et al., 1995; Mason et al., 1998). These studies have all largely been done using leptin promoter constructs transfected into cell lines. However, leptin expression is ~100 times lower in *in vitro* differentiated adipocyte cell lines compared to tissue levels (Mandrup et al., 1997a). Surprisingly, these adipocyte cell lines abundantly express the master regulator PPARɣ, C/EBPβ, delta and alpha and their targets. Furthermore, these transcription factors are also expressed *in vivo* in adipose tissue. This supports that the adipocyte-specific expression of leptin is controlled by a different transcriptional mechanism than the conventional adipocyte regulators (Soukas et al., 2001b).

Although the abovementioned experiments have shed light into some of the trans-acting factors which can affect the transcription of a reporter gene attached to leptin promoter *in vitro,* whether these factors regulate leptin gene expression *in-vivo* is not established.

Fat tissue biology

Fat tissue was long believed to be an inert depot for the storage of energy. This view was upended in 1994 by the discovery of the hormone leptin, which is secreted by adipocytes and signals to the brain to control body weight (Zhang et al., 1994). The intervening 15 years have led to the discovery of a complex cross-talk between adipose tissue and other organs that regulate metabolism and body weight (Farmer, 2006b). Nonetheless, our understanding of the biology of fat itself remains limited. Understanding how adipose tissue mass is generated and regulated *in vivo* has major implications for the treatment of obesity and metabolic diseases.

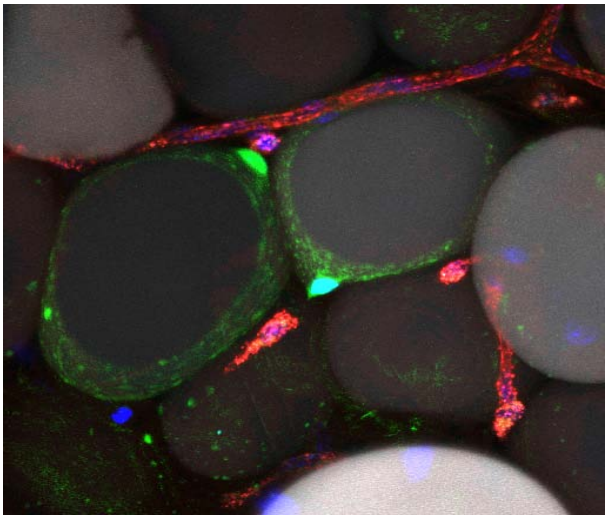

Figure 6: Adipose tissue is composed of different cell types

This figure shows the adipose tissue whole mount microscopy from transgenic animals expressing GFP only in adipocyte nuclei. Red shows the capillaries. Note the GFP- DAPI+ stained nuclei, which represents the non-adipocyte cells.

Adipose tissue is a complex and essential endocrine/energy-storing organ. Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells, and immune cells (Rosen and MacDougald, 2006) (Figure 6). Together, these components function as an integrated unit. Adipose tissue not only responds to afferent signals from CNS and other systems but also expresses a wide variety of peptides, known as adipokines, which function at both the local (autocrine/paracrine) and systemic (endocrine) level. Up to this date many secreted factors are discovered, including leptin, resistin, plasminogen activator inhibitor-1, and adiponectin. Through this interactive crosstalk, adipose tissue is involved in many biological processes including energy metabolism, immune function and neuroendocrine function.

Fat is an integral organ to the whole organism. As with other essential organs, excess amount (obesity) as well as lack of it (lipodystrophy) cause adverse metabolic consequences. Excess adipose tissue, obesity, is associated with hyperglycemia, insulin resistance, dyslipidemia, hypertension, and inflammatory states (Kopelman, 2000a). Conversely, adipose tissue deficiency or lipodystrophy is also associated with features of the metabolic syndrome in both humans and rodents (Gesta et al., 2007) (Hegele, 2000).

In mammals there are different fat depots, having different roles (Kissebah and Krakower, 1994). The main fat organs are subcutaneous and visceral adipose depots. These depots are responsive to energy stores and many hormonal/metabolic changes in an organism. There are also structural fat depots that can be found in many other areas, including in the retro-orbital space, on the

face and extremities, around breasts/thighs and within the bone marrow. Many of these areas are not responsive to energy level of the body. In addition, some adipose organs are responsive to sex hormones, such as adipose tissue in the breasts and thighs, whereas other depots, such as scapular fat, are responsive to glucocorticoids (Gesta et al., 2007). The evolution of the complexity of different depots possibly emerged with the endothermic animals, where there is a need of energy for strict thermoregulation (Gesta et al., 2007). Next section discusses the evolution of fat depots.

Species	 *Caenorhabditis elegans*	 *Drosophila melanogaster*	 *Carcharodon carcharias*	 *Cyprinus carpio*	 *Xenopus laevis*	 *Gallus gallus domesticus*	 *Mus musculus*	 *Homo sapiens*
Fat storage	Stored in intestinal cells	Stored in the "fat body"	Stored in liver	Stored in WAT	Intra-abdominal WAT (no sub-cutaneous WAT)	Subcutaneous and internal WAT	Subcutaneous and internal WAT	Subcutaneous and internal WAT
Leptin	No	No	No	Yes	Yes	Yes	Yes	Yes
BAT	No	No	No	No	No	No	Present throughout life	Present at birth; reduced in adults
UCP	UCP-like protein (ucp-4)	No	?	UCP-1 in liver	UCP-4 in oocytes	Avian UCP in muscle	UCP-1 in BAT	UCP-1 in BAT
Thermo-regulation	**Ectotherm**	**Ectotherm**	**Ectotherm**	**Ectotherm**	**Ectotherm**	**Endotherm** Shivering and nonshivering thermogenesis	**Endotherm** Shivering and nonshivering thermogenesis	**Endotherm** Shivering and nonshivering thermogenesis

Figure 7 : Evolution of fat tissue

Fat tissue distribution has changed in the course of evolution. (Gesta et al., 2007)

Evolution of Adipose Tissue

Almost all species, from simple invertebrates to Homo sapiens store their excess energy in the form of lipids (Figure 7). However, the cell type that stores the lipid differs from organism to organism relative to the complexity. For instance, *C. elegans* store fat in intestinal epithelium and sharks store fat in the

hepatocytes (McKay et al., 2003; Van Vleet et al., 1984). With the evolution of vertebrates, a special organ for storing fat appears- the adipose.

Adipose tissue distribution and fat storage changed dramatically with the process of evolution. As explained above, even small invertebrates such as worms and flies store lipids at some point in their life cycles; however adipose tissue evolved with the emergence of boney fish, teleostei. Of note, intra-abdominal white adipose tissue co-evolved with the adipocyte hormone- leptin- in boney fish (Figure 7). With the emergence of endothermic animals, differentiation of subcutaneous and internal WAT occured in higher species, such as birds and mammals. This further complexity possibly started due to the need to strictly stabilize the body temperature in endothermic animals. It is important to note that even though leptin seems to have coevolved with the adipose organ, adipose tissue is not the only organ to express leptin in the exothermic vertebrates (Gesta et al., 2007). Furthermore, there is very little evidence that leptin affects these exothermic animals the same way as it does mammals. Leptin, in exothermic animals such as frogs, fish and several reptiles, has been found to act on other functions such as the metamorphosis and development of these animals (Gorissen et al., 2009; Huising et al., 2006). This evidence suggests that the role of leptin as the signal for energy stores might have evolved with endothermia.

Adipogenesis

Adipogenesis is defined as the differentiation of adipocytes, which are committed for adipogenic lineage, into mature adipocytes (Rosen and

MacDougald, 2006; Rosen and Spiegelman, 2006). During the last 20 years, with the availability of adipogenic cell lines, a great deal of new information was learned about adipogenesis and transcriptional events regulating adipocyte differentiation (adipogenesis). Although many cell types may contain lipids, the unique property of adipocytes is that they can store a much greater volume of lipids, and further can release of these calories for use by other organs. For this reason, studying adipocyte differentiation specifically has clinical value to understand several metabolic diseases including obesity and lipodystrophy.

Adipocytes are possibly differentiated through 2 phases. The first phase is the commitment of a pluripotent stem cell to the adipocyte lineage, also known as the determination step (Rangwala and Lazar, 2000). After the determination, stem cell loses the potential to differentiate into other cell types and committed to adipocyte lineage. In the second phase, which is known as terminal differentiation, the pre-adipocyte takes characteristics of the mature adipocyte. The molecular regulation of differentiation is more extensively studied than determination because most studies make use of adipogenic cell lines that have a restricted potential to differentiate into other cell types (Rangwala and Lazar, 2000).

Predetermination type of the above-mentioned *in vitro* model systems include the NIH-3T3 and the C3H10T1/2 cell lines, which are supposedly pluripotent and can therefore be differentiated into many different lineages such as adipocytes, osteocytes, or chondrocytes (Rosen and MacDougald, 2006).

Other cell line models, such as the 3T3-L1 and 3T3-F442A are already committed to the adipogenic pathway, therefore considered to be preadipocytes (Green and Meuth, 1975). These cell lines were originally established by Green and colleagues, which have greatly facilitated our knowledge of the molecular mechanisms controlling adipogenesis (Green and Meuth, 1975). Confluent 3T3-L1 preadipocytes are differentiated upon exposure to the adipogenic inducers fetal bovine serum (FBS), dexamethasone, isobutylmethylxanthine, and insulin**.** 3T3-L1 preadipocytes differentiate into adipocyte through three steps. First, the cells go through a growth arrest at confluence, which is required for the initiation of differentiation events. Secondly, after addition of the hormone cocktail, cells synchronously enter into S phase of the cell cycle, through one or two rounds of mitosis, a step also known as clonal expansion. Finally PPARɣ, C/EBPα, and many of the adipocyte markers are expressed and the cells get their characteristic rounded morphology and accumulate lipid droplets, also known as terminal differentiation (Rangwala and Lazar, 2000).

The validity of the 3T3 preadipocyte system as an appropriate model of adipocyte formation has been accepted by many researchers due to the similarity of genes expressed *in vitro* vs *in vivo* adipocytes. Furthermore, injection of 3T3-F442A preadipocytes into mice gives rise to normal fat pads (Mandrup et al., 1997a). Despite this knowledge, the lack of a robust *in vivo* adipogenesis model hinders the comparison. The preadipocyte cells and *in vivo* characteristics of the cell types are unknown.

Molecular processes involved in adipogenesis

Differentiation of preadipocytes into adipocytes is regulated by a complex and temporally regulated network of transcription factors that coordinate expression of hundreds of proteins responsible for establishing the mature fat-cell phenotype (Figure 8). To this date, two principal adipogenic factors, PPARɣ and C/EBPβ, are thought to be the late and early master regulator of the adipogenic process respectively (Lefterova and Lazar, 2009). PPARɣ especially is considered the master regulator of adipogenesis, because precursor/preadipocyte cells are incapable of expressing any known property of the fat cell phenotype without PPARɣ (Tontonoz et al., 1994a; Tontonoz et al., 1994b; Tontonoz et al., 1994c; Tontonoz et al., 1994d).

Much of our knowledge of the adipogenic network and the importance of PPARɣ and C/EBPβ come from studies performed in established preadipocyte cell lines as discussed previously. Even though animal data for these two genes gave several indications of their role, (Jones et al., 2005b; Tanaka et al., 1997) it is especially hard to knock out these genes at the right temporal point due to the lack of the early markers and some of the genes such as C/EBPs have other roles in other cell types and development, causing embryonic/ postembryonic abnormalities when knocked out (Bai et al., 2006).

The late master regulator PPARɣ was discovered by Bruce Spiegelman and colleagues by studying the regulators of aP2 gene (Graves et al., 1992; Tontonoz et al., 1994a). Spiegelman group identified an enhancer fragment that

regulates aP2 expression and consequently isolated the factor from adipose extracts using this fragment. This factor turned out to be PPARɣ, which is expressed in a fairly fat specific fashion. PPARɣ is expressed in two forms, namely g1 and g2. Among these forms, g2 is found selectively in adipose tissue, whereas g1 is found also in other tissues. PPARɣ is a nuclear receptor and like other PPARs heterodimerize with RXR and activates its target promoters (Mueller et al., 2002). Strong activation of PPARɣ requires the binding of an unknown endogenous agonist. When overexpressed, PPARɣ is sufficient to drive adipogenesis and no factor has been discovered promoting adipogenesis in the absence of PPARɣ. *In vivo*, inducible knockout of PPARɣ in differentiated adipocytes leads to adipocyte death followed by generation of new adipocytes (Jones et al., 2005b). This suggests that PPARɣ is also important for maintenance of the differentiated adipocyte state (Tontonoz et al., 1994d).

Several C/EBP family members, including CEBPa, C/EBPβ and C/EBPδ were also shown to be crucial for adipogenesis (Figure 8). The temporal expression of these factors during adipocyte differentiation indicates a cascade where early induction of C/EBPβ and C/EBPδ leads to induction of C/EBPα and PPARɣ (Lefterova and Lazar, 2009).

Among the early factors C/EBPβ seems to be indispensible for adipogenesis in immortalized pre-adipocyte lines (Hamm et al., 2001; Tang et al., 2004). C/EBPβ-deficient mice have reduced adiposity (Tanaka et al., 1997). C/EBPβ promotes adipogenesis at least in part by inducing C/EBPα and PPARɣ

(Zhang et al., 2004b). Consistent with this, C/EBPβ- deficient MEFs do not express C/EBPα and PPARɣ.

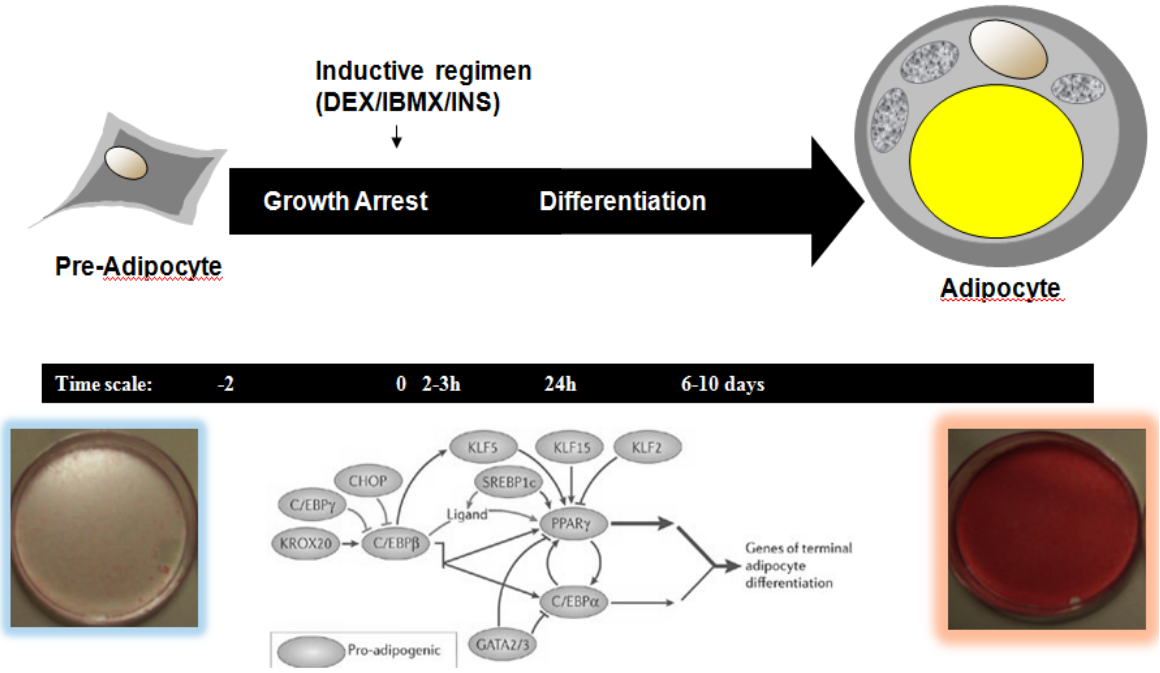

Figure 8: Transcriptional Regulation of Adipogenesis

Adipogenesis occurs through expression of a complex and temporally regulated transcriptional network. Adapted from Rosen, 2006

Apart from C/EBPs and PPARɣ, there are other factors that are known to be involved in adipogenesis. Among these, KLF factors seem to have variety of roles. The range of KLF genes that are expressed in adipose tissue, the variability in their expression patterns during adipocyte differentiation, and their effects on adipocyte development and gene expression indicate that a cascade of KLFs function during adipogenesis (Rosen and MacDougald, 2006). Another recent study has characterized expression changes in all nuclear-receptor family members in 3T3-L1 adipogenesis (Fu et al., 2005). Several members of this

family, including the glucocorticoid receptor, are known to affect adipogenesis, but this study indicates that many others could potentially regulate this process. GATA-binding transcription factors GATA-2 and GATA-3 also play a critical role in the molecular control of the preadipocyte-adipocyte transition (Tong et al., 2000). GATA-2 and GATA-3 are expressed in white preadipocytes, and their mRNAs are downregulated during adipocyte differentiation. One other transcription factor, CREB, is constitutively expressed prior to and during adipogenesis (Zhang et al., 2004a). Overexpression of a constitutively active CREB in 3T3-L1 preadipocytes is necessary and sufficient to initiate adipogenesis, whereas overexpression of a dominant-negative CREB alone inhibits adipogenesis. CREB phosphorylation, an event necessary for adipogenesis, is possibly among the earliest modifications in adipocyte differentiation upon hormone induction at least *in vitro* (Reusch et al., 2000b; Zhang et al., 2004a).

During the terminal phase of differentiation, activation of the transcriptional cascade leads to increased activity, protein, and mRNA levels for enzymes involved in triacylglycerol synthesis and degradation. Along with the lipid accumulation, synthesis of adipocyte-secreted products including FABP4, adipsin, resistin, and adipocyte-complement-related protein (Acrp30) begin, producing a highly specialized endocrine/energy-storing cell that will play key roles in various physiological processes (Rosen and MacDougald, 2006).

Adipose tissue development *in vivo*

Up to this date, origins of adipose tissue in terms of its location and possible progenitor cell types have not been fully characterized and precise lineage tracing studies have not been performed. Even though adipose is generally accepted as having a mesodermal origin, this theory has not been tested or studied and is based on the fact that fibroblastic cell lines and MEF cells can differentiate into adipocytes *in vitro* (Rosen and MacDougald, 2006).

White adipose tissue can hardly be detected macroscopically/microscopically during embryonic life and at birth in rodents (rat, mouse) due to the lack of cell markers and lack of lipid accumulation. The development of white adipose tissue has been studied extensively in pig fetuses in early 1980s (Hausman et al., 1981). These studies show that approximately the beginning of the second third of the gestation period in pigs, an extensive vasculature and developing fat cell clusters can be observed, which are surrounded by extensive stroma (Ashwell et al., 1987; Hausman, 1985; Hausman et al., 1981; Hausman et al., 1984; Hausman and Thomas, 1984). The embryonic development of human adipose tissue takes place earlier than that of the pig in various sites (Fritsch and Kuhnel, 1992; Labbe et al., 1989).

Based on the above-mentioned studies, adipose tissue appears and develops in the areas where it remains after birth. Initially, a dense mass of mesenchymal cells with no lipid accumulation occurs. Vascularization and accumulation of lipid possibly follows this process. Interestingly, after the development of fluorescent markers and microscopic analysis, there is almost no

study on the development of adipose tissue up to this date. The idea that developmental and patterning genes might play a role in the differential development of various adipose tissue depots has been suggested by several recent studies (Vohl et al., 2004) (Gesta et al., 2006). These studies show that several patterning genes mainly from Hox families might have roles in *in vivo* adipose development.

The adipose organ is composed of cells with different functions that most likely arise from different developmental lineages. Although the field of adipocyte biology has made great progress *in vitro*, we still lack markers capable of distinguishing preadipocytes that can define various stages in the differentiation process *in vivo*. Specifically, cell types, markers, morphology and anatomic location of these cell types before and after the determination step are not known. Identification characterization of these different cell types could provide new treatments for obesity and its complications. Even though it is widely controversial, some groups suggest that these preadipocytes might derive from blood lineage (Crossno et al., 2006). The defining experiments including a FACS sorting and characterization of different preadipocyte lineage from adipose tissue are lacking.

Regarding the preadipocyte markers, the only widely accepted marker of preadipocytes is preadipocyte factor 1 (Pref-1; also known as DLK-1 or *Drosophila* Homolog-like 1) (Villena et al., 2002). Pref-1 is expressed at high levels in both white and brown preadipocytes, and expression markedly

decreases upon differentiation. Pref-1, however, is not unique to the preadipocyte and is also expressed in other tissues such as placenta, pituitary, adrenal cortex, fetal liver and pancreatic islet cells. Pref-1 is synthesized as a transmembrane protein and is cleaved to generate a soluble form that acts to inhibit adipocyte differentiation. Mice lacking Pref-1 show accelerated fat deposition, (Moon et al., 2002). Despite this, it is not known what type of preadipogenic cell types express Pref-1.

Adipogenesis and Diseases

The increase in adiposity in obese individuals may result from an increase in both adipocyte number and size of individual fat cells (Rosen and MacDougald, 2006). Disproportionate increase in the visceral adipose depots in some individuals is thought to be linked to development of certain metabolic disorders. Understanding the mechanisms regulating fat tissue formation should have implications for treatment of obesity.

It is generally regarded in clinical medicine that adipocyte number of a person is determined just after birth and stays constant through life. This notion however is not based on any experimentation. In fact, several rodent models of obesity have increased adipocyte size, numbers or both (Cleary et al., 1979). Adipogenesis probably continues throughout life at a low rate until energy-storage demands promote further differentiation. A very interesting data came recently from Spalding and colleagues on this issue. Spalding and colleagues' results show that fat cells have a high turnover such that new cells are

continually being born to replace their dead predecessors. The average age of a fat cell seems to be about 10 years in both lean and obese individuals, and the number of fat cells as a proportion of all cells remains constant in each weight group. But the total number of new fat cells is higher in obese subjects, suggesting that they are replenishing an existing larger pool. This suggests that adipocyte hyperplasia might have more impact on obesity than recently appreciated (Spalding et al., 2008).

One other disease related to adipocyte differentiation is lipodystrophy. When adipocytes are absent, in these patients, lipids accumulate in muscle, liver and other peripheral locations. This causes insulin resistance and steatosis that leads to cirrhosis (Herranz et al., 2008; Reue and Peterfy, 2000; Thiebaut et al., 2000). In addition, due to the lack of adipose, some of these patients have extremely low levels of leptin and other adipokines, which was lately considered to be the cause for insulin resistance and diabetes. Consistent with this, leptin treatment of patients with congenital lipodystrophies improved these metabolic abnormalities associated with their disease (Oral et al., 2002; Wolfsdorf et al., 2002). Several genetic causes of lipodystrophy have been discovered in the past few years. Some of these mutations include adipogenesis related genes such as PPARɣ and AGPAT2 (Rosen and MacDougald, 2006). Similarly, overexpression of dominant negative C/EBP and mutation of lipin-1 causes lipodystrophy in genetically engineered mice (Peterfy et al., 2005).

CHAPTER 2:

Materials and Methods

Animal Experiments:

7 to 8 week old C57Bl/6 mice were purchased from the Jackson Laboratory, individually caged, and allowed to acclimate. All experiments were performed according to Rockefeller University CBC guidelines under protocol number 06055.

Leptin treatment procedure is performed as previously described using Alzet subcutaneous pumps. Wild type animals are treated leptin at 2.5 µg/hour and ob/ob animals are treated 150ng/hour. Animals were PBS (6) or leptin (6) treated as described (Cohen et al., 2002). Animals were sacrificed and fat tissues were isolated. Fat tissue from each animal were dissociated by 1.5mg/ml collogenase in Krebs-Ringer buffer as described.(Soukas et al., 2001a) RNA was isolated from stromavascular fraction and TAQMAN RT-PCR was performed.

For leptin withdrawal experiment, PBS (PBS control group) or leptin at 2.5 µg/hour (Leptin-FF and Leptin-NC groups) or was administered for 8 days by subcutaneous implanted osmotic pump (Alza, Palo Alto, CA). On day 8, leptin or PBS treatment was withdrawn by removing pumps under inhaled anesthesia. Body mass and food intake were monitored daily at 1200h. Animals were free-fed (Leptin-FF and PBS groups) or maintained at the level of food intake prior to leptin withdrawal (Leptin-NC group). Mice were sacrificed at 1200 h on days 6 and 8 of leptin treatment (PBS and Leptin groups) and 1, 2, 3, and 4 days after

pumps had been withdrawn (PBS, Leptin-FF and Leptin-NC groups). Serum and peri-uterine white adipose tissue were collected and kept at -80 °C for later analyses. Serum leptin (R&D Systems, Minneapolis, MN) and insulin (Crystal Chem, Chicago, IL) were measured by ELISA according to the manufacturers protocols.

BAC modifications and in-vitro / *in vivo* imaging:

Modification of BACs has been performed as described (Warming et al., 2005) (Gong et al., 2003). Firefly luciferase is from Promega. Generation of transgenic mice following microinjection was based on standard techniques described by Hogan and colleagues (Hogan B. , 1994). In each case, leptin luciferase animals were killed and tissues; fat, spleen, kidney, brain, colon, liver, intestine, heart were isolated and lysed in passive lysis buffer (Promega). Luciferase activity was measured using Luciferase Assay Kit (Promega). Protein normalization was performed after protein measurements using the BCA (Pierce) kit according to the manufacturer's instructions.

In vivo imaging of transgenic animals was performed using the Xenogen IVIS Lumina imaging system. Anesthetised animals were injected intraperitonally with the luciferase substrate, luciferin (200 µl of stock 15 mg/ml in PBS). After 15-20 min, the animals were placed in a dark imaging chamber under isoflurane anesthesia. Photon emission resulting from the luciferin/luciferase reaction was detected with a sensitive CCD camera. This photon image was superimposed on a normal video image of the mouse with Living Image 3.0 software (Xenogen).

White Adipose Tissue Histology and Glycogen Quantitation:

Tissue fragments were embedded in paraffin wax (Paraplast Plus, Fisher Scientific, Morris Plains, NJ). Sections were PAS stained and hemaxoxylin counterstained according to standard protocol (Sigma). To demonstrate specificity of glycogen staining, several sections were pre-incubated for 5 minutes at 37 °C in 5 mg/ml α-amylase (Sigma) after re-hydration prior to PAS staining. Glycogen was quantitated with the amyloglucosidase method.(Keppler, 1974) For periodic acid-schiff (PAS) staining, to avoid loss of water soluble glycogen, small pieces of tissue (50 mg) were incubated overnight in alcoholic fixative (90% ethanol, 10% formaldehyde).

Plasmids:

For overxpression studies, KLF4 cDNA was cloned by RT-PCR and an HA epitope was added at the C terminus. It was subsequently inserted into pMSCVpuro (Clontech). Other pMSCV vectors are as used in Chen et al (Chen et al., 2005a). FLAG-KLF4 is courtesy of Douglas Boyd.

For knockdown of KLF4, Krox20 or C/EBPβ expression in 3T3-L1 cells, the DNA-based, retroviral vector-mediated siRNA technology was used. The targeted sequence was determined by the Whitehead Institute siRNA designing tool and verified by BLAST searches to ensure specificity. Two complementary oligos for each targeted sequence were then designed according to the protocol of Clontech's RNAi-Ready pSIREN-RetroQ system, annealed, and ligated into the BamHI/EcoRI-linearized pSIREN-RetroQ vector. The resulting plasmid, upon

packaged into retrovirus, allowed stable expression of siRNA hairpin for a specific gene. All sequences for the siRNA constructs are as described in Chen et al (Chen et al., 2005a).

Retroviral infections and 3T3-L1 differentiations:

Recombinant pMSCV or pSIREN-RetroQ viral packaging was achieved by transfection of the plasmid into Phoenix ecotropic packaging cells (cultured in DMEM with 10% FBS in 5% CO_2) using Fugene6 (Roche). Viral supernatants were supplemented with 8 µg/ml polybrene and added to cells for infections for 18–24 hr. Cells were selected with 2µg/ml puromycin or 100 µg/ml hygromycin (Sigma), expanded, and seeded for differentiation experiments. 3T3-L1 cells (ATCC) were maintained in DMEM with 10% FBS (Invitrogen) in 5% CO_2. Stable cell lines from retroviral transduction were cultured to confluence and exposed to the differentiation cocktail (1 µg/ml insulin, 0.25 µg/ml dexamethasone, 0.5 mM IBMX). After 48 hr, cells were maintained in medium containing 1 µg/ml insulin until day 8 for harvest. Plates were oil-red O stained as described earlier (Soukas et al., 2001a). In experiments, where each component is added separately or in groups, the concentrations are as in the standard hormone cocktail.

Coimmunoprecipitation

293T cells grown in DMEM supplemented with 10% FBS were transiently transfected using Fugene6 (Roche) according to the manufacturer's recommendations. Transfected cells were harvested in BA300 buffer (BA0 with 300mM NaCl) after 36h, and lysates were used for coimmunoprecipitation as

described (Dou et al., 2005). Briefly, lysate expressing indicated plasmids were immunoprecipitated by M2 agarose beads (Sigma) and washed extensively for 3 times. Beads were denatured with SDS-PAGE loading dye, and supernatants were separated on 12% SDS-PAGE gels and western blotting was performed by anti-HA. (Sigma-H6908)

RT-PCR analysis

Total RNA was isolated from cells by QIAGEN RNA prep kit following TRIZOL reagent (Invitrogen). Real Time PCR was performed by the Taqman system (Applied Biosystems) according to the manufacturer's instructions. Oligos were designed by the PrimerExpress software. Amount of expression was normalized with cyclophilin for cell culture experiments and with ELK3 for preadipocyte fraction experiments. Sequences of the Taqman primers and probes are available in Supplementary methods.

Transfection and reporter assays.

C/EBPβ reporter was kindly provided by Daniel Lane. Deletions were made by PCR and cloned into pGL3 plasmid. (Primers are available upon request.) The reporter plasmids along with effector plasmids were transfected using Fugene6 into 293T cells at 80-90% confluence. The pRL-TK (Promega) that carries Rennila luciferase was also cotransfected as an internal control for transfection efficiency. Cells were harvested after 20-24h and luciferase activities analyzed using the Promega Dual-Luciferase assay kit as recommended by the manufacturer.

GST pull-down:

KLF4 was cloned by PCR from pMSCVpuro-KLF4 and subsequently inserted into pGEX-T1 plasmid to express GST-KLF4 protein. GST-KLF4 protein is isolated as described (Morlon and Sassone-Corsi, 2003). *In vitro* transcription and translation of the Krox20 protein was performed by using the TNT T7 quick-coupled transcription-translation kit (Promega).

GST pull-down assays were performed by incubating GST fusion proteins immobilized on GST beads with *in vitro*-translated ^{35}S-labeled Krox20 or Luciferase. After washing, bound proteins were isolated by eluting the proteins with 2× SDS buffer, separating them on SDS/PAGE, and developing by autoradiography.

CHIP assays

ChIP assay Kit (Upstate Biotechnology, Lake Placid, NY) was used according to the method recommended by the manufacturer, with minor modifications. In brief, a plate of cells was fixed in the presence of1%formaldehyde for 10 min at room temperature. The reaction was stopped by the addition of glycine at a final concentration 0.125 M. A soluble chromatin fraction containing fragmented DNA of 500–2,000 bp was obtained after cell lysis and sonication. The fraction was diluted ten times and precleared by using Protein A agarose slurry.

Samples were incubated with Rabbit anti-KLF4 (santa cruz) or rabbit-IgG as a control, later mmunoprecipitated and DNA was used for PCR.

SDS-PAGE and Immunoblotting:

Proteins were separated by SDS-PAGE using pre-cast 4-20% or 12% SDS gels (Bio-Rad) and transferred to nitrocellulose membranes. Western blot has been performed as described (Boyer-Guittaut et al., 2005). Briefly, total protein lysate (20–50 µg) was loaded on SDS-PAGE for each sample. Rabbit anti-KLF4, rabbit anti-C/EBPβ, and mouse anti-TBP were from Santa Cruz (H-180, C-19, and N-12, respectively). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from Amersham(GE).

Gel shift assays

EMSA was performed by incubating 5 ug of Nuclear extracts or 100ng of purified protein at room temperature for 20 min in a 30-µl binding reaction mixture containing 10 mM HEPES, pH 7.9, 80 mM KCl, 5% glycerol, 30 ng of poly(dI-dC), 75 ng of denatured salmon sperm DNA, 10 mM dithiothreitol, and 30,000 cpm of ^{32}P-labeled double-stranded DNA probe. Following incubation, reaction mixtures were loaded on a 5-7% polyacrylamide gel, dried, and subjected to autoradiography.

A 500-fold molar excess of unlabeled probe was added for competition as indicated in the appropriate figures. For competition experiments, proteins were preincubated for 20 minutes at room temperature with unlabeled probe at specified -fold excess. Labeled probe was then added and incubated for 20 min at room temperature.

Whole mount staining

Mice were sacrificed by cervical dislocation, after which the epididymal fat was removed using sterile techniques and minced into small pieces (~2-3 mm) using a scalpel. The tissue pieces were then washed and incubated with LipidTox, and/or *Griffonia simplicifolia* isolectin GS-IB4 conjugated with Alexa Fluor (Molecular Probes) before observation. Nuclei were counterstained with Hoecst 33342 (Molecular Probes). *Griffonia simplicifolia* IB4 isolectin is reportedly a useful histochemical probe that specifically labels endothelial cells in many species and tissues, including adipose tissue.

Confocal microscopy

A confocal laser scanning microscope (LSM510 Meta; Carl Zeiss) equipped with 10x dry, 40x dry, and 63x oil objectives was used to collect stacks of images at regular intervals along the optical axis. The tissue was excited using multiple color laser lines, and the emission was collected through appropriate narrow band-pass filters. Each image was produced from an average of 8 frames after which the acquired images were processed to produce a surface-rendered 3-dimensional model. In preliminary experiments, adipose tissue was observed using 5-μm-thick stacks of images obtained at 0.5-μm intervals and 50-μm-thick stacks obtained at 2-μm intervals.

CHAPTER 3:

Transcriptional Networks in Early Regulation of Adipogenesis

Introduction

Two decades of research have revealed that adipogenesis is controlled by a complex network of transcription factors, including numerous transcriptional activators, coactivators and repressors (Farmer, 2006a). When confluent 3T3-L1 and 3T3-F442A cell lines are exposed to a cocktail of hormonal stimulants, these cell lines accumulate lipid and develop the characteristic morphology of mature adipocytes (Green and Meuth, 1975). The earliest inductive event in this process is transcriptional activation of two of the C/EBP family of transcription factors, C/EBPβ and C/EBPδ, which then stimulate expression of PPARɣ, the late major transcription factor (Cao et al., 1991; Lane et al., 1999; Wu et al., 1995; Yeh et al., 1995). PPARɣ is necessary and sufficient for adipocyte differentiation (Tontonoz et al., 1994d). Less is known about what controls C/EBP gene expression. The identification of the factors that regulate these important early genes in preadipocytes would provide additional insight into the mechanisms regulating the initiation of fat cell differentiation.

In order to study the earlier transcription factors, it was considered that these factors might be included in a panel of transcription factors that are expressed at a much higher level in white adipose tissue *in vivo* than in 3T3-L1 cells *in vitro*. This approach yielded two early transcription factors, Krox20 and KLF4. However, a role of KLF4 in adipogenesis has not been previously

demonstrated. KLF4, also known as GKLF/ZIF, is highly expressed in differentiated, post-mitotic cells of the skin and the gastrointestinal tract and it has been suggested to have variety of roles as differentiation-proliferation switch and control of the cell cycle (Garrett-Sinha et al., 1996; Shields et al., 1996; Takahashi and Yamanaka, 2006; Yoon et al., 2003). KLF4 knockout mice die around 12 hours after birth due to defects in skin development with a failure of normal basement membrane formation (Segre et al., 1999). In these mice, the skin's fat layer is disrupted, causing the loss of skin barrier function and rapid loss of body fluids (Segre et al., 1999).

I thus considered the possibility that KLF4 might play a role in regulating the development of adipose tissue and tested this possibility by studying the functional role of KLF4 during the differentiation of 3T3L1 adipocytes.

Results

Several transcription factors are expressed highly in *in vivo* adipose tissue compared to *in vitro* differentiated adipocytes

A previous microarray analysis has been done by Soukas et al in order to characterize factors that might regulate leptin gene expression by comparing *in vivo* versus *in vitro* adipocytes (Soukas et al., 2001a). However, since adipose tissue also contains other cell types (stromavascular fraction), we considered the possibility that early factors that are expressed in the stromavascular fraction might be included in this panel of transcription factors that are expressed at a much higher level in white adipose tissue *in vivo* than in 3T3-L1 cells *in vitro*

(Soukas et al., 2001a). Among these transcription factors, Krox20 has been characterized as one of the earlier factors, validating this microarray approach (Chen et al., 2005b) (Figure 9).

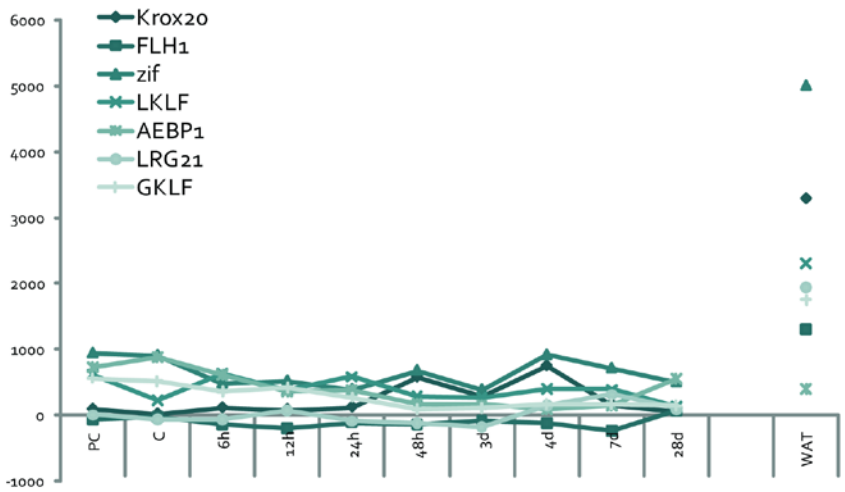

Figure 9 *In vivo* enriched transcription factors.

This figure shows the *in vivo* adipose-tissue enriched transcription factors. Left side of the graph shows the expression of each transcription factor during 3T3-L1 differentiation time course. WAT: White adipose tissue

One of the other transcription factors that is induced *in vivo* is KLF4, a member of a large family of zinc-finger proteins that is known to play important roles in differentiation and proliferation (Ghaleb et al., 2005). Several other KLFs have previously been implicated in the regulation of adipogenesis. KLF6 and KLF15 have both been shown to promote adipogenesis and KLF15 also upregulates GLUT4 expression (Boquest et al., 2005; Halaas et al., 2005). In addition, another KLF factor, KLF5, is necessary for adipocyte differentiation and acts by transactivating the PPARɣ2 promoter (Oishi et al., 2005). All these KLF

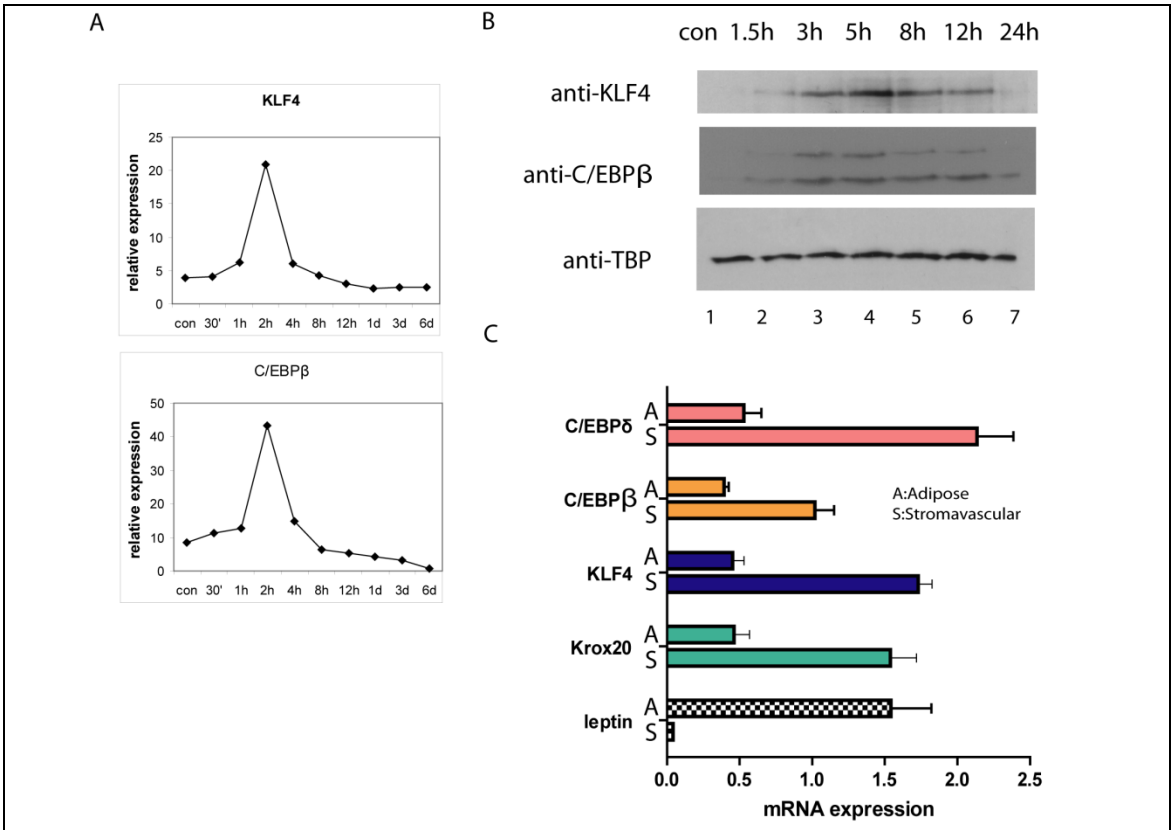

Figure 10 Expression of KLF4 during 3T3-L1 differentiation

A) The expression of KLF4 during differentiation of 3T3-L1 cells was analyzed using Taqman RT-PCR and (B) Western blots. Cells were harvested at the indicated times. Cyclophilin was used as an internal control for Taqman analysis.
C) Expression of early genes in stromavascular fraction and adipocyte fraction. Taqman RT-PCR was performed in stromavascular and adipocyte fractions of the adipose tissue disintegrated by collagenase. Leptin levels (enriched in the adipose fraction) were checked as a control of the preparation. Error bars are SEM.

factors are thought to function through different coactivators or repressors (Farmer, 2006a).

KLF4 is an early-expressed and necessary factor during 3T3-L1 differentiation

To assess if KLF4 is an early factor, KLF4 gene expression was assayed at numerous times early during 3T3-L1 differentiation that was induced using the standard IBMX, dexamethosone, and insulin cocktail. Quantitative RT-PCR

revealed that KLF4 mRNA appeared 30 min post-induction and peaked at 2 hr post-induction. Protein levels for KLF4 were consistent with the mRNA levels (Figure 10). This result shows that KLF4 is expressed very early in the course of 3T3-L1 differentiation with a similar pattern to other previously described early transcription factors (Figure 10) including Krox 20, C/EBPβ and C/EBPδ. These same factors were also enriched in the stromal/pre-adipocyte fraction of the adipose tissue compared to the adipocyte fraction (Figure 10C).

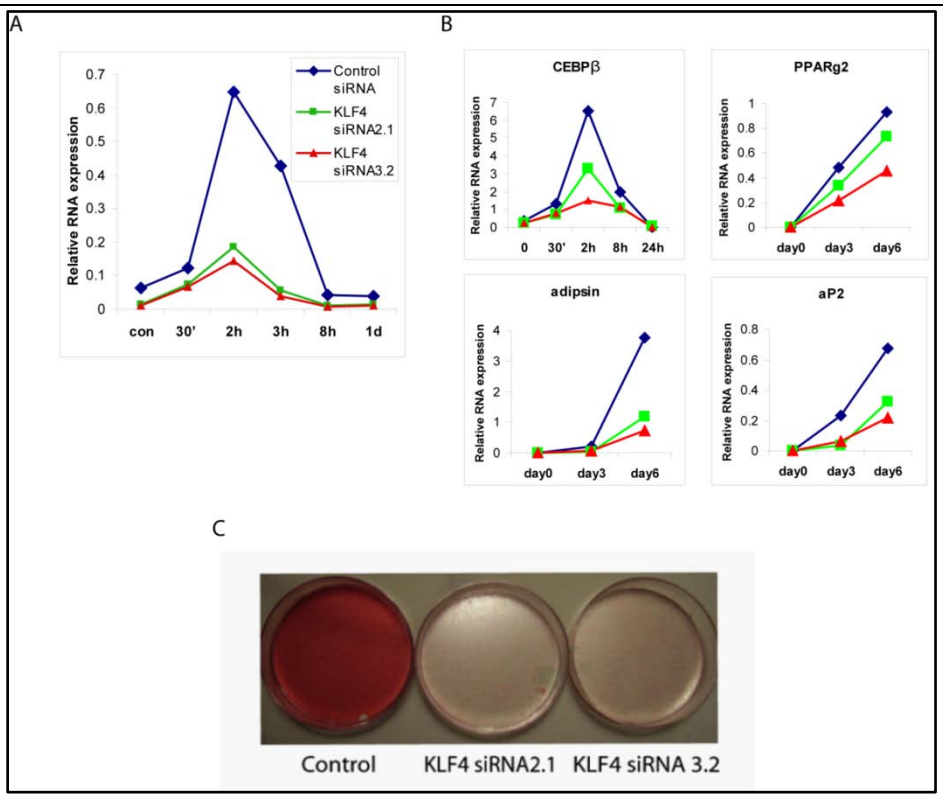

Figure 11 KLF4 is necessary for adipogenesis

KLF4 knock-down impairs adipogenesis. 3T3-L1 cells were infected with pSIREN-RetroQ-derived retroviruses carrying either siRNA oligos for KLF4 or control oligo, selected for puromycin resistance, expanded as mixed population and induced to differentiate using MDI cocktail. The upper panel (A) shows the efficiency of the 2 knock-down plasmids against KLF4 by Taqman assays. (B) Right panel shows expression levels of fat markers aP2, PPARɣ2, adipsin and an early gene, C/EBPβ in control and KLF4 knock-down 3T3-L1 cell lines at different time points during differentiation.
C) ORO staining of KLF4 knock-down and control hairpin cell lines at day 8.

The role of KLF4 in adipogenesis was tested using retroviruses carrying siRNA for KLF4, to knock down its expression. Two independent hairpin siRNA sequences for KLF4 (cloned into pSIREN-RetroQ-puro retroviral plasmid) were effective for knocking down KLF4 expression (Figure 11). 3T3-L1 cells were infected with the two KLF4 siRNA-containing retroviruses along with a control hairpin. After selection with puromycin, we established stable cell lines with constitutive siRNA expression. Note, these cell lines were a mixture of all cells expressing the selectable marker and were not clonal. These stable cell lines were cultured to confluence and differentiated using the standard induction cocktail for 8 days. Oil red O staining revealed that both KLF4 knock down cell lines accumulated significantly less lipid than the control cell line. (Figure 11C) RNA levels of adipogenic regulators such as adipsin, ap2 and PPARɣ2 were also reduced consistent with the oil red-O staining. In addition, early C/EBPβ expression was also reduced in KLF4 knock down cell lines compared to control cells (Figure 11B). These results suggest that KLF4 is necessary for normal differentiation of 3T3-L1 cells and acts upstream of C/EBPβ and PPARɣ. Of note however, the reduction of PPARɣ gene expression was not proportional to the decrease in adipogenesis suggesting that KLF4 might partially promote the differentiation of adipocytes independent of PPARɣ.

KLF4 transactivates C/EBPβ at a conserved -1.45kb to -1.1 kb region of the C/EBPβ promoter

The data shown above suggested that KLF4 might regulate C/EBPβ transcription. To test this hypothesis, a virus expressing a KLF4-HA-protein and

a control plasmid were cotransfected into 293T cells with a promoter-luciferase reporter plasmid (B3K), which contains 3kb of the C/EBPβ promoter(This construct was generously provided by Daniel Lane). These showed that KLF4 transactivated the C/EBPβ promoter (B3K) by ~10 fold, in a dose dependent manner (Figure 12A).

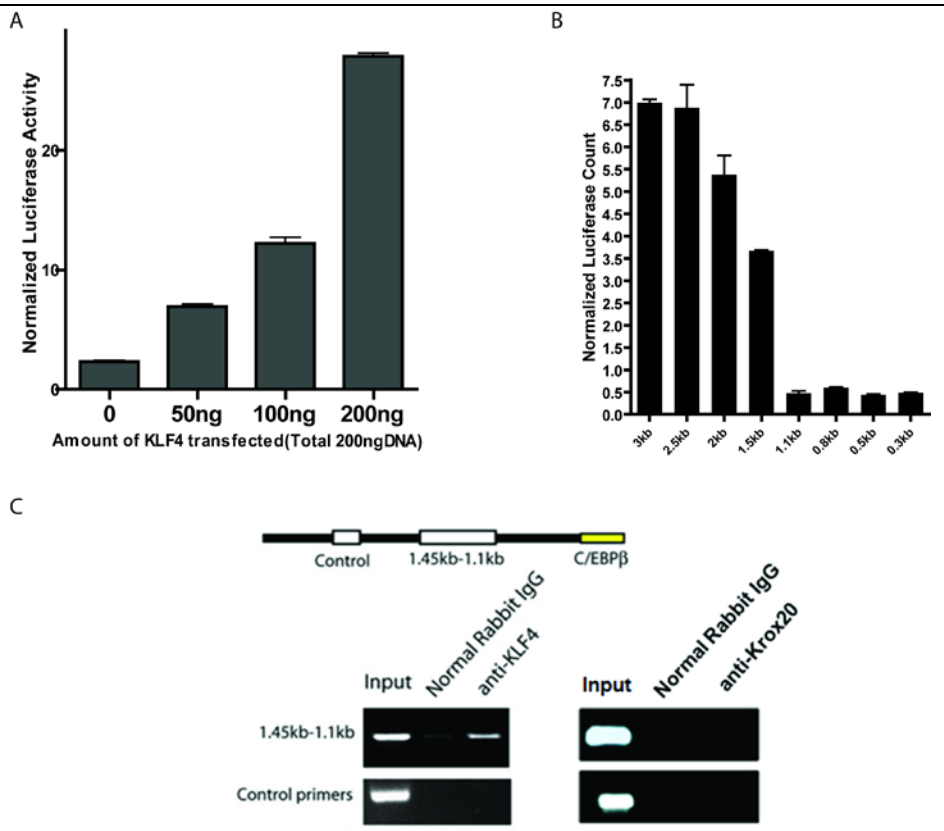

Figure 12 KLF4 transactivates the C/EBPβ promoter

A) Transactivation of C/EBPβ plasmid by KLF4
B) A deletion series derived from of a luciferase reporter construct (B3K), carrying 3kb promoter region of C/EBPβ (generously provided by Daniel Lane) was cotransfected with 250ng of pMSCV-KLF4 and pRL-TK(Renilla). Results were expressed as firefly luciferase activity normalized for renilla luciferase activity.
C) CHIP analysis of KLF4 binding to the target region on C/EBPβ promoter. 3T3-L1 cells were fixed 2.5h post-differentiation and chromatin samples were subjected to CHIP assays by using a KLF4 antibody or normal rabbit IgG as a control. An upstream region (-2.5kb) in C/EBPβ promoter was also checked as negative control.

To further narrow down the region(s) responsible for the transactivation of KLF4, a series of 5' deletions of the C/EBPβ promoter was generated and analyzed for the induction in response to KLF4. Transactivation by KLF4 dropped by more than 5 fold, when a region from -1.45kb to -1.1kb was deleted (Figure 12B). Deletion of sequences from -3kb to -1.5kb region resulted in a further 2-fold decrease in the activation.

To assess whether KLF4 directly activated the C/EBPβ gene, I next assayed the binding of KLF4 to the endogenous C/EBPβ promoter in 3T3-L1 cells using chromatin immunoprecipitation assays 3T3-L1 cells were differentiated for 2.5 hours at which time DNA-protein complexes were cross-linked and CHIP assay was performed by using an anti-KLF4 antibody, or rabbit IgG as a control. Chromatin precipitation using the anti-KLF4 antibody caused a significant enrichment of the C/EBPβ promoter sequence indicating that the KLF4 transcription factor is directly bound to the C/EBPβ promoter (Figure 12C). Further characterization of the region between -1.45 to – 1.1 kb of the C/EBPβ promoter was performed using gel shift assays with fragments of the promoter and bacterially expressed Klf4. Seven 55bp oligos spanning the 1.45kb-1.1kb region were incubated with recombinant GST-KLF4 protein. GST-KLF4 shifted 3 of these oligos and this was correlated with the results of a cotransfection of Klf4 with a series of C/EBPB promoter deletions between -1.45 to – 1.1 kb. (Figure 13) Same binding could not be seen by Krox20. Taken together, these findings suggest that KLF4 transactivates C/EBPβ gene by binding directly to several sites on 1.45kb-1.1kb region of the promoter. However the sequences of these

oligonucleotides were different and DNAse footprinting failed to reveal a clear consensus sequence (data not shown). This suggests that the Klf4 binding site may be highly degenerate.

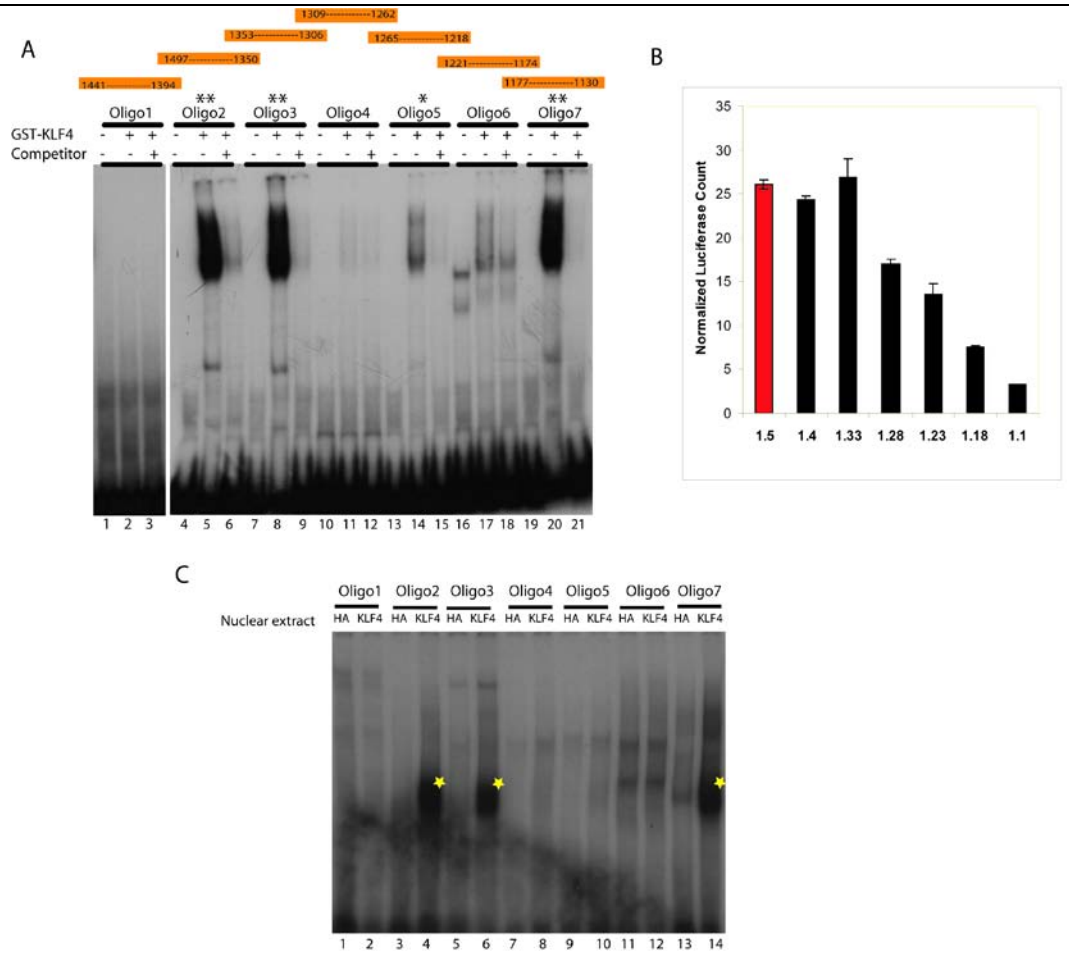

Figure 13: KLF4 binds C/EBPβ promoter

Gel shift assay with 293T nuclear extracts overexpressing KLF4 (C) or purified GST-KLF4 (A) Seven 55bp oligos spanning the 1.45kb-1.1kb region were checked for their ability to bind to nuclear extracts or recombinant GST-KLF4 protein. B) A deletion series between 1.45-1.1 kb fragment derived from of a luciferase reporter construct (B3K) was cotransfected with 250ng of pMSCV-KLF4 and pRL-TK (Renilla). Results were expressed as firefly luciferase activity normalized for renilla luciferase activity.

KLF4 and Krox20 interact and transactivate the same site of the C/EBPβ promoter

Krox20 transactivates the C/EBPβ promoter via sequences between 1.45kb-1.1kb which is identical to that reported above for Klf4 (Figure 14B). The fact that KLF4 and Krox20 also have similar expression kinetics led us to examine whether the ability of these transcription factors to activate C/EBPβ promoter is synergistic. Plasmids directing the expression of Krox20 and KLF4 were co-transfected into 293T cells using the same C/EBPβ promoter-luciferase reporter. Transfection of KLF4 or Krox20 alone led to a 6-fold and 4-fold induction respectively, while simultaneous transfection of both plasmids increased the luciferase activity 10-fold (Figure 14C).

Since Krox20 (Chen et al., 2005a) and KLF4 bind to the same region and can both activate transcription of C/EBPβ, I considered the possibility that these proteins might bind to one another. Flag-tagged KLF4 and HA-tagged Krox20 were ectopically expressed in 293T cells for 2 days and the lysate was immunoprecipitated using anti-FLAG M2 agarose beads (Sigma), washed extensively with BA300 (300mM NaCI) and immunoblotted using an ant-HA antibody. In this assay, immunoprecipitation of KLF4 using an antibody to the Flag tag pulled down Krox20 (Figure 15). In addition studies using standard mammalian 2-hybrid assay provided functional evidence that Krox20 and Klf4 can bind to one another and activate gene expression (data not shown). A cross-species comparison of the C/EBPβ promoter further indicated that the

1.45kb-1.1kb region is the only region that is significantly conserved among mammals (Figure 14C).

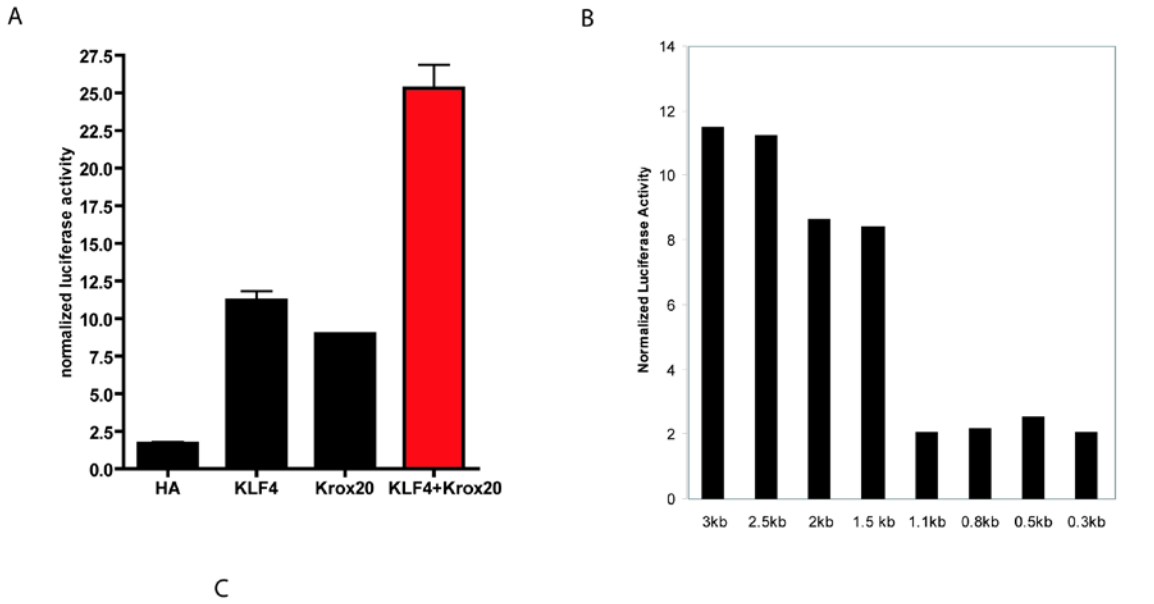

Figure 14: Effect Krox20 and KLF4 on C/EBPβ promoter

A) KLF4 and Krox20 expression plasmids were cotransfected with the C/EBPβ luciferase reporter construct. An additive effect for transactivating C/EBPβ promoter was shown.

B) A deletion series derived from of a luciferase reporter construct (B3K), carrying 3kb promoter region of C/EBPβ (generously provided by Daniel Lane) was cotransfected with 250ng of pMSCV-Krox20 and pRL-TK (Renilla). Results were expressed as firefly luciferase activity normalized for renilla luciferase activity.

C) Conservation in 1.45-1.1 kb promoter region of C/EBPβ gene (UCSC genome browser)

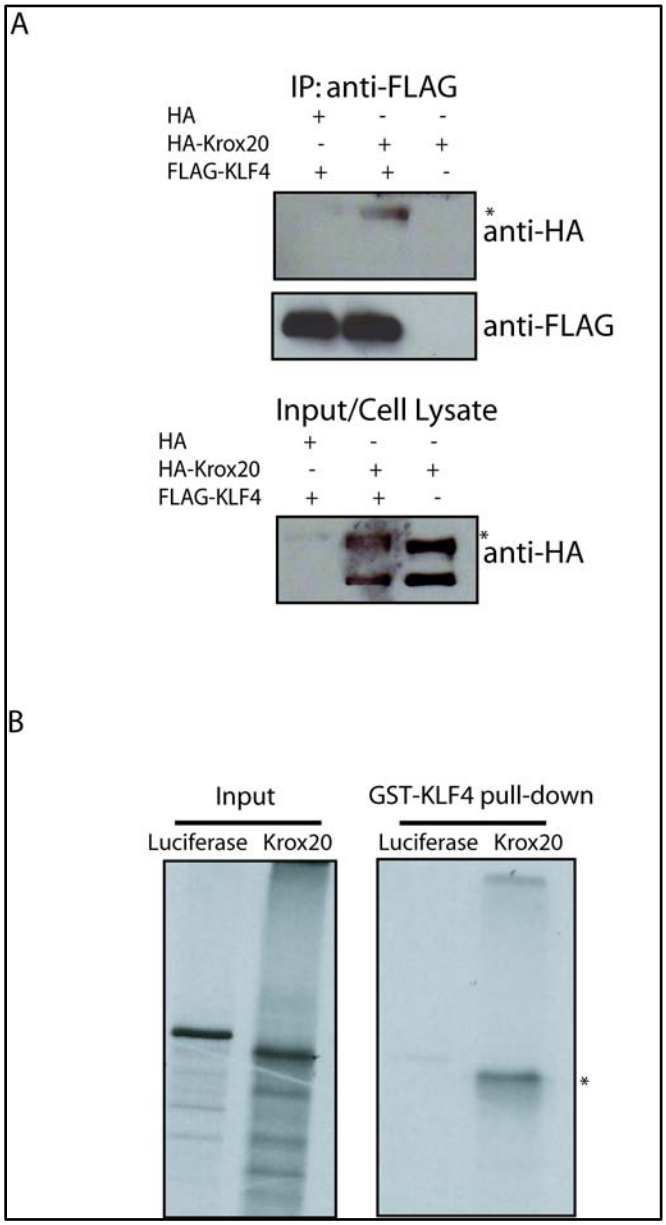

Figure 15: Interaction of KLF4 and Krox20

A) Coimmunoprecipitation of KLF4 with Krox20. FLAG-KLF4 and Krox20-HA were cotransfected into 293T cells. After 2 days, cells were lysed and co-immunoprecipitated using an anti-FLAG antibody and immunoblotted against HA. The data shows the specific binding of KLF4 to Krox20. B) GST pull-down assays. Krox20 and luciferase plasmids were *in vitro* translated in presence of S35, mixed with purified GST-KLF4 and pulled-down by GST beads. Krox20 but not luciferase was pulled down by GST-KLF4.

Krox20 and C/EBPβ overexpression partially overcomes the knockdown of KLF4

The data suggested that Klf4 acted upstream of C/EBPβ. If true, expectation would be that overexpression of C/EBPβ or Krox20 should bypass the effect of KLF4 knockdown. To test this, 3T3-L1 cells were infected with retroviruses carrying KLF4 siRNAs and either Krox20-HA or C/EBPβ. These data confirmed that the negative effect of a KLF4 knockdown on adipogenesis can be ameliorated by the overexpression of either Krox20 or C/EBPβ as assessed using Oil red O staining and measuring the level of expression of aP2, adipsin and PPARɣ2 (Figure 16).

KLF4 expression is dependent on IBMX but not on Dexamethasone or Insulin

These data show that KLF4 and Krox 20 cooperate to activate C/EBPβ transcription and establish them as among the earliest if not the earliest factors induced during adipogenesis. If this is true, both factors might be expected to respond directly to one of the components of the adipogenic cocktail and not the others. To test this, confluent 3T3 cells were exposed individually to each of the three components of the induction cocktail: insulin, dexamethasone, and the phosphodiesterase inhibitor IBMX. Insulin and dexamethasone concentrations did not change KLF4 levels at 2 hr post-induction, the time at which KLF4 is maximally induced in response to the three-component cocktail. On the other hand, IBMX increased KLF4 levels 4 to 5 fold (Figure 17) and increasing

concentrations of IBMX proportionally increased C/EBPβ and KLF4 gene expression (Figure 17B).

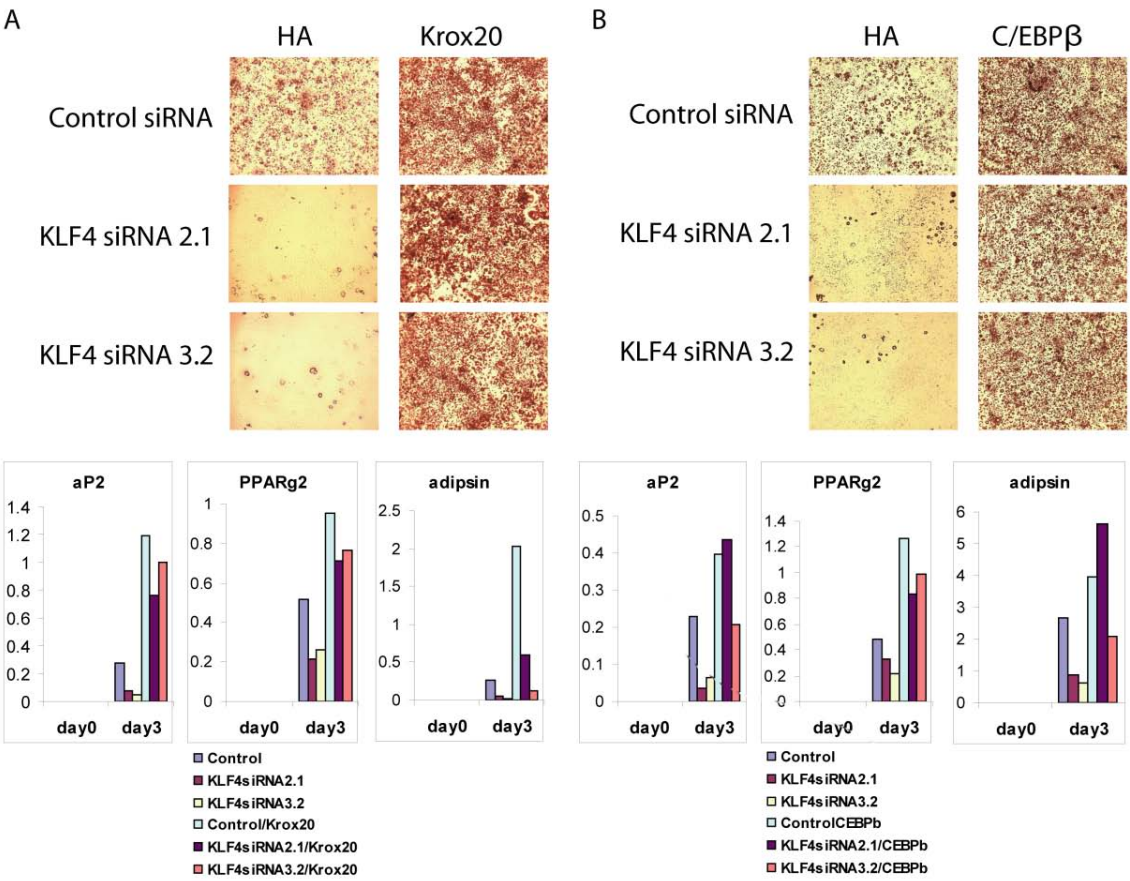

Figure 16 Krox20 and C/EBPβ overexpression partially overcomes the knockdown of KLF4

3T3-L1 cells were coinfected with pMSCVhyg-derived retroviruses carrying either Krox20-HA/ C/EBPβ or control insert and pSIREN-RetroQ-derived retroviruses carrying either siRNA oligos for KLF4 or control oligo, selected for hygromycin and puromycin resistance, and induced to differentiate till day 7.

A) Overexpression of Krox20 overcomes the knockdown of KLF4. Upper panel shows the oil red O staining and lower panel shows the TAQMAN analysis of 3 late genes, aP2, adipsin and PPARɣ2.

B) Overexpression of C/EBPβ overcomes the knockdown of KLF4. Upper panel shows the oil red O staining and lower panel shows the TAQMAN analysis of 3 late genes, aP2, adipsin and PPARɣ2.

To further assess the effect of IBMX on KLF4 in 3T3-L1 cells, 293T cells were transfected with a luciferase reporter driven by 2 kb KLF4 promoter. When the transfected cells were exposed to IBMX, 3 to 4 fold increase in luciferase activity was observed (Figure 17C).

KLF4 and Krox20 are regulated through a negative feedback loop

Overall these data suggest that KLF4, Krox 20 and C/EBPβ compose a network of transcription factors that play a key role in orchestrating the early events of adipogenesis. The interaction among these factors was checked by analyzing their expression in cells in which only one of these transcription factors was knocked down by stable expression of their corresponding siRNA. The C/EBPβ knockdown cell lines showed a robust 2 fold increase in Krox20 and KLF4 expression levels at 2 hours post-induction, suggesting that C/EBPβ expression normally inhibits its upstream activators via a negative feedback mechanism. These data add further evidence that it is a downstream target of KLF4 and Krox20 (Figure 18A).

Consistent with the hypothesis, a 3T3L1 cell line that over expressed C/EBPβ showed a robust early decrease of KLF4, and to a lesser extent in Krox20 expression levels (Figure 18B). A negative effect of C/EBPβ on KLF4 expression was also seen using the KFL4-luciferase reporter. Co-expression of the KLF4-luciferase reporter with C/EBPβ in 293T cells reduced KLF4 expression as much as 30% and was dose-dependent. This inhibition was even more robust, i.e.; 70%, when these cells are incubated with IBMX (Figure 19).

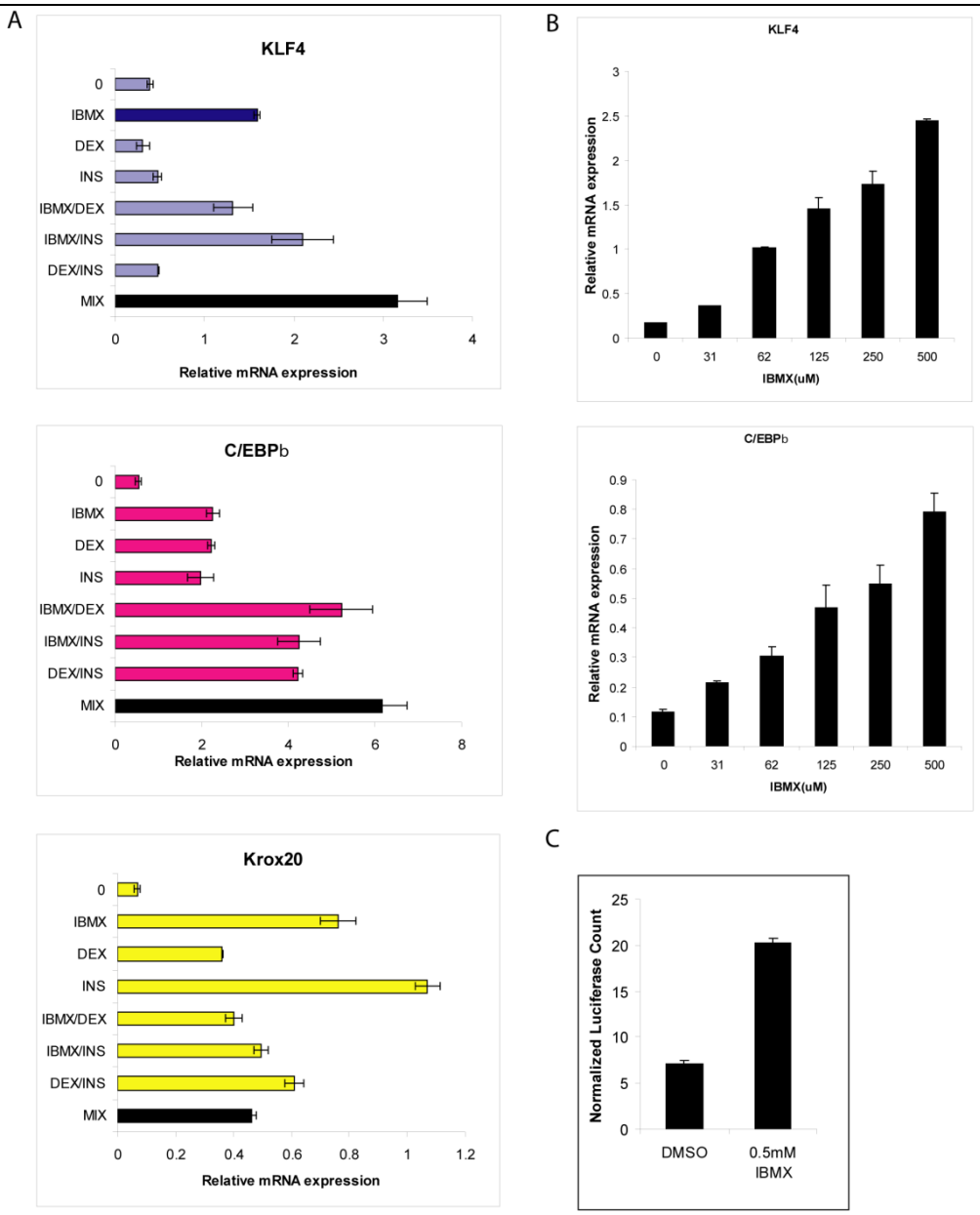

Figure 17 KLF4 expression is dependent on IBMX

A) Individual components of the induction cocktail (IBMX,dexamethasone and insulin) were added onto confluent 3T3-L1 cells alone or in combination. Cells were harvested 2h post-treatment for Taqman analysis of KLF4. Panel shows the effect of each component on gene expression. B) The left panel shows dose-dependent induction of KLF4 and C/EBPβ. Confluent 3T3-L1 cells were treated by increasing amounts of IBMX (0 to 0.5 mM). Cells were harvested 2h-post induction for Taqman Analysis.
C) IBMX can transactivate C/EBPβ promoter in 293T cells *in vitro*. 293T cells were transfected with KLF4 luciferase reporter plasmid carrying 2kb upstream of KLF4 promoter. 16 hours later, the cells were either treated with DMSO or 0.5mM IBMX for 8 hours. After treatment, cells were harvested for luciferase assays.

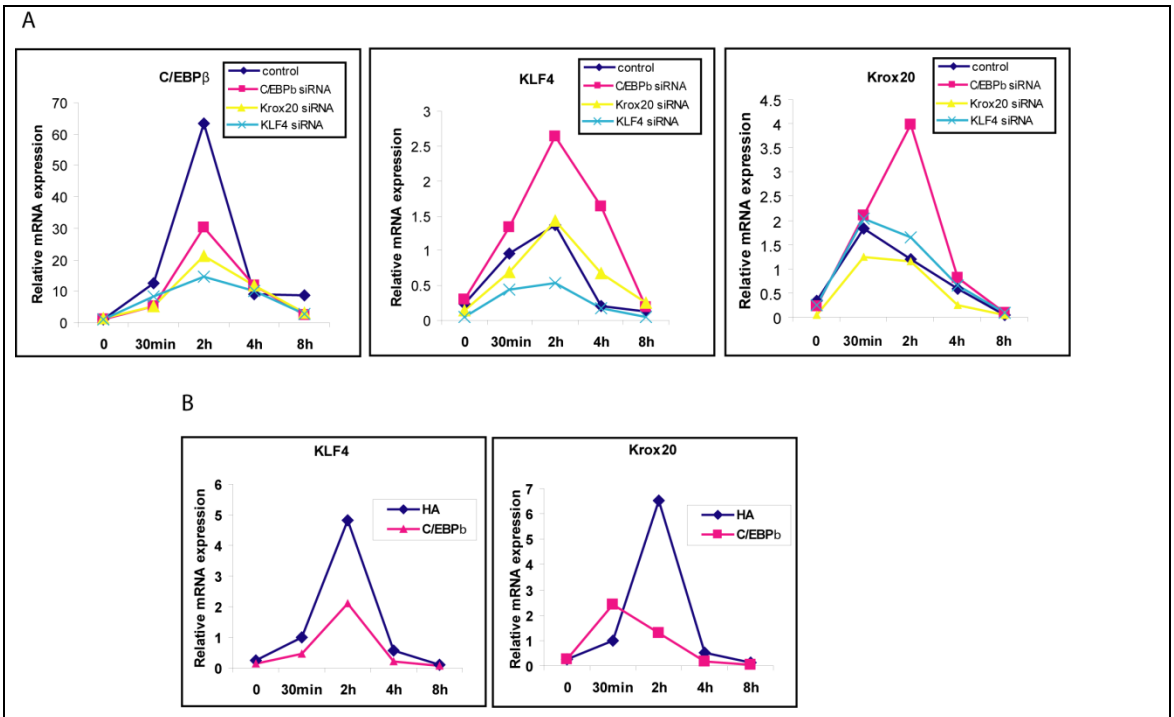

Figure 18: KLF4 and Krox20 are regulated by C/EBPβ through a negative feedback loop

3T3-L1 cells were infected with retroviruses expressing C/EBPβ knockdown or overexpression constructs. Cells were selected, differentiated and the effect of C/EBPβ expression on KLF4 was observed.
A) KLF4 and Krox20 levels are upregulated in C/EBPβ knockdown 3T3-L1 cells.
B) KLF4 and Krox20 levels are downregulated in C/EBPβ overexpressing 3T3-L1 cells

Overall, these results suggest a potential negative feedback mechanism whereby C/EBPβ actively down-regulates its upstream regulators and add further evidence to the possibility that KLF4 and Krox20 act upstream of C/EBPβ. Overall, these data indicate that the level of expression of Klf4 is tightly controlled, a finding that is consistent with the observation the overexpression of Klf4 leads to cell cycle arrest in preadipocytes (and other cell types) further suggesting that Klf4 must be expressed at tightly regulated levels in a narrow window during adipogenesis.

p300 co-activates KLF4/Krox20 dependent C/EBPβ expression

In order to assess the effect of possible coactivators of this early regulatory network, several co-activators including GRIP-1, SRC-1,TRAP220 and p300 were cotransfected along with KLF4, Krox20 and both. This experiment showed that p300 has the most co-activation potential in this context (Figure 20).

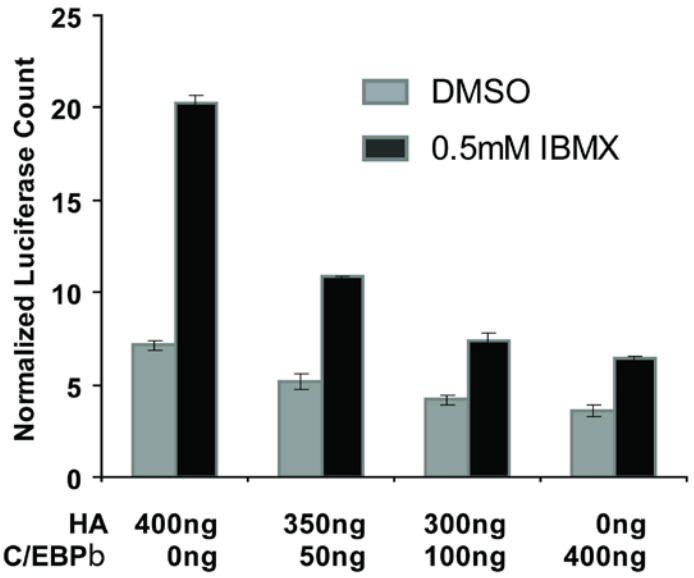

Figure 19: KLF4 transcription is inhibited by C/EBPβ.

293T cells were cotransfected with increasing amounts of C/EBPβ plasmid along with a luciferase reporter plasmid carrying 2kb promoter of KLF4. After 16 hours the cells were treated with DMSO or 0.5mM IBMX. Cells were harvested after 8 hours for luciferase assays.

Leptin treatment down-regulates early genes including KLF4 in the preadipocyte fraction

Leptin treatment of *ob/ob* mice reduces food intake and body mass, and has been suggested to decrease adipogenesis particularly in ob mice compared to PBS treated controls. Fat tissue from 12-day leptin or PBS treated ob animals was isolated and RNA was prepared from the preadpocyte fraction. The

expression levels of KLF4, Krox20, C/EBPβ and C/EBPβ were measured using specific TAQMAN assays. All of these early genes were downregulated by approximately 2-fold in the preadipocyte fraction in 12 day leptin treated animals compared to 12d PBS treated animals (Figure 21).

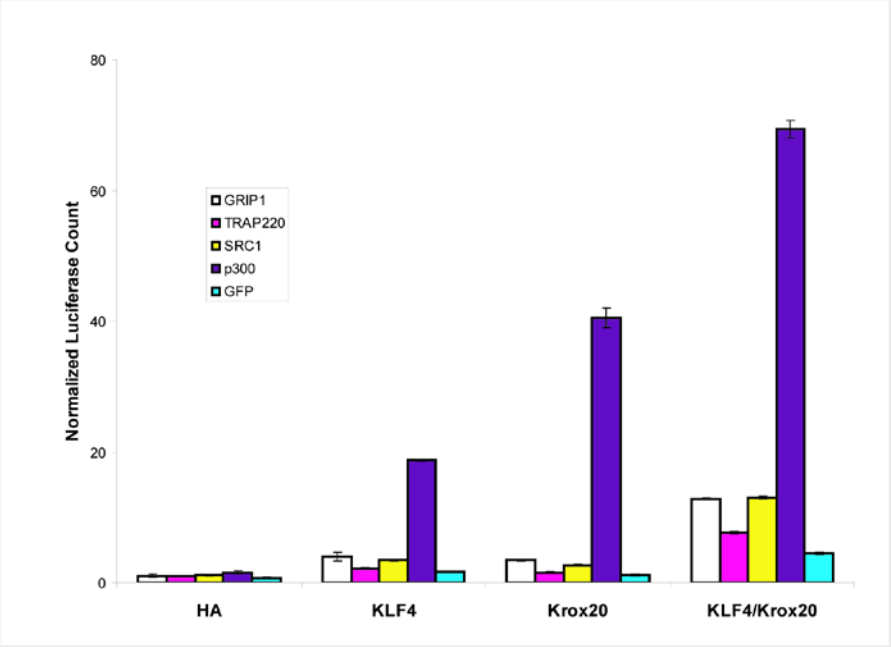

Figure 20: p300 is the predominant coactivator of KLF4 *in vitro*.

The KLF4 and Krox20 plasmids were transfected into 293T cells along with the luciferase reporter carrying 3kb C/EBPβ promoter and a series of coactivators. Results were normalized by Renilla Luciferase.

Discussion

Adipogenesis is controlled by a cascade of transcription factors that act to induce the expression of gene products necessary for the acquisition of the characteristic morphology and specialized functions of adipocytes (Farmer, 2006a). Previously C/EBPβ has been shown to be among the first transcription factors activated during adipogenesis and it is considered to be one of the initiators of a transcriptional cascade, which in turn activates C/EBPβ and

PPARɣ2 (Rosen et al., 2002). These latter genes then activate the genes that mark the fully differentiated adipocyte phenotype. However, the upstream factors regulating C/EBPβ expression have not been characterized.

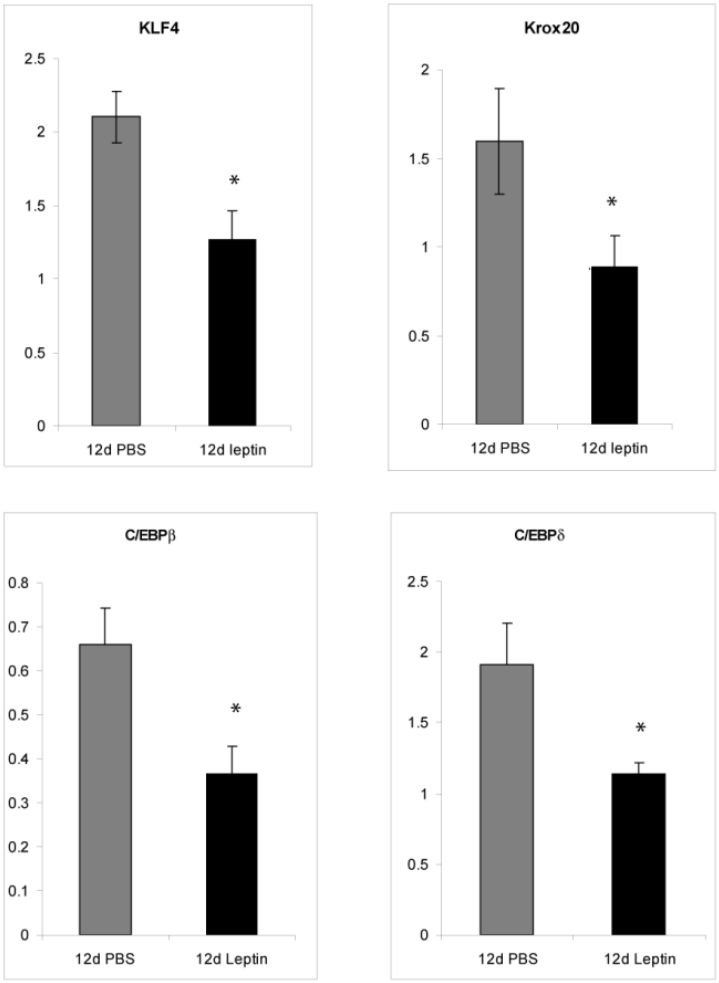

Figure 21: Leptin treatment reduces expression of early genes in stromavascular fraction.

6 ob/ob animals were treated with leptin or PBS. Animals were sacrificed after 12 days leptin/PBS treatment and stromavascular fraction was collected for Taqman assay.

KLF4 gene expression is induced early during adipogenesis in response to IBMX, one of the components of the standard induction cocktail. KLF4 knockdown cell lines showed a marked decrease in adipogenesis as assessed

using Oil Red O and reduced expression of differentiated fat markers such as aP2, adipsin and PPARɣ2. However, the decrease in PPARɣ2 expression seems to be less prominent than for the other two markers suggesting that Klf4 might have PPARɣ2-independent effects. KLF4 knock-down cells also showed reduced expression of C/EBPβ, adding functional evidence to support the conclusion that KLF4 is upstream factor of C/EBPβ in the transcriptional network controlling adipogenesis. Consistent with this, KLF4 is able to transactivate a luciferase reporter driven by 3kb C/EBPβ promoter in a dose dependent manner, and a deletion series further localized the critical region to between −1438 and −1134 base pairs. CHIP assays and gel shift assays confirmed the binding of KLF4 to C/EBPβ promoter at this conserved critical region. KLF4 seems to bind several sites along this region as assessed by footprinting studies. The extensive homology of this region of the C/EBPβ promoter suggests that these promoter sequences are functionally important. Note that, Krox20 could not be shown to bind C/EBPβ promoter in both Chromatin IP assays and gel shift assays. This suggests that Krox20 may be acting on C/EBPβ promoter through KLF4. It is also possible that Krox20 binding to C/EBPβ promoter requires an interaction with other unidentified partners.

Krox20 has previously been shown to activate C/EBPβ promoter in the same promoter region (Chen et al., 2005a). Klf4 and Krox20 trans-activation is additive with KLF4, suggesting that both factors cooperate to activate C/EBPβ expression in adipogenesis. In addition, overexpression of either Krox20 or C/EBPβ seems to bypass KLF4 knockdown phenotype, further supporting the

role of Krox20 and KLF4 to activate C/EBPβ. It was also shown that, in cooperation with p300 these two factors potently transactivate C/EBPβ promoter. Consistent with this, ribozyme-mediated targeting of p300/CBP suppresses differentiation of 3T3-L1 preadipocytes into adipocytes, indicating that p300 is also necessary for adipogenesis (Takahashi et al., 2002). p300 has been previously shown to interact directly with KLF4 by using pull-down assays (Feinberg et al., 2005). These data suggest that p300 might play a role in early phases of adipogenesis via interactions with Krox20 and KLF4 potentially with other proteins to form a protein complex that induces C/EBPβ and coordinates some (though not all, see below) of the key early events during adipogenesis.

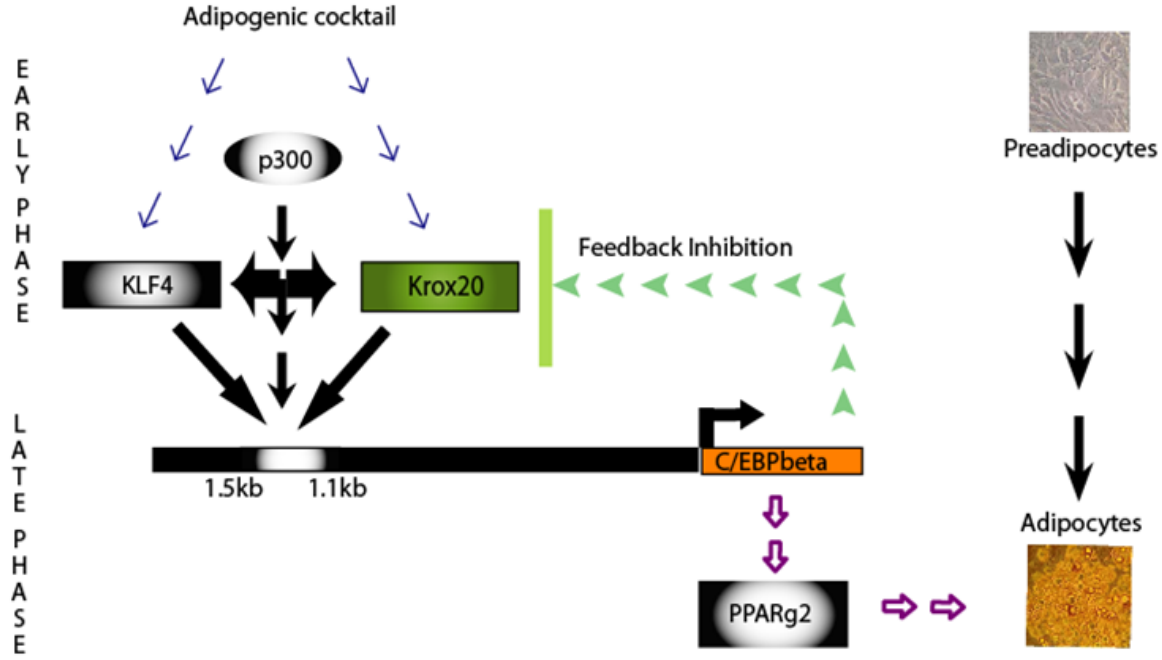

Figure 22: Early transcriptional network in adipogenesis

KLF4, Krox20 and possibly p300 are a part of early transcriptional network acting on the early master regulator of adipogenesis, C/EBPβ. C/EBPβ, then, activates PPARɣ, leading to the transcription of adipogenic genes.

A knockdown of C/EBPβ increases the expression of KLF4 and the overexpression of C/EBPβ reduces KLF4 expression. These data indicate that C/EBPβ acts downstream of KLF4 and reduces its expression by feedback inhibition. Ectopic expression of KLF4 results in cell-cycle arrest in several cell lines including NIH3T3 cells and its transcriptional targets are involved in differentiation and cell-cycle inhibition (Chen et al., 2001; Chen et al., 2003; Shields et al., 1996). Consistent with this, over-expression of KLF4 in 3T3L1 cells leads to slow proliferation even before differentiation and these cells differentiate extremely poorly (data not shown). These results also suggest that the levels of KLF4 must be tightly controlled either quantitatively and/or temporally.

KLF4 is specifically activated by IBMX, an inducer of cAMP, but not by dexamethosone or insulin, the other two components of the adipgenic cocktail. IBMX by itself can partially induce adipocyte differentiation in 3T3L1 cells *in vitro* but IBMX treatment does not fully recapitulate the effects of the entire cocktail (Yeh et al., 1995). This suggests that other factors activated by dexamethosone and insulin, in combination with KLF4, are necessary for the activation of the fully differentiated adipocyte phenotype.

It is not known whether the failure of IBMX to fully activate adipogenesis is the result of a failure to fully induce the quantitative expression of downstream mediators such as C/EBPs or if additional, as yet unidentified factors are required. The identification of these dexamethosone and insulin induced target genes would resolve this issue and advance our understanding of the earliest

events of adipogenesis. It is not known however whether KLF induction by cAMP is a direct effect via PKA phosphorylation of CREB family transcription factors or if another mechanism contributes (Reusch et al., 2000a; Zhang et al., 2004a). The fact that CREB phosphorylation is required for early events in adipogenesis is consistent with this possibility (Reusch et al., 2000a). However, cotransfection of CREB with a KLF4-promoter-luciferase reporter construct in the presence of forskolin, did not show any increase in KLF4 expression (data not shown) suggesting that the effect of CREB on Klf4 expression is likely to be indirect.

The role of KLF4 *in vivo* could be further tested in fat specific knockouts of KLF4 however the interpretation of such data could be complicated by the fact that the expression of KLF4 might not be required at later times in adipogenesis such as when the aP2 promoter is turned such as in aP2-cre or leptin-cre mice (unpublished data) (Jones et al., 2005a). In other words, an aP2 directed knockout of KLF4 would not be informative, if KLF4 is not required for the maintenance of adipose tissue. The low levels of KLF4 in fully differentiated adipocytes are consistent with this possibility. Thus promoters that direct cre expression early in adipogenesis i.e. in preadipocytes is likely to be required to assess the inductive role of KLF4 in tissue specific knockouts. Because such promoters are not known or available, the delineation of a role for KLF4 *in vivo* might require an ES cell complementation strategy analogous to that recently developed to study pancreatic development (Stanger et al., 2007).

In summary, KLF4, here, is identified as an essential player in adipocyte differentiation (Figure 22). These findings put KLF4 among a group of key proadipogenic early transcription factors, including Krox20 and CREB (Reusch et al., 2000a; Zhang et al., 2004a). It seems that there is an earlier transcriptional regulatory network before C/EBPβ expression at least *in vitro*.

CHAPTER 4:

Characterization of Adipogenesis *in vivo*

Introduction:

Obesity can be a result of both hypertrophy, an increase in adipocyte size, and hyperplasia, an increase in cell number. In the previous section, an early adipogenic transcriptional network including Krox20, Klf4 and C/EBPβ was identified. However, those experiments were limited to the *in vitro* adipogenic processes (Fischbach et al., 2004) (Mandrup et al., 1997a). Unfortunately, the information regarding *in vivo* adipogenesis is very limited. On the way to study adipogenesis *in vivo*, a major issue is identifying the progenitor cells that make up the fat cells in adipose tissue.

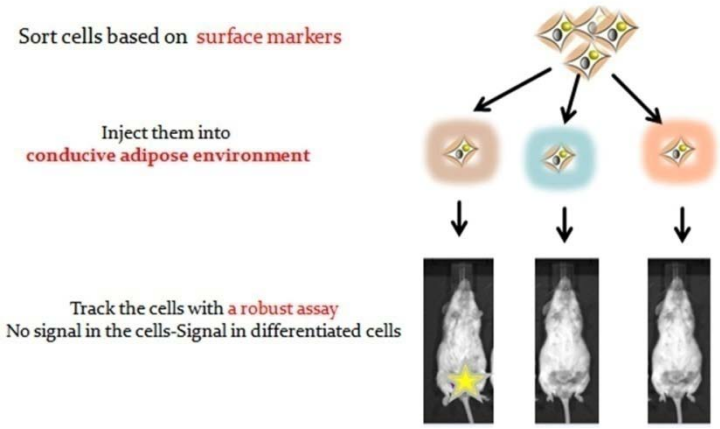

Figure 23 Scheme for identification of adipocyte progenitor cells

SVF fraction is sorted based on several surface markers and injected in a conducive hyperadipogenic environment for growth. Cells can be tracked using an *in vivo* imaging assay without killing the animal.

An experimental scheme for identifying the adipocyte progenitor fraction is as follows. Firstly, stromavascular fraction can be sorted using various cell

markers by fluorescence-activated cell sorting (FACS). Next, these cells can be transplanted into an *in vivo* model, preferably with a conducive adipogenic environment. Lastly, there is a need to monitor the growth of adipocytes from these cells (Figure 23). This scheme is analogous to previous assays employing precursor cell transplantation *in vivo* for the study of hematopoietic, mammary epithelial, and spermatogonial stem cells (Brinster and Zimmermann, 1994; Deome et al., 1959; Ford et al., 1956).

Results

A Lipodystrohic Model contains hyper-adipogenic/proliferative fat pads

Lipodystrophy is a medical condition characterized by complete loss of fat depots and usually associated with hepatomegaly, hyperglycemia, insulin resistance, hyperlipidemia and hypermetabolism (Chinen, 2001; Hegele, 2000). One model of genetic lipodystrophy in rodents is A-ZIP lipodystrophic mouse model. A-Zip lipodystrophic mice have a severe, generalized lack of WAT (Moitra et al., 1998), and retain very small WAT depots at the sites where mature WAT depots typically develop in wild type animals (Figure 24). These mice express a dominant-negative transcription factor (A-Zip) that inhibits C/EBP transcription factors under the control of the aP2 (FABP4) promoter (Moitra et al., 1998). The transgene encodes a peptide, carrying an acidic portion, which binds to the B-ZIP leucine zipper transcription factors stronger than their corresponding

DNA binding site. Thus, this dominant negative peptide blocks the DNA binding of B-ZIP transcription factors such as C/EBPs.

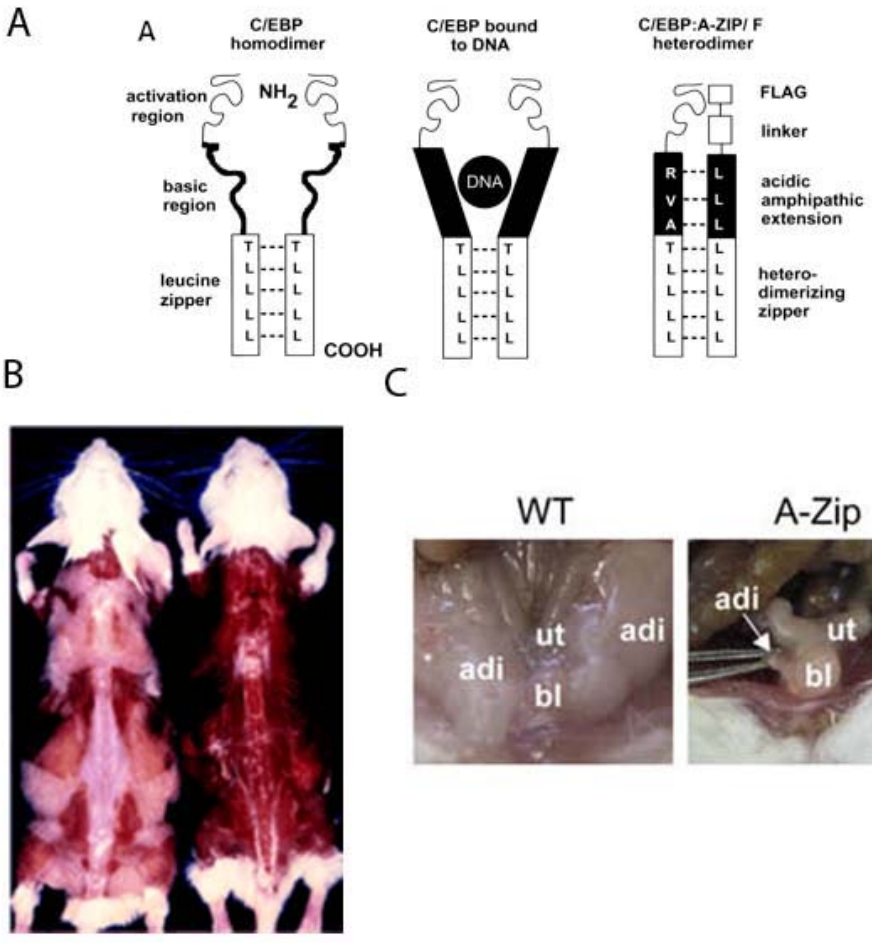

Figure 24 A-ZIP lipodystrophic animals

A) Depiction of the dominant negative transgene of A-ZIP animals. Dominant negative peptide carries an acidic extension which binds to C/EBP factors with high affinity, preventing their binding to DNA. B) Overall morphology of lipodystrophic animals. The animal on the right side is lipodystrophic. Note the lack of any visible fat. C) Ventral view of the lower abdomen of female littermate wild-type (WT) and A-Zip mice at 12 weeks of age. The bladder (bl), uterus and fallopian tubes (ut), and parametrial WAT (adi) are indicated

Since aP2 is expressed relatively late in adipogenesis it was reasoned that there might be an expansion of precursor cells upstream of this block in differentiation caused by the expression of the transgene. It was also possible

that by blocking C/EBP and downstream factors, the negative feedback loop that inhibits KLF4 and Krox20 will not work, resulting in induction of these genes.

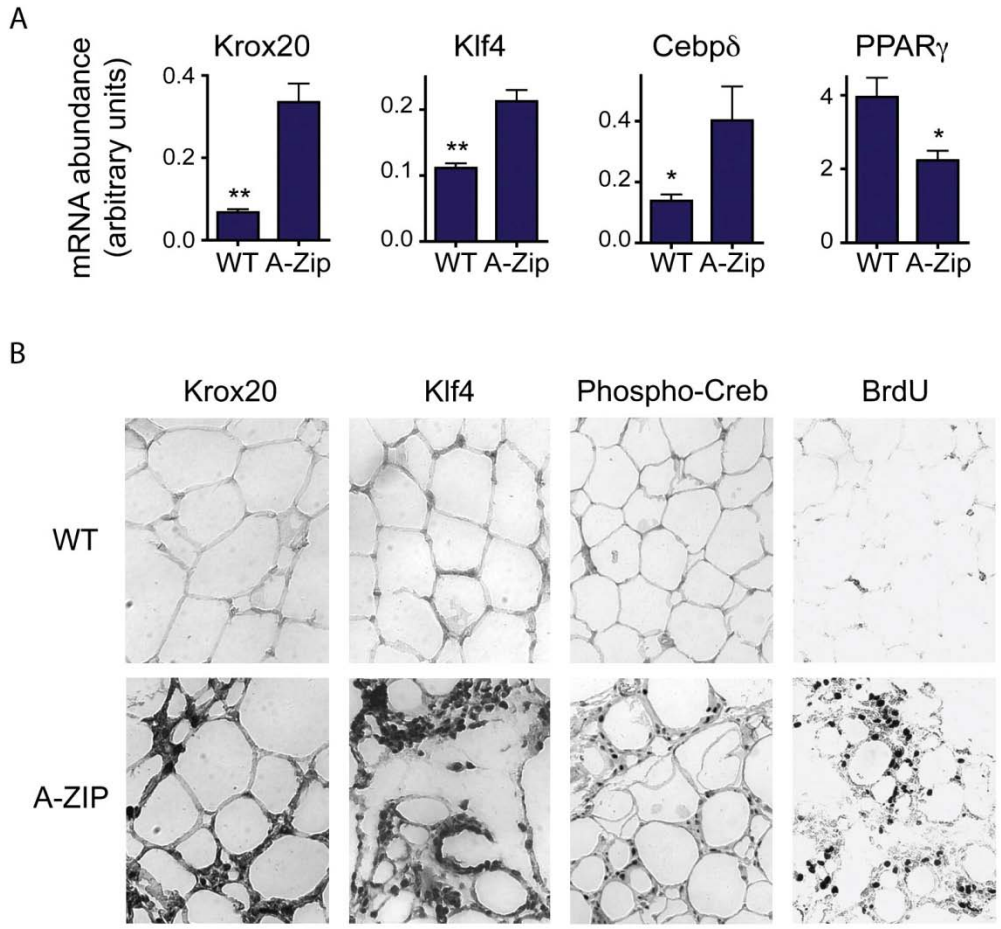

Figure 25: Early genes are upregulated in A-ZIP remnant adipose organ

A) Taqman RT-PCR was performed for the markers of early adipogenesis, Krox20, Klf4, and Cebpδ, as well as PPARɣ. RNA was isolated from adult A-Zip and WT parametrial adipose tissue. Results are shown as mean ± SEM, n = 3 (*, $p < 0.05$; **, $p < 0.001$). B) Immunostaining of parametrial WAT from A-Zip and WT mice for the early markers of adipogenesis Krox20, Klf4, and phospho-CREB, as well as the proliferation marker BrdU, is shown. For BrdU incorporation analysis, mice were treated with BrdU for 7 days prior to immunostaining.

As explained above, it was hypothesized that inhibition of the formation of mature adipocytes in the WAT depots of the A-Zip animals would lead to an increase of proliferation and differentiation of precursor cells upstream of the block in differentiation. If true, cells in the stromal fraction of these depots should

continue to express genes induced early during adipogenesis (upstream of the block induced by the transgene) and continually proliferate in a manner similar to the clonal expansion phase of adipogenesis in preadipocyte cells lines induced to differentiate *in vitro*. To test this, expression of genes typically induced early during adipogenesis were analyzed in the A-Zip WAT depots. It was found that RNAs for early markers of adipogenesis, Klf4, Krox20 and C/EBPδ, were increased in A-Zip WAT depots relative to wild type WAT by 191%, 498% and 291%, respectively, while the expression of PPARγ, a marker of mature adipocytes, was decreased to 56% of the levels in wild type tissue (Figure 25A). Consistent with this, immunohistochemistry for Krox20 and Klf4 indicated very low expression of these factors at the protein level in wild type WAT with a significant increase in the numbers of Klf4 and Krox20 positive cells in A-Zip WAT (Figure 25B). In lipodystrophic WAT, there was also a similar increase in the level of CREB phosphorylation, one of the earliest events observed during adipogenesis *in vitro* (Zhang et al., 2004a) (Figure 25B).

Above data suggests that A-ZIP adipose is hyperadipogenic. Since preadipocytes proliferate through clonal expansion *in vitro*, proliferation was tested in lipodystrophic WAT. Briefly, A-Zip mice were treated with BrdU for seven days and fat tissue sections were analyzed by immunohistochemistry. Analysis of BrdU incorporation showed that A-Zip adipose tissue contains large numbers of proliferating cells compared to wild type adipose in which there are barely detectable levels of BrdU incorporation, as expected due to its quiescent

state (Figure 25B). The percent of BrdU positive cells was significantly higher in lipodystrophic fat pads compared to wild type fat pads (Data not shown).

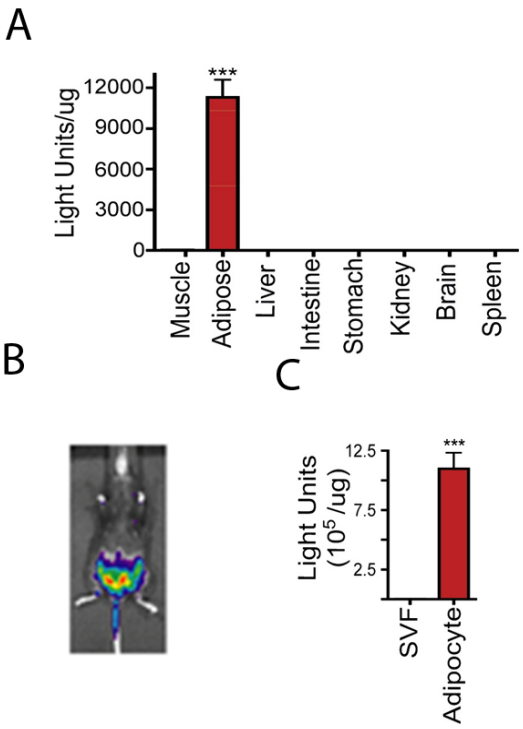

Figure 26: Leptin-luciferase BAC transgenic animals

Luciferase activity in tissues (A) and fractionated WAT isolated from *leptin-luciferase* mice.(C) Luciferase can also be detected in the animals, using a CCD camera.(B) The indicated tissues were isolated, and luciferase activity was determined. Data shown are normalized to total protein in each lysate. Results are shown as mean ± SEM; ***, $p < 0.001$ (n = 3).

These results, all together, suggest that A/ZIP mice can provide a conducive environment for the injection of candidate progenitor cell fractions, due to its hyperadipogenic/hyperproliferative environment.

A luciferase transgenic model to study adipogenesis *in vivo*

In order to follow the fate of adipose tissue under different conditions, a transgenic animal, in which luciferase is expressed under the control of leptin gene regulatory sequences, was generated. Leptin is highly enriched in adipose tissue and luciferase was chosen as the reporter since it can be quantified *in vitro* biochemically and *in vivo* by imaging anesthetized animals using a CCD camera. *In vivo* imaging allows one to monitor luciferase levels in the same animal over time, without having to sacrifice animals.

Leptin-luciferase transgenic animals were generated using BAC number RP24-69D4 from UCSC database. This BAC contains 22 kb of 5' sequence and 160 kb of 3' sequence relative to the leptin transcription start site. The firefly luciferase-polyA (Promega) was inserted into the ATG site of the leptin BAC via homologous recombination and transgenic mice were generated by pronuclear injection of the modified BAC. All subsequent studies use the D7-1 line, which has nine copies of the modified BAC incorporated as determined by real time PCR analysis (data not shown); similar data were obtained for the other line.

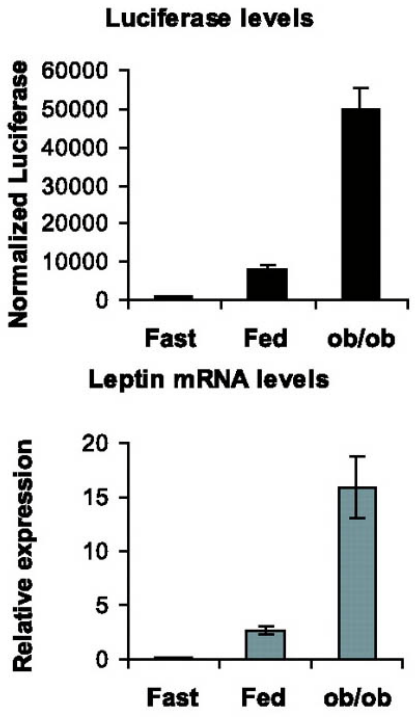

Figure 27: Luciferase regulation recapitulates leptin regulation in leptin-luciferase animals

Luciferase activity recapitulates regulation of leptin. The relative expression of luciferase versus leptin was compared among fed, fasted, and ob/ob animals. (*Lower*) Endogenous leptin expression levels from fasted, fed, and ob/ob animals were measured by Taqman real time PCR normalized to cyclophilin levels. (*Upper*) Luciferase activity of adipose tissue lysates from fed, fasted, and ob/ob animals normalized to protein content (mean ± SEM).

Tissue specific expression of luciferase in leptin-luciferase transgenic animals was confirmed using biochemical assays from lysed tissues and *in vivo* imaging. Luciferase activity, normalized to the amount of total protein, was present specifically in adipose tissue at high levels with undetectable levels in other tissues, other than intestine

(Figure 26A and B). It is not clear if the small signal of luciferase expressed in this tissue is in epithelial cells or in resident adipocytes.

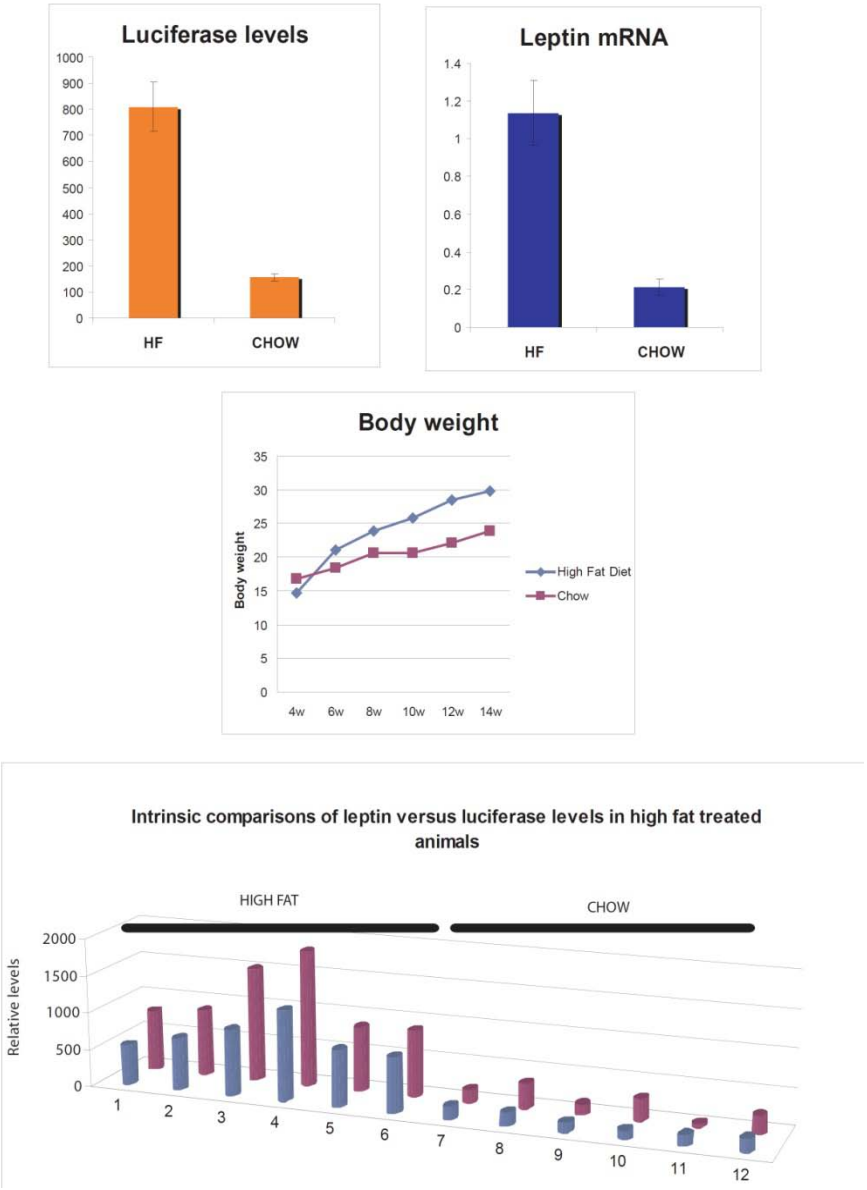

Figure 28: Regulation of luciferase upon high fat feeding in leptin-luciferase animals

Luciferase and leptin mRNA levels of leptin-luciferse animals fed on a high-fat diet versus normal diet. (Upper Panel) Animal-to-animal comparisons of leptin mRNA versus luciferase levels in high fat treated animals (Lower Panel)

To confirm that the leptin-luciferase transgene was correctly regulated quantitatively, it was next tested whether luciferase expression was correlated with leptin mRNA levels in obese and lean animals. Leptin levels are highly correlated with cellular lipid content and its mRNA levels can vary hundreds of fold among fasted vs. obese animals (Maffei et al., 1995a). Relative expression of luciferase vs. leptin was compared among fed, fasted and *ob*/*ob* visceral fat pads. There was a hundred fold greater level of luciferase activity in extracts from *ob*/*ob* adipose tissue vs. extracts from fasted animals, which fully recapitulated the mRNA levels for endogenous leptin expression (Figure 27). These results were also confirmed *in vivo* using a CCD camera to monitor luciferase activity. (see Regulation of leptin gene expression chapter) Another evidence for correlation between luciferase levels and leptin mRNA levels came from high-fat feeding experiments. Fold induction of leptin gene expression in high-fat fed leptin-luciferase animals versus chow-diet fed animals was highly similar to that of luciferase levels. In addition, leptin mRNA correlated tightly with luciferase levels within subjects, ie. fatter animals having higher luciferase levels (Figure 28). Same fold inductions have been observed also in luciferase mRNA levels, which suggest that luciferase expression in the leptin-luciferase mice depends mostly upon transcriptional regulation (Data not shown).

It should be noted that SVF fraction does not express leptin. Preadipocytes express leptin when differentiated to adipocytes. For this reason, use of leptin-luciferase animals provides a robust tool to track possible adipocyte progenitor cells and differentiation ability into mature adipocytes. Since leptin

correlates very well with the amount of lipid stored, the signal increases over time as the differentiated adipocyte accumulate more and more lipid (Figure 26C).

Isolation of adipocyte progenitor cell using markers

WAT is composed of multiple cell types including adipocytes, vascular cells and populations of fibroblast like cells that have not been well characterized (Cinti, 2005). To identify potential adipocyte progenitor cells, the stromal-vascular fraction (SVF) of wild type WAT was isolated (Rodbell, 1964) and FACS was performed to isolate distinct cell populations based on the differential expression of well characterized cell surface markers expressed by stem cell populations in other tissues. The cell sorting strategy that was used in these studies is shown (Figure 29) and described below. Briefly lineage markers (Lin) and CD34, CD24 and Sca1 are used to isolate the progenitor cell fractions. Among these markers, CD34 was previously suggested to be enriched in adipocyte progenitor cells (Miranville et al., 2004). CD24 and Sca1 were both implicated to have roles in stem cell biology as shown by previous studies (Lian et al., 2007; Okada et al., 1992; Rao et al., 1995; van de Rijn et al., 1989).

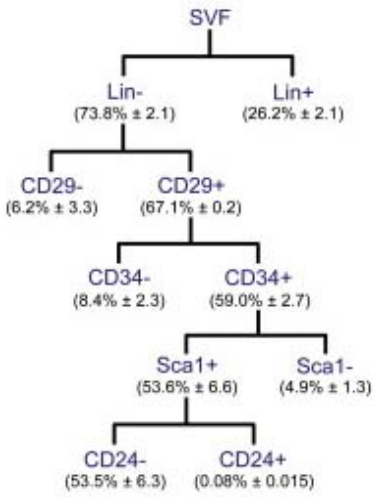

Figure 29: Scheme for FACS analysis of SV fraction

The scheme for the FACS analysis is shown as a dendrogram of the hierarchy developed for cell sorting. The percentage yield of each population compared to the starting number live single cells derived from the SVF is indicated.

The FACS strategy was used to enrich for adipocyte precursors that were fully competent for adipogenesis *in vitro*. First, endothelial cells and hematopoietic cells were depleted from the SVF based on staining of CD31, CD45, and Ter119, generating a Lineage negative (Lin-) population. Second, the cell populations depleted of the lineage markers were separated based on staining for CD34 and CD29, as described above. The Lin-:CD34+:CD29+ subpopulation was further separated based on staining for stem cell antigen 1 (Sca-1) and CD24 (Shackleton et al., 2006; Spangrude et al., 1988; Wilson et al., 2007).

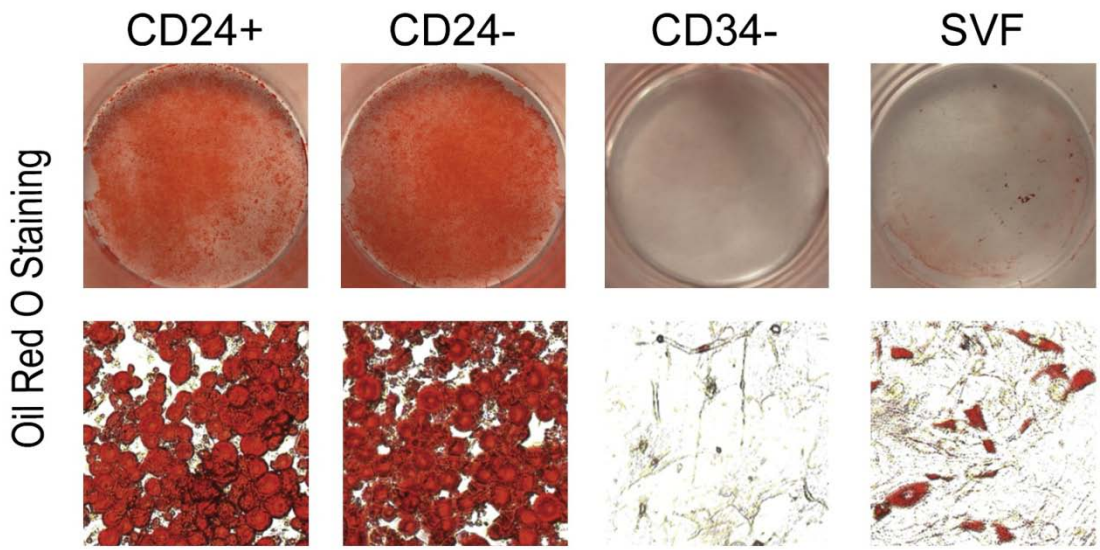

Figure 30: *In vitro* differentiation of adipocyte progenitor fractions

The sorted $CD24^{+}$, $CD24^{-}$, and $CD34^{-}$ cells and unsorted SVF were cultured and induced to differentiate with IBMX, glucocorticoid, and insulin. The cells were then cultured for 12 days prior to staining with oil red O. Macroscopic (upper panel) and microscopic (lower panel) views of the oil red O-stained dishes are shown (upper panel).

The Lin-:CD29+:CD34+:Sca-1+ population comprises approximately half of the cells present in the SVF (53.5%), but only a small population of cells also

expressed CD24 with the total number of Lin-:CD29+:CD34+:Sca-1+:CD24+ (CD24+) cells constituting in total ~0.08% of the cells in the SVF (Figure 29).

As the first step, these cell populations were tested based on their differentiation ability *in vitro*. CD24+, Lin-:CD29+:CD34+:Sca-1+:CD24- (CD24-) and Lin-:CD29+:CD34- (CD34-) subpopulations can differentiate into adipocytes *in vitro* using the standard protocol. After exposure to an adipogenic cocktail *in vitro* both populations, CD24+ and CD24-, developed into adipocytes with large, unilocular lipid droplets that stain with Oil Red O (Figure 30). Similar differentiation levels were found for both the CD24+ and CD24- subpopulations when insulin alone was used for *in vitro* differentiation (Data not shown). Note that while both CD24- and CD24+ populations could differentiate into adipocytes, the CD34- cells did not differentiate *in vitro*. These data showed that the FACS strategy that was employed had indeed enriched for adipocyte precursor cells.

In vivo adipogenic ability of different SVF fractions

Previous data suggested that the adipose tissue of lipodystrophic animals may provide a microenvironment that is conducive to support the proliferation and survival of preadipogenic cells. Furthermore, leptin-luciferase animals provides the tool to monitor the ability of the $CD24^{+}$ cells to develop into adipocytes, since the expression of luciferase in these mice is restricted to mature adipocytes within adipose tissue. Luciferase activity can be followed noninvasively *in vivo* by imaging with a CCD camera. Since cells of the SVF do not express the luciferase reporter, transplant of donor cells from the *leptin-*

luciferase BAC transgenic mice allows to follow the development of mature adipocytes (as marked by luciferase activity) from the donor cells noninvasively *in vivo*.

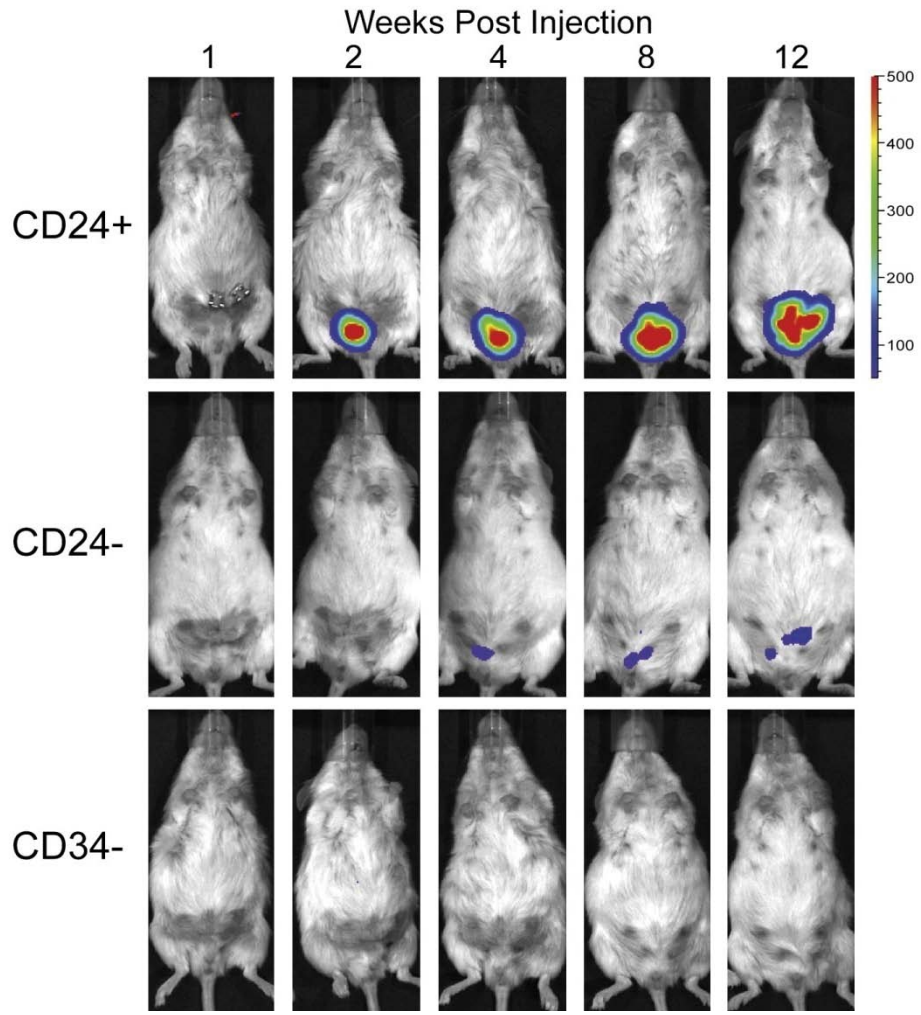

Figure 31: CD24+ cells reconstitute adipose tissue in lipodystrophic animals

Luciferase imaging of adipocyte differentiation in the A-Zip transplantation model at indicated time points after injection of 50,000 donor cells from the $CD24^{+}$, $CD24^{-}$, or $CD34^{-}$ cell populations.

SVF cells from *leptin-luciferase* mice were isolated, FACS sorted and injected in the fat depots of A-Zip mice. Mice injected with the $CD24^{+}$ cell population derived from *leptin-luciferase* animals expressed luciferase in the

injected depot beginning at 2 weeks after injection with the expression of luciferase gradually increasing through the end of the 12 week time course. In contrast, injection of other cells did not yield high luciferase expression levels. This result suggested that the $CD24^-$ subpopulation is capable of adipogenesis *in vivo* but that the differentiation or proliferative potential of these cells is limited (Figure 31).

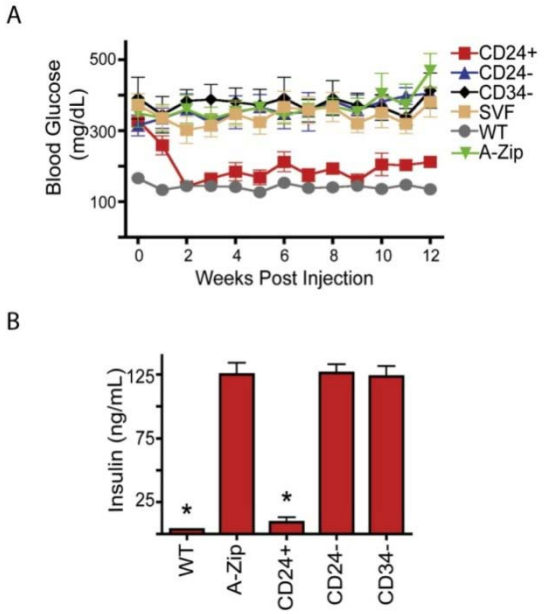

Figure 32: Injection of CD24+ fraction corrects hyperglycemia and hyperinsulinemia in lipodystrophic animals

A) Plasma (glucose) from A-Zip mice injected with the $CD24^+$, $CD24^-$, $CD34^-$, or SVF cells, as well as WT and A-Zip controls. Results are shown as mean ± SEM. Correction of blood glucose by the $CD24^+$ population is significant ($p < 0.05$) at week 2 and remains significant through the 12 week experimental endpoint ($n = 5$). Results are shown as mean ± SEM.

B) The plasma (insulin) at the 12-week endpoint of A-Zip mice that were injected with the $CD24^+$, $CD24^-$, or $CD34^-$ cell populations and noninjected wild-type and A-Zip controls is shown. Results are shown as mean ± SEM; *, $p < 0.05$ ($n = 5$).

A-ZIP lipodystrophic animals are extremely hyperinsulinemic and hyperglycemic, which can be ameliorated by leptin treatment or adipose transplantation. If the cells are differentiating into functional adipocytes, the expectation would be that CD24+ injected animals will show amelioration of high glucose and insulin levels. For this reason, plasma glucose and insulin levels in A-Zip mice injected with the $CD24^+$, $CD24^-$, or $CD34^-$ cell populations were compared to noninjected A-Zip and wild-type control animals. Blood glucose was normalized after injection of the $CD24^+$ population into the A-Zip animals in those animals where an adipose depot had formed. None of the other cell populations showed this capacity (Figure 32). The decrease of blood glucose levels was significant within 2 weeks of injection and was sustained for 12 weeks. Plasma insulin levels also were normalized in these $CD24^+$-injected animals (Figure 32). These data indicate that the $CD24^+$ cells generate functional WAT when injected into the WAT depot of A-Zip mice.

Discussion

This section introduces a novel way of identification of the unknown adipocyte progenitor cell population *in vivo*. Through FACS analysis, these studies demonstrate that primary cells from the adipose tissue stromal fraction can proliferate and differentiate into functional, mature adipocytes to reconstitute a fully functional adipose depot *in vivo*, which is enough to ameliorate lipodystrophic phenotype.

One critical aspect of the study is the identification of A-Zip lipodystrophic mice as hyperadipogenic/hyperproliferative model. WAT of A-Zip animals showed increased expression of early markers of adipocyte differentiation and high rates of proliferation, suggesting that presumptive WAT provides a niche conducive for promoting early events in adipogenesis. Indeed, CD24+ cells, when injected into wild type adipose depots, do not give rise to new adipocytes, which can be explained by the quiescence of the adult adipose depots (data not shown) (Greenwood and Hirsch, 1974; Greenwood et al., 1974). In this regard, A-ZIPs provide as the injection environment for identification of the CD24+ progenitor cells, making them a rare useful model for same type of studies.

Another important implication of this work is that CD24+ cells can produce metabolically functional adipocytes, secreting the adipokines to ameliorate lipodystrophic phenotype. It has previously been shown that the transplant of normal adult WAT into A-Zip mice is required for correction of their insulin resistance and hyperglycemia (Gavrilova et al., 2000). This also sets a preliminary model for treatment of lipodystrophy. The same type of cells, if they exist in humans, can be used as a stable source of fat cells in lipodystrophic patients, providing a constant source of leptin and other adipokines.

The identification of an adipocyte precursor cell population provides a means for identifying tissue factors in wild-type and lipodystrophic WAT that regulate adipocyte proliferation and differentiation. Furthermore, the availability of the *leptin-luciferase* mice as donors in the A-Zip transplant assay, which allows

the noninvasive monitoring of adipogenesis *in vivo*, should facilitate the identification of conditions that optimize engraftment of adipose tissue precursors.

CHAPTER 5:

Embryonic Development of Adipose Tissue

Up to this date, embryonic development of adipose tissue in rodents has not been fully characterized. One caveat to study adipose tissue development is that the adipocyte and lipid markers are thought to be expressed after the accumulation of lipids. However, it is very likely that the adipocyte character and commitment occurs before the accumulation of lipids. In this regard, lipid markers cannot be used to localize where adipose tissue develops. In this section, location of adipose tissue is determined using a leptin-luciferase transgenic animal (Birsoy et al., 2008). Identification of location of the developing adipose tissue helped to study the gene expression and morphology of these tissues.

Results

Location and Morphology

In order to study the embryonic development of adipose tissue, the first step is finding the location of adipose tissue development. As explained above, the location cannot be determined using lipid markers. In parallel, early markers of adipogenesis usually have significant roles in other cell differentiation processes, so commonly expressed in other places. For instance C/EBPs have roles in hematopoiesis (Jones et al., 2002). Many KLF factors have roles in epithelial cell differentiation (Katz et al., 2002; Swamynathan et al., 2007; Zheng et al., 2009). In addition, there is almost no distinct morphology for “lipidless” embryonic adipose to the naked eye.

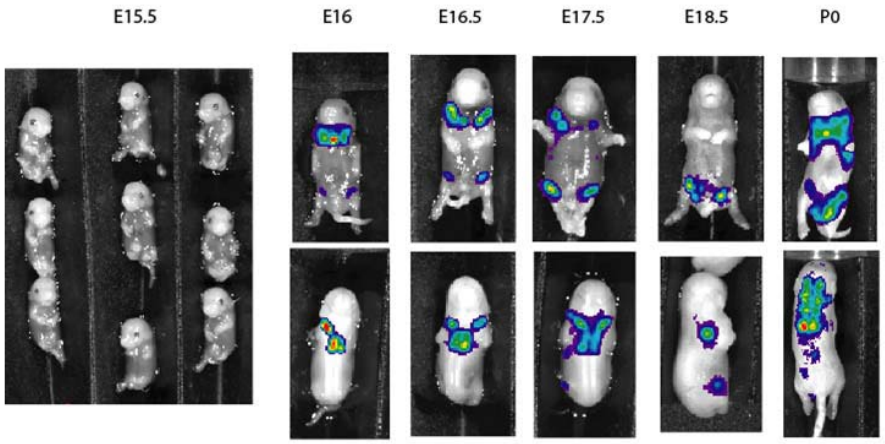

Figure 33: Embryonic development of adipose tissue

Leptin-luciferase animals were sacrificed at the indicated embryonic time points. Embryos were imaged for assessing the location of possible adipose tissue structures.

The tool to study adipose tissue development came from a leptin luciferase animal (see previous section) (Figure 26), where luciferase is expressed in context of leptin regulatory sequences. Since leptin expression is highly fat specific, any luciferase signal during embryogenesis is also thought to be fat specific. Indeed, during development, luciferase signal can be detected in leptin-luciferase mouse embryos (Figure 33). The signal is extremely low and the animals should be exposed 10 minutes as opposed to 30 second exposure of an adult leptin-luciferase animal. However, even this low level expression is enough to detect the exact localization of the possible adipose tissue development sites. Luciferase can be detected in E16.0 embryos and no E15.5 embryo showed any detectable luciferase signal. The source of the luciferase signal emanates from the subcutaneous areas as well as interscapular areas, consistent with the adult adipose locations (Figure 33). One advantage of finding the locations of

developing adipose tissue sites is that these sites can now be dissected and the morphology and gene expression patterns be studied.

Microscopic analysis of AT development between E16.5-P0

Since the location of developing adipose tissue sites can be dissected, next these small tissue fragments were analyzed by using whole-mount technique under confocal microscopy. Vascularization, lipid accumulation and overall morphology have been checked using different dyes as explained below.

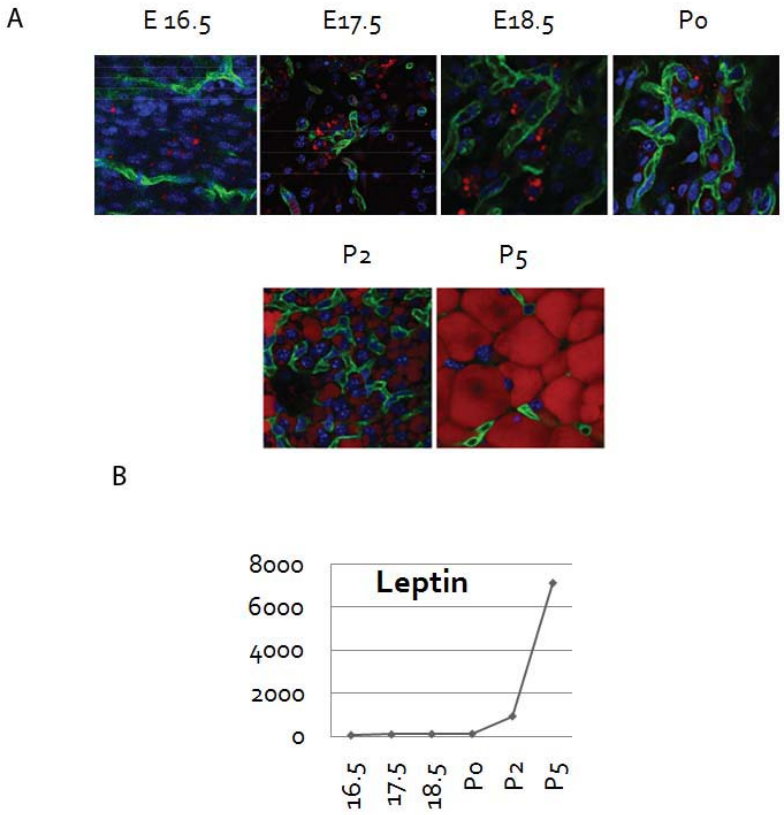

Figure 34: Morphology of adipose tissue during development

Figure shows the fat accumulation and vascular structure of adipose tissue during development. Tissues were dissected using dissection microscope. Green shows the vasculature, red shows lipid and blue shows the nuclei staining. (A) Panel B shows the real time PCR analysis of leptin through embryonic development. Note that the lipid accumulation is very correlative with the leptin expression. Also note that lipid starts to accumulate after birth.

The results show that lipid accumulation does not happen until after birth. Just after birth, lipid starts to accumulate very rapidly in the adipocytes as assessed by lipid-tox staining at P0. Real-Time PCR analysis of leptin shows that big increases for leptin expression occur rapidly after birth around P2-P5, which is consistent with the fact that lipid accumulation is highly correlated with the expression of leptin (Figure 34). In addition, note that, real time PCR analysis shows extremely low leptin expression levels before birth, which suggests that the CCD camera system is very sensitive to luciferase signal detection.

Gene expression analysis of adipose tissue development

Embryonic adipose tissue fragments can be dissected and analyzed as explained above. As a part of the characterization, developing adipose fragments were digested by collagenase and sorted in order to analyze the percentage of different adipocyte populations. Of importance, P1 (CD24+,CD34+) population showed significant change during development. Preliminary results show that this population, which was identified to be the adipocyte progenitor cell population, increases around E18.5 just before birth (data not shown).

Microarray analysis was also performed on the developing subcutaneus adipose tissue. Figure 35 shows the change in early genes and late genes during development of adipose tissue. Note that, compared to 3T3-L1 differentiation, this data is *in vivo* and leptin expression can be detected at high levels. Interestingly, late genes such as PPARγ, CEBPa, FABP4, Adipsin and Adiponectin were all found to be expressed at their maximum level around E18.5,

before the start of lipid accumulation. On the other hand lipid accumulation seems to be very consistent with the leptin expression levels (Figure 35). Early genes such as KLF4, Pref1 and KLF5 are downregulated starting E16.5 to P0, suggesting that early adipogenic processes along with the clonal expansion is mostly completed before birth.

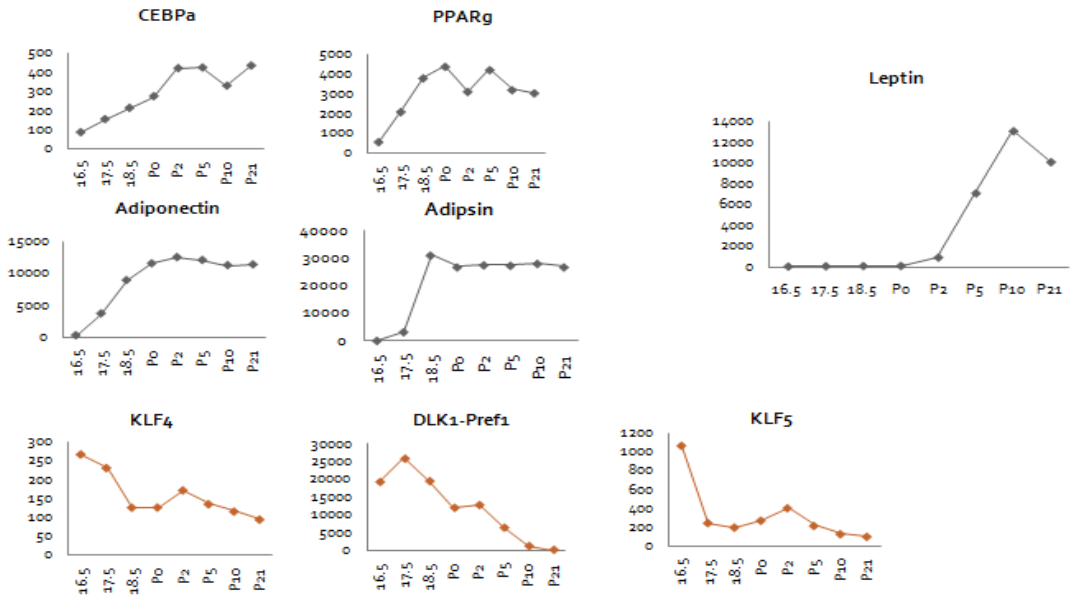

Figure 35: Gene Expression Analysis of Developmental Time course

Late genes such as PPARɣ, CEBPa, Adiponectin and Adipsin reach their maximum levels around P0. On the other hand leptin levels plummet with lipid accumulation after birth. Many early genes were found to be downregulated through development.

Discussion

In summary, above data provides the first thorough characterization of embryonic development of adipose organ in rodents. This data suggests that *in vivo* adipose tissue development may be different than *in vitro* adipocyte differentiation in terms of genes and their temporal expression patterns.

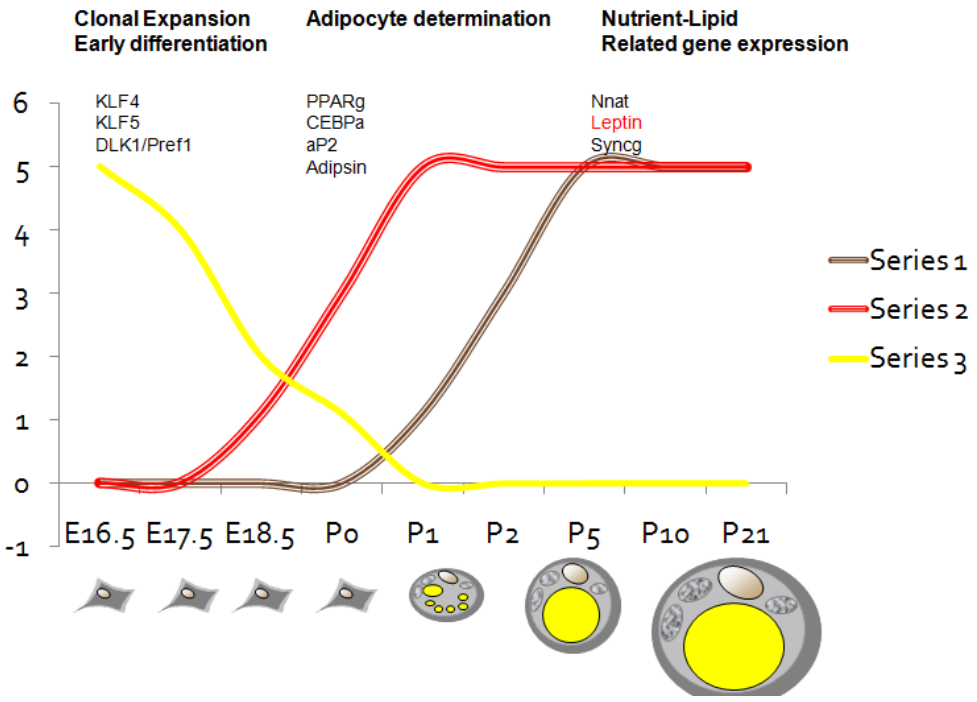

Figure 36: Distinct transcriptional networks regulating adipocyte differentiation *in vivo*

During development, luciferase signal can be detected in three major locations; posterior subcutaneous, anterior subcutaneous (neck) and interscapular region. Subcutaneous regions are consistent with the localization of white adipose tissue in adults. However, interscapular signals suggest that these signals may be coming from developing brown adipose tissue. One caveat of identifying if the signal is coming from brown or white adipose tissue is that, brown adipose tissue contains white adipocytes. Furthermore, interscapular region contains a separate white adipose tissue located laterally to the brown adipose tissue. This suggests that close proximity and heterogenous nature of brown fat may result in the luciferase signal in the interscapular region. In addition, since leptin is a signal for energy stores, leptin's presence in brown adipocytes seem physiologically irrelevant. However, it is

possible that brown adipocytes may express low amounts of leptin due to the presence of lipid, as suggested by previous reports (Cinti et al., 1997; Oliver et al., 2000).

Our data suggest that there are several distinct networks of genes that are expressed during fat cell differentiation. One group includes the genes that are upregulated during embryogenesis till the birth of the animal (Figure 36). This group of genes contains adipocyte-related transcription factors such as PPARɣ, C/EBPα and also secreted factors such as adiponectin and adipsin. The second group of genes is upregulated with the accumulation of lipid. These genes include leptin and other mostly uncharacterized genes. An important implication of this analysis is that genes that are previously thought to be associated with the adipocyte phenotype based on *in vitro* studies seem to be regulated independently of the accumulation of lipid. On the other hand genes in the latter cluster, including but not limited to leptin, are expressed correlative with the lipid accumulation. Clustering analysis of genes in this microarray data may lead to the identification of novel genes/pathways having roles in adipocyte function and development *in vivo*.

CHAPTER 6:

Regulation of Leptin gene expression

In mammals, homeostatic control of adipose tissue mass is regulated by leptin, which functions as the afferent signal in a negative feedback loop to maintain constancy of body fat content. Leptin is secreted in proportion to adipose tissue mass and in turn regulates food intake and energy expenditure. A fuller understanding of the elements of this system could have important implications for the patho-physiology and treatment of obesity. Adipocytes secrete leptin in proportion to their size meaning that as they accumulate more lipids, they secrete more leptin (Figure 37). This suggests the possibility that cellular lipid content is sensed and that a fuller understanding of the mechanisms controlling leptin gene expression could lead to the delineation of a lipid sensing mechanism in fat cells and possibly other cell types. To discover this mechanism, there is need to search for the region in the leptin gene promoter that controls the quantitative expression of the leptin gene; ie the elements that confer increased leptin expression with obesity and decreased expression after weight loss (see below). Since leptin is expressed at much higher levels in fat tissue *in vivo* than *in vitro* where it is not regulated in proportion to cellular lipid content, these studies require that gene expression be

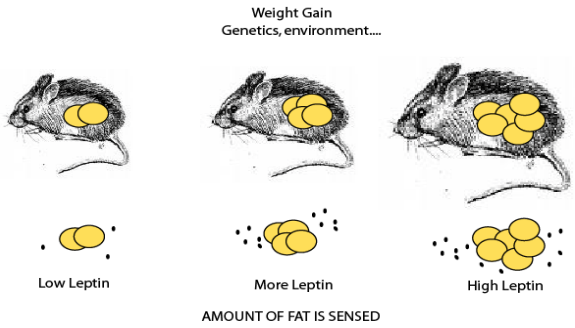

Figure 37: Amount of fat is sensed by adipocytes.

monitored *in vivo*. This part of the thesis deals with the regulation of leptin gene expression via a BAC transgenics approach.

Results

Leptin expression can be studied using a BAC transgenic model

The leptin gene is relatively unusual because it is expressed at much higher levels *in vivo* vs. *in vitro* in differentiated 3T3-L1 or F442 cells(Mandrup et al., 1997b). Since these cell lines abundantly express the master regulator PPARɣ, CEBPs, and their targets, this suggests that the adipocyte specific expression of leptin is controlled by a different transcriptional mechanism. Thus the delineation of the basis for the tissue specific expression of the leptin gene could reveal an *in vivo* specific developmental program. The fact that leptin is only expressed at high levels *in vivo* has necessitated that the leptin promoter be studied using transgenic mice. In order to confer all the regulatory regions, BAC transgenic mouse lines that express luciferase under the control of overlapping leptin regulatory sequences were generated.

Luciferase expression can be quantified *in vitro* as well as *in vivo* using a CCD camera (Greer and Szalay, 2002b). Thus, DNA sequences responsible for these regulatory processes can be generated by generating a series of promoter deletions and then monitoring luciferase expression 1) in different tissues, 2) in ob and fasted mice, 3) during development and 4) in response to hormones and drugs. It should be noted that same DNA sequences may be responsible for some or all of the different transcriptional responses the leptin gene exhibits.

The net result of these studies will be to localize one or more DNA sites responsible for conferring an ability of the leptin promoter to respond to intrinsic and extrinsic signals.

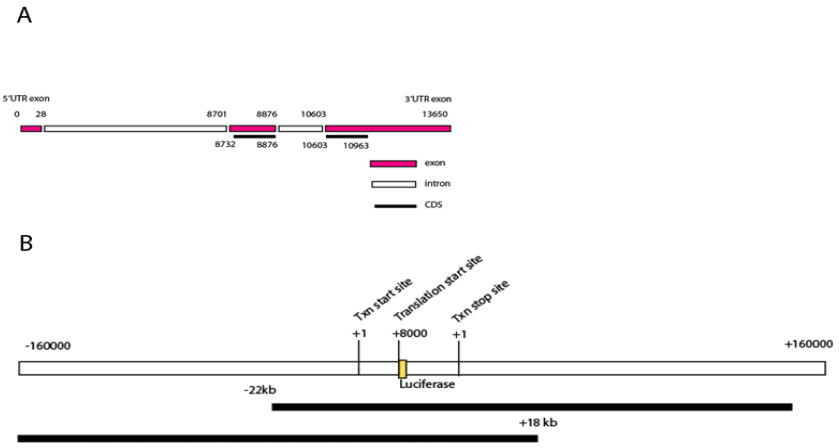

Figure 38: Leptin gene structure

Leptin regulatory elements are in -22kb to -18kb region

Gene regulatory elements are often found at a great distance from transcription initiation sites (Chandler et al., 2007; Choi and Engel, 1986a; Murisier et al., 2006). For this reason and because there is no prior knowledge of the sites of the gene regulatory elements that control leptin expression *in vivo*, we began by making BAC transgenic mice in which the luciferase was inserted into the second exon of the leptin gene at the site of the ATG initiation codon (Figure 38A). Two overlapping BACs were modified in this manner using homologous recombination in bacteria as previously described (Gong et al., 2002). The first BAC extended from –22 to + 140 kB and the second extended from –160 kB to + 18 kB (Figure 38B).

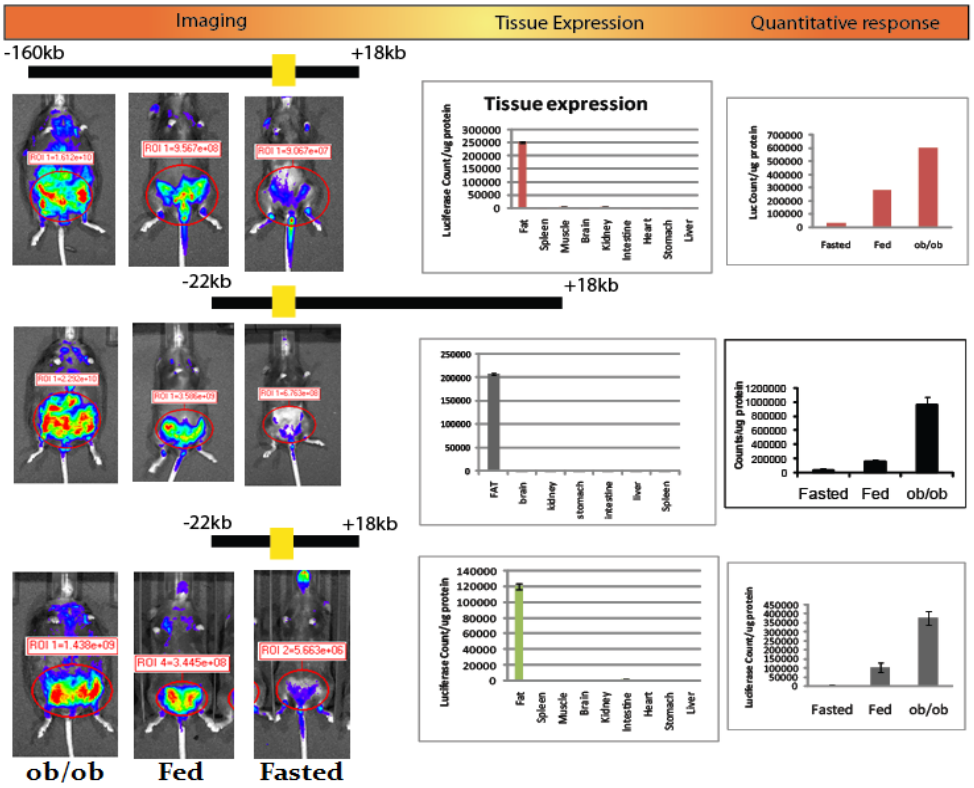

Figure 39: -22kb - +18kb construct is enough for fat-tissue specificity and fat-sensing regulation of leptin

Images on the left side show the luciferase activity of the corresponding leptin-luciferase BAC transgenic line at different metabolic conditions. Each line was mated to ob animals to get obese luciferase animals or fasted. Imaging was done using IVIS lumina CCD camera using ob, fed and fasted mice.

Middle panel shows tissue expression of luciferase of the corresponding leptin-luciferase BAC transgenic line. Briefly, each tissue was dissected and lyzed and luciferase activity was asssesed using a luminometer.

Right panel shows the fat sensing regulation of the corresponding line upon fasting and obesity. Fat tissue was dissected from 5 animals for fasted, obese and fed group and luciferase activity was measured in fat using a luminometer, normalized by protein concentration. This result is consistent with the imaging experiments. (Left Panel)

These BACs were injected into fertilized eggs from C57BL/6J mice and founder mice were bred to generate F1 animals. The animals generated using the BAC that extended from –22 to +140 kB were characterized first. Protein extracts were made from a variety of tissues and analyzed for luciferase activity

in the presence of luciferin the standard substrate. These studies showed very high luciferase levels in adipose tissue with undetectable levels in the other tissues that were tested. In this and subsequent experiments, the luciferase activity was related to the amount of total protein (Figure 39).

The relative expression of luciferase vs. leptin was also compared among fed, fasted and ob mice. The latter were made by mating the Leptin-Luciferase BAC transgenes to ob/+ animals followed by intercrossing. In this case, there was a several hundred fold greater level of leptin RNA and luciferase in the ob vs. fasted animals (Figure 39).

The second set of transgenic animals made using the BAC that extended from –160 kB to + 18 kB also gave the same results. These animals also show fat specific luciferase expression with a marked decrease after fasting and a marked increase when the animal is mated to ob/ob animals. Since both BACs show coordinate regulation of leptin and luciferase over the same dynamic range these data narrow the sequences required for fat specific and quantitative regulation to between –22 kB and +18 kB of the gene. A -22kb-18kb animal was also made to ascertain the region. Indeed this transgenic animal showed consistent results with the previous animals (Figure 39).

Luciferase can also be quantified *in vivo* by imaging anesthetized animals using a CCD camera(Greer and Szalay, 2002a; Welsh and Kay, 2005). *In vivo* imaging allows one to follow luciferase levels in the same animal over time (without having to sacrifice mice) and also provides the basis for experiments to

follow the fate of adipocyte stem cells as explained before. To monitor *in vivo* luciferase activity, animals were anesthetized with isoflurane, and injected with 15 mg/ml D-Luciferin (Xenogen, Alameda, CA) i.p. Luciferase activity was imaged using the IVIS Lumina machine (Xenogen) and the data were analyzed using the Living Image software (Xenogen)(Franke-Fayard et al., 2006). Of importance, the software integrates signal over surface area of tissue so approximately the same amount of adipose tissue is assayed in comparisons of fed, fasted and ob mice.

This data provided the basis for the characterization of a series of promoter deletions to fine map the cis regulatory elements of the leptin gene, as a starting point for identifying interacting trans-factors.

Ultraconserved sequences around leptin gene

As a general rule of thumb for studying promoters of genes, researchers make use of comparative DNA sequence analysis to identify conserved sequences in the vicinity of the gene of interest to guide the making of promoter deletions. Leptin gene has evolved with emergence of vertebrates. However, its function as a lipostat sensor seems to have evolved with endothermic animals, especially mammals. For this reason, vicinity of leptin gene has been checked for high conservation among mammals.

Fortunately, the chromosomal region in the vicinity of the ob gene has been extensively sequenced in a number of organisms. Genomic sequences around leptin gene from several mammalian animals were aligned by using

VISTA program for finding conserved regions, which might be candidates for possible transcription binding sites. Genomic sequences have been retrieved from UCSB genome browser. This analysis identified 2 ultraconserved regions, one being in the -17kb upstream of leptin, the other being in the first intron (Figure 40).

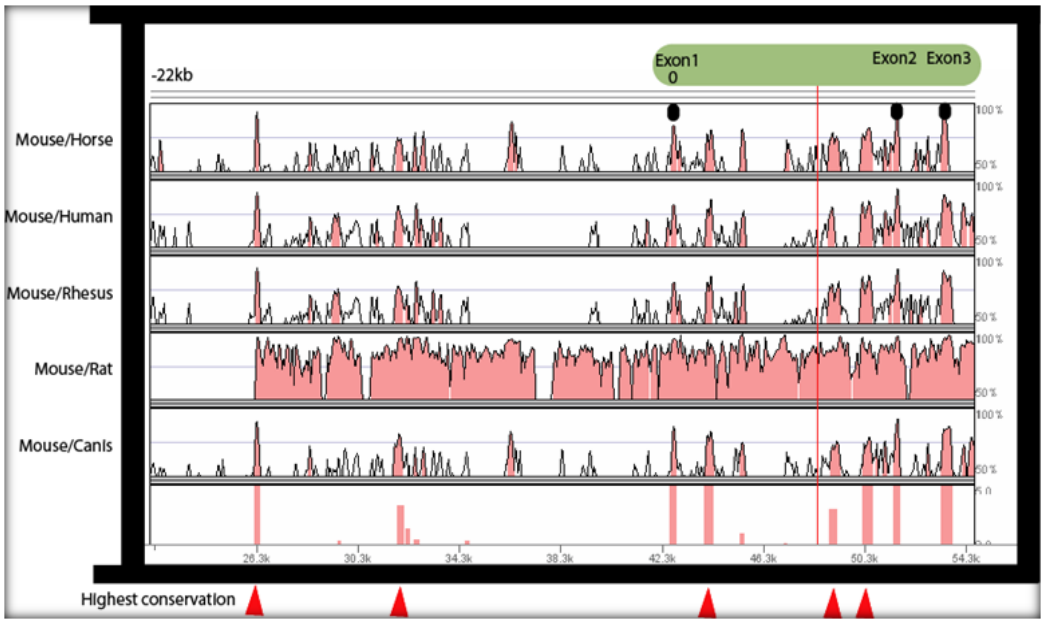

Figure 40: Conservation of sequences around leptin gene

Data has been retrieved using UCSC genome browser. 0 indicates the TSS of leptin. Note the high conservation between -17kb and +3kb.

-22-0 kb regulatory sequences carry fat specificity and fat sensing

Since, the 5' upstream sequence of a gene is usually regarded as the most critical region for transcriptional regulation, 22kb upstream region of the leptin gene were used to make transgenic animals as the next step. Note that this construct does not carry any introns, since the luciferase is inserted in the first exon. -22-0kb transgenic animals, carrying 22kb upstream region, conferred fat tissue specific expression and the expression is regulated by fasting and

obesity (Figure 41). On the other hand; -762bp-0 transgenic animals, carrying 762 bp upstream sequence, showed no fat tissue specificity in all two founders, both having ovary/testis specific luciferase expression (Figure 42). One issue with the –22-0kb animals is that the quantity of luciferase expression was 50-100 times less compared to the transgenic lines where luciferase was inserted into the translation start site, namely -160-18kb, -22-140kb and -22-18kb (Data not shown). This low expression can be ameliorated by adding a chimeric intron; however it might also suggest the existence of an enhancer region in the intron.

These data suggest that 22kb upstream sequence is enough to drive fat-specific expression and lipid sensing properties of leptin. Further, 762bp promoter is not enough to drive fat-specific regulation of leptin.

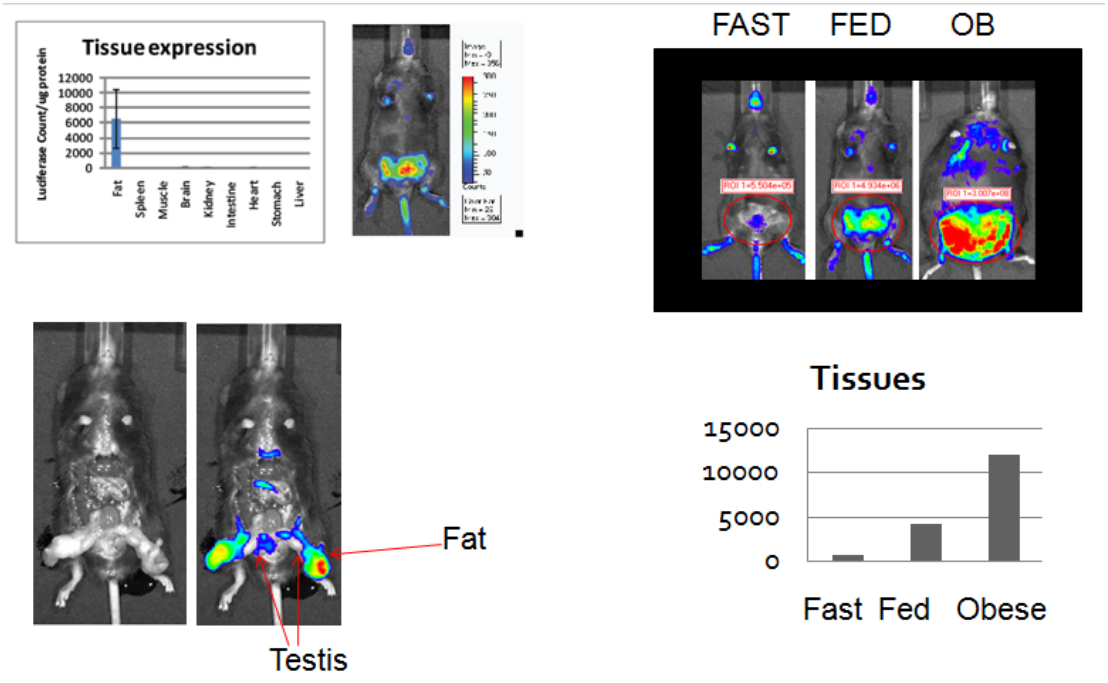

Figure 41: Luciferase expression in -22kb-0 Luciferase animals

Expression of luciferase was assessed by *in vivo* imaging using an IVIS CCD camera.

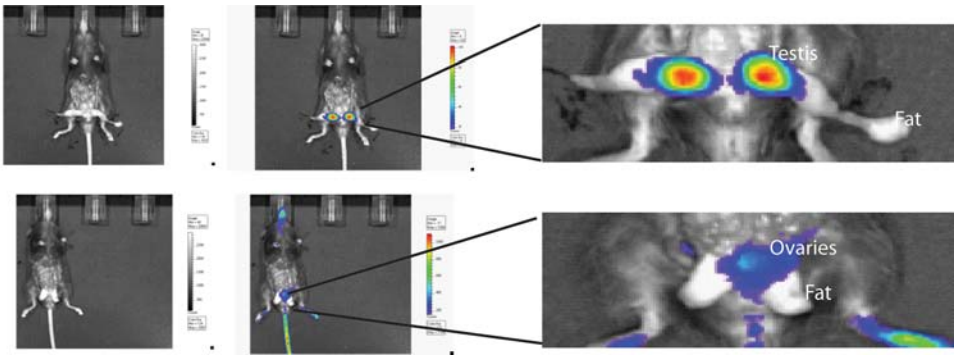

Figure 42: Luciferase expression in -762bp-0 Luciferase animals

Expression of luciferase in ovaries and testis was assessed by *in vivo* imaging using an IVIS CCD camera. No expression was observed in the fat tissue

-17kb conserved region histones are hyperacetylated and hyper monomethylated

Patterns of histone modifications influence transitions between chromatin states and the regulation of transcriptional activity. Different histone marks have been proposed to have different effects on transcriptional activity. Some of these marks are enriched in enhancers, and some in promoters as some are associated with active transcription whereas others are associated with repression. There are many reports regarding the histone marks at distant enhancers by several different groups. H3K4 methylation is often linked with transcriptional activity in many contexts. Specifically, H3K4 trimethylation is associated with promoter regions of almost all active genes (Choi and Engel, 1986b). Recently several groups also found associations between active enhancer regions and H3K4dimethylation and/or H3K4monomethylation (Bernstein et al., 2006; Robertson et al., 2008; Roh et al., 2007). In addition,

there seems to be a direct correlation between active transcription and histone acetylation (Ruthenburg et al., 2007). In contrast, H3K27 trimethylation is associated with promoters of inactive genes. In an earlier study, Litt et al. performed a “chromosome walk” on DNA sequences for the beta globin gene, testing previously known to be active or inactive regions, by performing Chip assays using antibodies against H3K4 marks (Litt et al., 2001).

Since the elements conferring leptin regulation is thought to be between -22kb and -762 bp region, it was hypothesized that the only ultraconserved DNA stretch in this region may be functionally important. For this reason, chromatin immunoprecipitation analysis has been performed around the -17kb region. Basically, fat tissue was fixed, lyzed and nuclei were isolated. Nuclei later were lyzed and sonicated and immunoprecipitated with the corresponding antibodies. H3K9Ac and H3K4monomethyl antibodies were used for immunprecipitation. For analysis, sybr green real time PCR analysis was used. PCR fragments with 800 bp intervals were designed around -17kb region and input and IP samples were analyzed by Real Time PCR. The results show that -17kb region was indeed enriched for H3K9Ac and H3K4monomethyl marks (Figure 43). Both of these marks before were shown to be enriched in enhancer regions using whole genome CHIP studies.

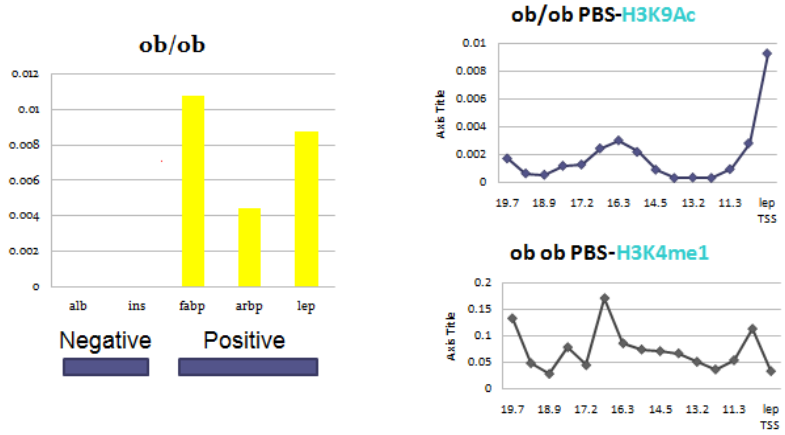

Figure 43: Chip analysis of -17kb conserved region

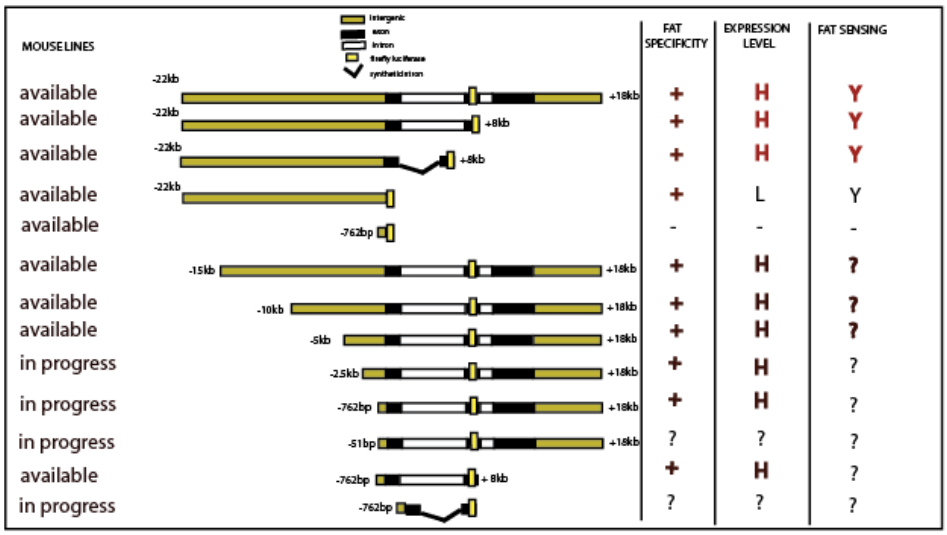

Figure 44: Deletion analysis of leptin-Luciferase BAC transgenic animals

Table shows the current state of the characterization of BAC transgenic constructs.

Fat specificity shows if the corressponding luciferase-BAC transgenic line recapitulates fat-specific expression of leptin.

Expression level shows the luciferase expression of the corresponding BAC transgenic animal. "?" indicates that the work is in progress. "H" indicates high expression of luciferase as assessed by *in vivo* imaging using IVIS CCD camera. "L" indicates low expression.

Fat sensing shows if luciferase expression in the animals recapitulate the lipid-sensing regulation of leptin. "Y" indicates that the animals recapitulate the fat sensing property of leptin. This is assessed by crossing the animals to obese (ob/ob) animals versus fasting the animals for 48 hours.

Further deletion analysis of leptin promoter

As the next step, more deletion constructs were made to refine the region. As shown in figure 44, deletions from -17kb down to -762bp constructs in the context of +18kb downstream have been made, assuming that the element is between -22kb to -762bp region. Surprisingly -762bp-18kb (also 762bp-8kb) transgenic animals conferred fat tissue specific expression of luciferase. Furthermore, this expression was at high levels. Note that only -762 bp region alone was not enough to confer any fat-specific expression. These results suggest that leptin regulation might be redundant or a complex regulation is taking place which will be discussed in the final discussion section (Figure 44).

Search for trans-factors regulating leptin gene expression

One approach to find possible transfactors and mechanisms that regulate leptin expression is by using microarray analysis. DNA microarray represents a powerful tool in biomedical discoveries. Harnessing the potential of this technology depends on the development and appropriate use of data mining and statistical tools. Leptin is regulated differently in different metabolic states. This range of regulation is extremely helpful for microarray analysis, since it will restrict the number of hits to a minimum due to the availability of multiple comparisons.

Leptin is adipocyte-specific such that when the adipose tissue is dissociated by collagenase digestion, the pelleted stromavascular fraction does not have any detectable leptin mRNA compared to adipocytes. The assumption

would be that any factor that confers fat specificity should be enriched in adipocytes. For this reason, a DNA chip analysis was performed to identify genes that are enriched in the adipocyte fraction compared to stromavascular fractions. Interestingly, only a handful transcription factors were enriched in adipocyte fraction. These transcription factors include, CHREBP(MLXIPL), which is known to be a glucose sensor transcription factor that is also expressed in several other organs such as liver, (Dentin et al., 2005; Mason et al., 1998) PPARɣ/RXR, which is thought to be the master regulator of adipogenesis, C/EBPα, a gene that was shown to regulate adipogenesis and leptin expression *in vitro*, (Mason et al., 1998) SREBP, a cholesterol sensing transcription factor (Kochan, 2003; Stelmanska et al., 2005) and DBP, a zinc finger factor with relatively unknown function (Mikhailov et al., 2008) (Figure 46).

Since -762bp-8kb region which includes 762 bp promoter and 8kb first intron, confers high expression in adipose tissue, the conserved regions and possible transcription factor binding sites were analyzed. Conserved region in 762 bp promoter contains a C/EBP binding site along with Cdx/USF binding sites. Ultraconserved region in the first intron contains C/EBP binding sites along with GATA binding sites, which were before shown to be important in adipogenesis. These transcription binding sites might provide useful with regard to their binding to the putative leptin regulatory elements (Figure 45).

Discussion

The *in vivo* analysis of leptin regulation suggests that leptin regulation is more complex than it was previously thought using *in vitro* systems (Chen et al., 1999; Hollenberg et al., 1997; Mason et al., 1998). -762 bp promoter, which was shown to contain elements for C/EBP factors, is not enough to confer leptin regulation (Mason et al., 1998). The cis-elements possibly involve a fat-specific regulatory domain along with an enhancer domain. The possible binding sites found in 762 bp promoter and ultraconserved sequence in the intron for C/EBP and GATA suggest that these transcription factors which were before shown to be expressed in adipocytes might indeed have roles in regulating fat-specific expression of leptin. However, the fact that these transcription factors are also expressed in 3T3-L1 adipocytes, cannot explain why leptin is not expressed in this system. It is likely that a novel combination of transcription factors are lacking in 3T3-L1 adipogenesis. These issues will be discussed more thoroughly in the final discussion chapter.

Dissecting cis-acting regulatory sequences using genomic clones and reporter genes has been performed mostly in cultured cells and *in vitro* systems rather than animals because transgenic methods are relatively inefficient, costly and regulatory regions for proper expression *in vivo* usually lie large distances from the targets and cannot be included in conventionl plasmids. A significant advance came from the discovery of modification of bacterial artificial chromosomes using BAC recombineering (Warming et al., 2005). This method has been used by several groups to identify cis-acting regulatory sequences and

locus control regions (Carvajal et al., 2001; Huang et al., 2000; Jamsai et al., 2003; Kaelin et al., 2004; Lehoczky and Innis, 2008; Oginuma et al., 2008; Smale, 2003; Yu et al., 1999). Dissection of cis-acting transcriptional regulatory elements using reporter genes in BAC transgenic mice can provide insights into eukaryotic gene regulation *in vivo*.

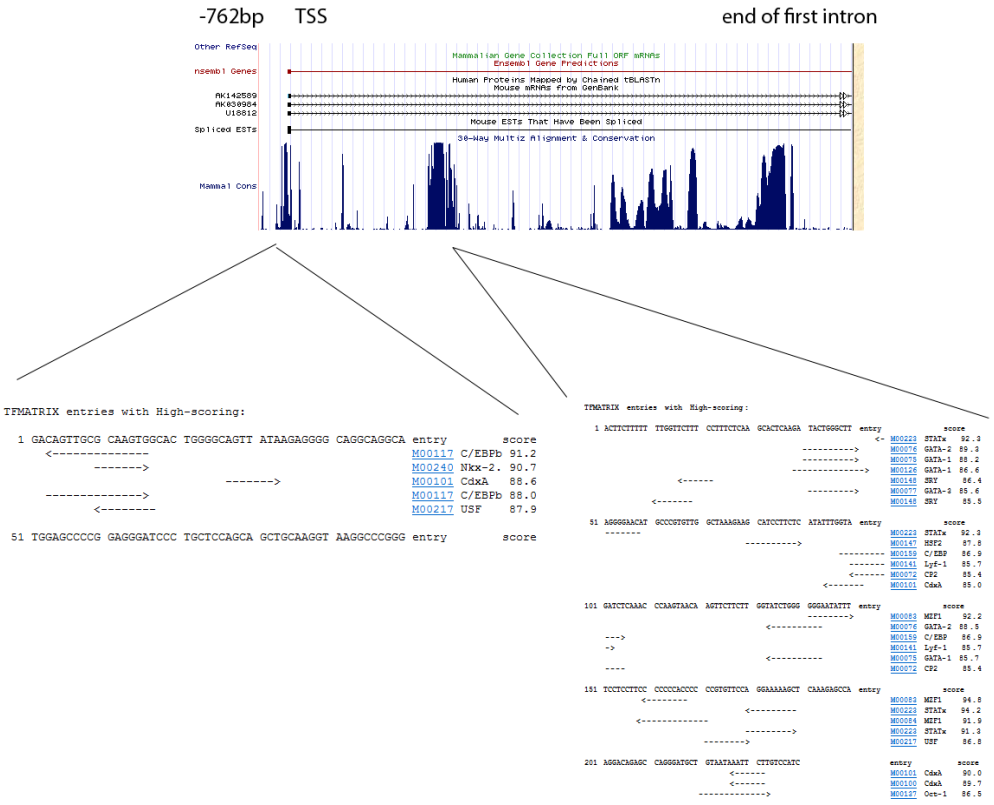

Figure 45: Conserved sequences and transcription binding sites in -762bp-+8kb sequence

		SVF	Adipocyte	
MLXIPL	5090343	54.6	1326.4	60.73260073
CEBPA	7380400	77	526.9	17.10714286
PPARG	3450465	27.3	220.7	20.21062271
RXRG	5290131	32.8	205.7	15.67835366
SREBF1	6840156	70	341.9	12.21071429
DBP	3180750	1565.3	4818.9	7.696447965

Figure 46: Transcription factors enriched in adipocytes compared to SVF fraction

Chapter 7:

Practical Applications of Leptin-luciferase Animals

Introduction

Leptin-luciferase BAC transgenic mouse line expresses luciferase under the control of leptin promoter elements, making it possible to assess the activity of the leptin gene *in vitro* biochemically, and *in vivo* by imaging of luciferase activity with a CCD camera (Figure 26-28) (Contag and Bachmann, 2002). Since leptin level is highly correlated with adipose tissue mass,(Ahima and Flier, 2000; Frederich et al., 1995; Jernas et al., 2006; Maffei et al., 1995a) the leptin-luciferase animal model also provides a novel means for assessing adipose tissue mass non-invasively *in vivo*. Possible applications for this line include the delineation of the embryonic origins of adipose tissue, as discussed in the previous chapters, and screens for modulators of adipose tissue mass. In addition half-life of luciferase (~3 hours) enables to study relatively short changes in gene expression (Leclerc et al., 2000). In this chapter, this strain was employed to study the response of adipose tissue before and after either food restriction or leptin treatment. These experiments enabled the identification of a novel cellular program that allows the conversion of glycogen intermediates to triglycerides in hypoleptinemic conditions.

Results

Fasting and refeeding induce dynamic responses in leptin-luciferase expression levels in adipocytes

The level of luciferase was monitored in animals during a three day fast and after refeeding. These experiments were performed in the same animal over time using the CCD camera. Luciferase activity fell gradually over the course of the fast and by three days there was a ~6-8 fold lower level of luciferase activity (Figure 47A). Luciferase activity returned to baseline by 6 hours after refeeding. Because 6 hours is not enough time to regenerate the adipocyte population, this extremely rapid recovery of the leptin-luciferase signal after refeeding suggested the possibility that lipid depleted adipocytes persist in adipose tissue after fasting.

Consistent with this, histological analysis at the conclusion of the three-day fast showed smaller adipocytes with a markedly lower cellular lipid content (Figure 47B). The identity of these cells as adipocytes was established by immunohistochemical staining with an antibody to aP2, an adipocyte specific marker (Figure 47B). However, note that there was residual lipid in some of the adipocytes after the fast, evident by significant luciferase activity and thus sought to identify conditions under which there was near complete depletion of cellular lipid in adipocytes. This was accomplished by treating animals with leptin.

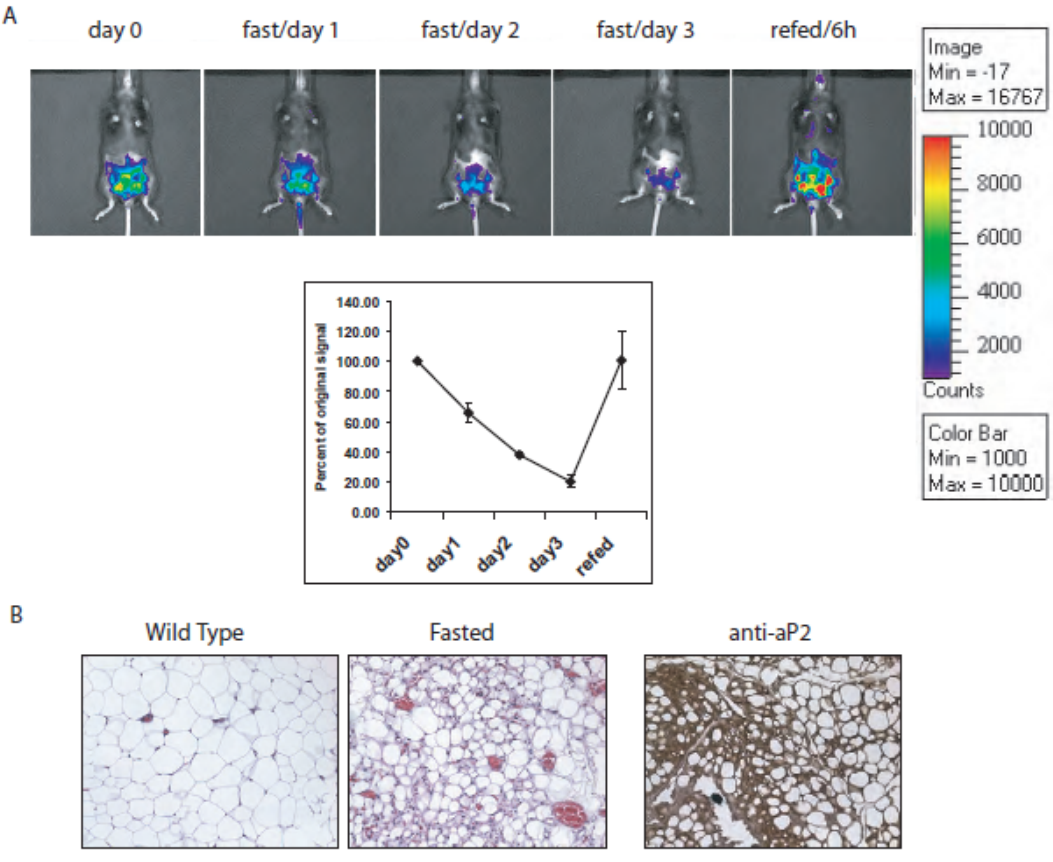

Figure 47: Time course luciferase activity in leptin-luciferase animals upon fasting/refeeding

(a) Time course luciferase activity in leptin-luciferase animals upon fasting and refeeding. The level of luciferase in animals was monitored during a three day fast and after refeeding . The data were collected for the same animal over time. (n=4) Luciferase activity fell gradually over the course of the fast. After refeeding, luciferase activity returned to baseline by 6 hours. (Upper panel) Representative time-course images of fasted and refed animals are shown. (Lower panel) Quantitation of *in vivo* signals by Living Image 3.0 software. (mean +/-SEM). (b)Histological and immunohistochemical analysis of adipose tissue of fasted mouse. H&E staining of wild-type (left) and fasted (middle) animals for comparison. Note that nuclei are approximately the same size. Immunohistochemical staining of the adipocyte specific protein, aP2, in fasted animals (right). The fasted fat pad consisted almost entirely of adipocytes expressing high levels of aP2 protein.

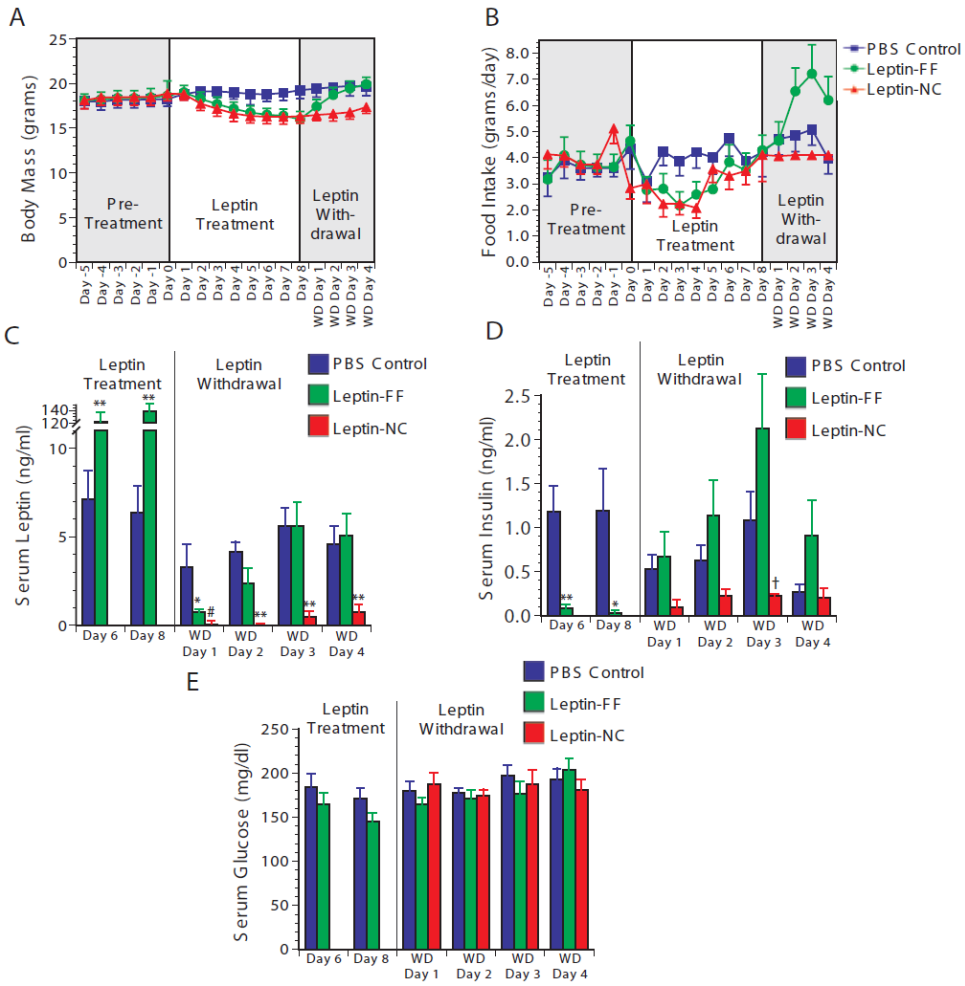

Figure 48 Body weight, food intake, serum leptin, serum insulin and serum glucose of acutely leptin deficient mice

(A) Leptin treatment resulted in a rapid and sustained decrease in body mass relative to PBS control animals (blue squares). When exogenous leptin was withdrawn (leptin withdrawal, Right), free-fed animals (Leptin-FF, green circles) rapidly gained back lost weight while animals maintained at normocaloric intake levels (Leptin-NC, red triangles) rebounded more slowly. (B) Leptin treatment decreases food intake only while body mass is being lost after which time levels return to normal. Following leptin withdrawal, hyperphagia ensued in leptin-FF animals, while it was prevented in Leptin-NC mice in order to control for effects simply due to an increase in food intake. (C) Serum leptin levels were 20 fold elevated during leptin treatment and dropped precipitously following leptin withdrawal relative to PBS control animals (blue bars). In leptin-FF mice (green bars), levels were low for two days (WD Day 1 and WD Day 2), while in leptin-NC (red bars) mice, low serum leptin persisted for four days. Statistically significant differences are noted (*, $P < 0.05$ vs. PBS; #, $P < 0.06$ vs. PBS; **, $P < 0.005$ vs. PBS). (D) Serum insulin levels are 12 to 20 fold lower than PBS controls following 6 or 8 days of leptin treatment (*, $P < 0.05$ vs. PBS; **, $P < 0.005$ vs. PBS). Following leptin withdrawal, insulin rose in Leptin-FF mice while maintaining mice on normocaloric intake prevented any rise in serum insulin (†, $P < 0.05$ vs. Leptin-FF). (E) Normal serum glucose levels were evident in all groups before and after leptin withdrawal.

Leptin treatment induces near complete delipidation of adipocytes, conserving adipocyte specific protein expression

Mice were treated with high dose leptin via subcutaneous pump for 8 days. Leptin treatment led to a complete depletion of all visible body fat which was associated with the complete absence of luciferase expression (Figure 49). Histology of the adipose depot after 8 days of leptin showed a dense population of cells with little or no detectable cellular lipid. However, these lipid-depleted cells still showed high levels of the adipocyte-specific protein aP2 expression suggesting that after leptin treatment the adipose tissue was populated by lipid depleted adipocytes (Figure 49).

After eight days the leptin osmotic pumps were removed. Following withdrawal of exogenous leptin, animals fed ad libitum became markedly hyperphagic and body fat mass returned to pretreatment levels over the course of four days (Figure 48). Luciferase levels were clearly detectable in animals post leptin withdrawal after one day; and after 4 days the luciferase signal returned to baseline levels (Figure 49).

The data from leptin treated animals were consistent with data from fasted animals and suggested that under both conditions the adipose depot is composed of lipid depleted adipocytes. The data further suggest that leptin treatment leads to the complete depletion of cellular lipid providing a unique opportunity to study the molecular programs that are responsible for the repletion of cellular lipid.

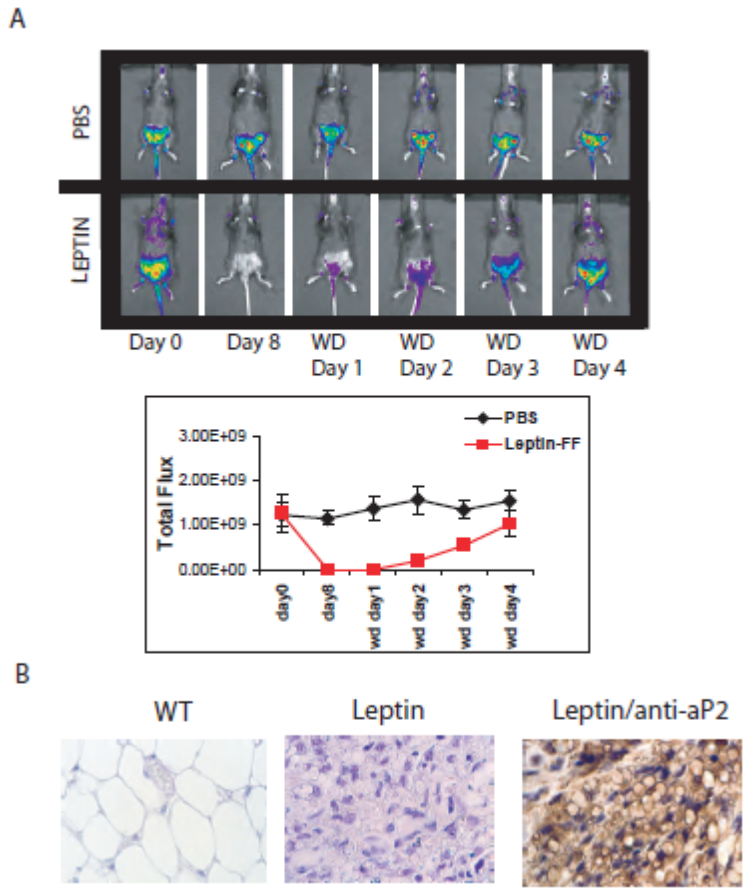

Figure 49: Leptin withdrawal experiment

(a) *In vivo* imaging of leptin-luciferase animals upon leptin withdrawal. PBS (n=6) or leptin at 2.5 µg/hour (n=5) was administered for 8 days by subcutaneous implanted osmotic pump. On day 8, leptin or PBS treatment was withdrawn by removing pumps under inhaled anesthesia. Animals were imaged before treatment, after 8 days of leptin treatment and daily for the 4 days following removal of leptin pumps. Leptin treatment resulted in a rapid and sustained decrease in leptin expression and fat depots relative to PBS control animals. Note that no significant luciferase activity was detected after leptin treatment. After exogenous leptin was withdrawn, luciferase activity in adipose tissues from free-fed animals returned to original levels in 4 days. (Upper panel) Imaging results of representative animals form PBS and leptin treated groups are shown. (Lower panel) Quantitation of *in vivo* signals by Living Image 3.0 software indicates a dramatic suppression of luciferase by exogenous leptin treatment (mean +/-SEM). (b)Preservation of adipocyte identity during chronic leptin treatment. Leptin treatment results in near complete depletion of triglyceride droplets from white adipose tissue. White adipose tissue of mice treated for 8 days with leptin consists of dense islands of cells as assessed by hematoxylin and eosin staining (middle). Note for comparison that nuclei are the same size. Immunohistochemical staining demonstrated high levels of the adipocyte specific protein aP2 in the cytoplasm of high numbers of cells in de-lipidated white adipose tissue (right, brown, peroxidase positive material). Fat pads of leptin treated animals consisted almost entirely of adipocytes expressing high levels of aP2 protein, despite lack of preservation of typical adipocyte morphology.

Recovery from Hypoleptinemia Increases Cellular Glycogen Prior to the Reappearance of Cellular Lipid

In order to study the cellular programs activated when fat mass is repleted, RNA was prepared from adipose tissue during leptin treatment and after 1-4 days post leptin withdrawal for use with oligonucleotide microarrays to follow the patterns of gene expression after leptin withdrawal. While preparing the RNA from these samples, we noted the appearance of large amounts of a white flocculent material after ethanol precipitation. This material had the gross appearance of glycogen. To confirm this, tissue glycogen levels were checked biochemically. On the first day post leptin withdrawal there was a massive increase in glycogen content in the adipose depot with a total glycogen content of 1 % of the total wet weight of the tissue (Figure 50). Treatment of acutely leptin-deficient white adipose tissue lysates with amyloglucosidase, an enzyme which specifically hydrolyzes glycosidic bonds (Keppler, 1974) liberated a large amount of soluble glucose, confirming the presence of high levels of glycogen. Periodic acid-schiff (PAS) staining of tissue sections showed dense staining in cells containing small lipid droplets confirming the presence of glycogen granules in adipocytes (Figure 50). The PAS stained material was further confirmed to be glycogen by pre-digesting tissue sections with α-amylase which eliminated PAS positive granules. Intense staining was visualized in a similar pattern to that shown previously for aP2 (Figure 50).

The total glycogen content decreased over the ensuing 3 days by which time adipose tissue mass had returned to the same level as prior to treatment.

Histological analysis over this period indicated enlarging adipocyte lipid droplets in the same cells that showed PAS staining for glycogen (Figure 50).

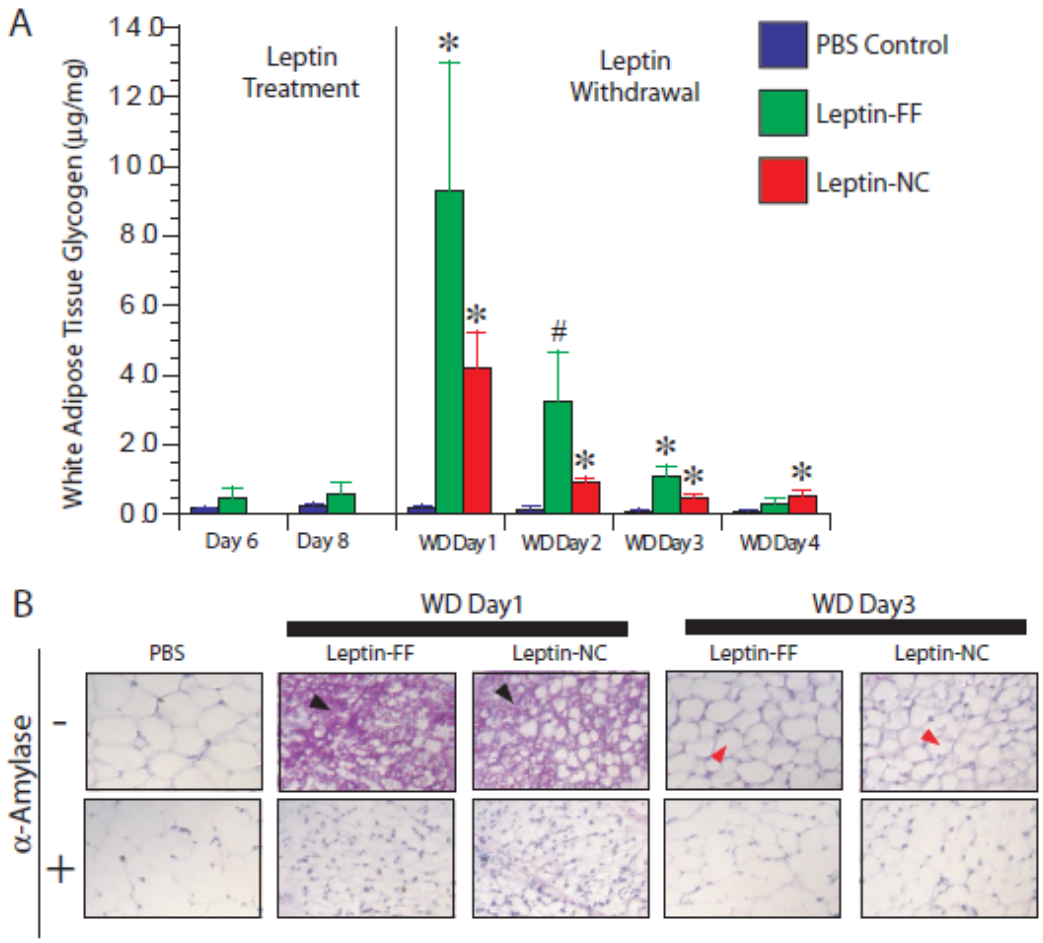

Figure 50: Accumulation of white adipose tissue glycogen during acute leptin deficiency

(a) White adipose tissue glycogen content rose 30 to 60 fold in leptin withdrawal animals(green and red bars) relative to PBS controls (blue bars) following withdrawal of exogenous leptin treatment, as measured by glucose liberated by amyloglucosidase (*, $P < 0.05$ vs. PBS; #, $P < 0.065$ vs. PBS). This increase occurred in both free-fed animals (Leptin-FF, green bars) and animals maintained at normocaloric intake levels (Leptin-NC, red bars), and increased glycogen content persisted for 3 and 4 days in these groups, respectively. (b) Periodic acid-schiff (PAS) staining of white adipose tissue on withdrawal day 1 and withdrawal day3. PAS staining on withdrawal day 1 indicated large amounts of cytoplasmic glycogen content in leptin-FF and leptin-NC tissue sections (black arrows). This contrasted sharply with control PBS treated white adipose tissue. On withdrawal day 3, glycogen accumulation decreased in parallel to the accumulation of lipid. (red arrows) a-Amylase pretreatment of tissue sections eliminated PAS positive granules in adipocyte cytoplasm, indicating specificity of staining for glycogen.

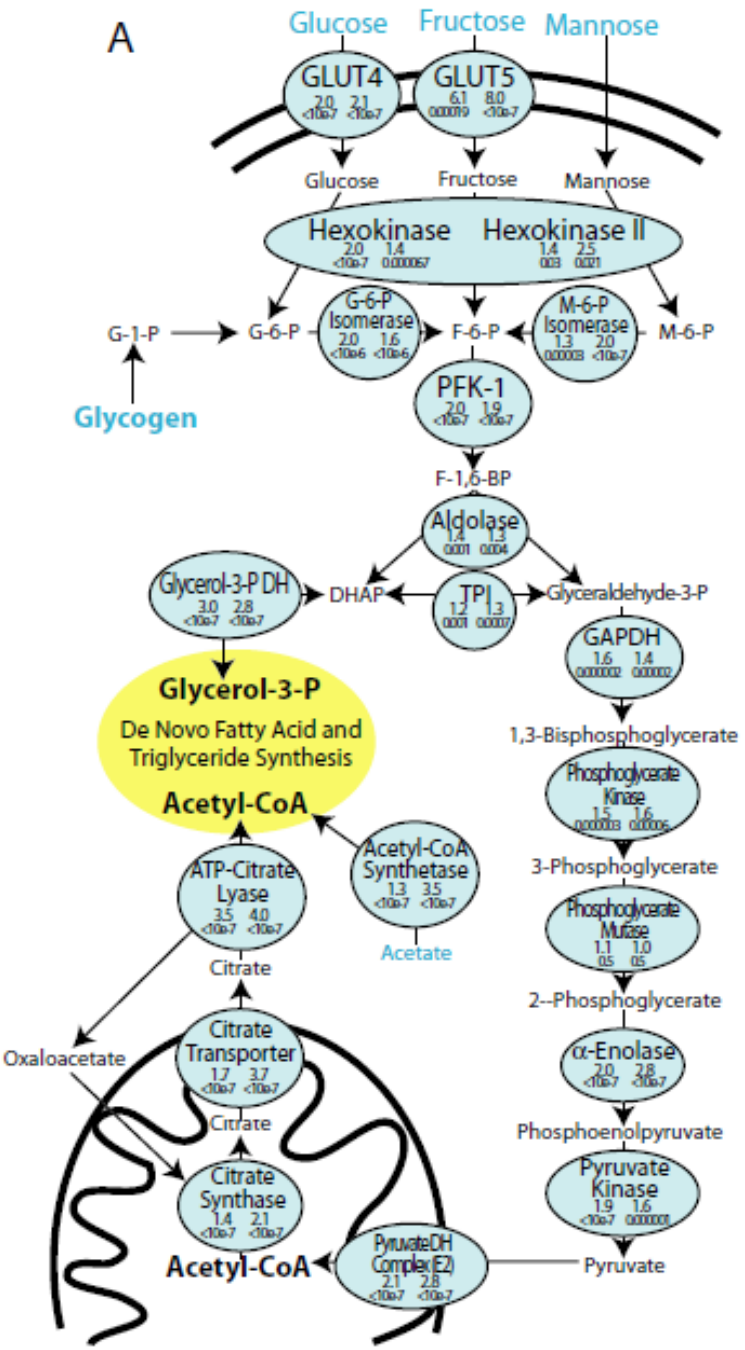

Figure 51: Pathway of acetyl-CoA and glycerol generating enzymes upregulated by acute leptin deficiency.

Acute leptin deficiency induced the pathway of genes (blue ovals) necessary to synthesize cytoplasmic acetyl-CoA and glycerol from simple sugar precursors. Fold change for each gene on day 1 following leptin withdrawal is shown below each enzyme name for leptin-FF (left) and leptin-NC (right) mice with the associated P value immediately below each fold change value. Potential sources of carbon for acetate or glycerol synthesis are in blue text.

The Transcriptional Program Associated with Recovery of Adipose Tissue Mass

These data suggested that the repletion of cellular lipid content and adipose tissue is achieved via a glycogen intermediate in lipid-depleted adipocytes. To study this further, transcription profiles of adipose tissue were generated by analyzing RNAs at 6 or 8 days of leptin treatment and 1, 2, 3, and 4 days after leptin withdrawal (Figure 47-48). To refine the analysis, RNA was prepared both from hyperphagic animals fed ad libitum after leptin withdrawal, i.e leptin-FF as well as from animals whose food intake was restricted to that which the animals ate voluntarily prior to leptin withdrawal, i.e leptin-NC animals (defined above). As an additional control, tissues from PBS treated animals were analyzed both before (day 6) and after (day 1 post-withdrawal) removal of the osmotic pumps. In all cases, RNA was labeled using biotin and hybridized to Affymetrix 11K oligonucleotide microarrays.

Selection and *k*-means cluster analysis (Soukas et al., 2000) of 683 genes changing significantly across experimental conditions identified 14 statistically distinct patterns of gene expression. Analysis clusters induced specifically by acute leptin deficiency revealed an enrichment of genes associated with gene ontology (GO) terms related to carbohydrate metabolism (Figure 51). Specifically, many genes induced in adipose tissue during weight gain play a role in glucose uptake, glycolysis, and acetyl-CoA and glycerol synthesis. An acute decrease in circulating leptin level induced nearly the entire complement of genes necessary to increase simple sugar flux into the adipocyte, and

subsequently convert these sugars into cytosolic acetyl-CoA and glycerol for *de-novo* fatty acid, triglyceride, and cholesterol biosynthesis (Figure 51). Average expression level for these mRNAs peaked on day 1 of acute leptin deficiency but remained elevated for four days following cessation of leptin treatment.

Leptin withdrawal also induced the expression of genes required for the storage of cytoplasmic triglycerides including genes involved in the synthesis of glycerol such as glycerol-3-phosphate dehydrogenase mRNA (3 and 2.8 fold in leptin-FF and leptin-NC mice, respectively). This enzyme catalyzes the conversion of dihydroxyacetone phosphate, a glycolytic intermediate, into glycerol-3-phosphate (or vice versa). Triglyceride storage is also augmented during acute leptin deficiency by a 70% and 30% induction of glycerol-3-phosphate acyltransferase (GPAT) in leptin-FF and leptin-NC animals, respectively. GPAT catalyzes the esterification of glycerol-phosphate and fatty acids for storage in adipose tissue.

Acute leptin deficiency specifically repressed four groups of genes that were induced or unchanged during chronic leptin treatment. These genes included both carnitine-palmitoyl transferase-I (CPT-I) and peroxisomal acyl-CoA oxidase (ACO) both of which are required for fatty acid oxidation. CPT-I, which enhances mitochondrial uptake of fatty acids for oxidation, is decreased in leptin-FF and leptin-NC mice 3.0 and 1.9 fold, respectively. ACO, which catalyzes enzymatic fatty acid breakdown, is 2.7 fold repressed in leptin-FF mice and 1.3 fold repressed in leptin-NC mice. White adipose phosphoenolpyruvate carboxykinase (PEPCK) mRNA, which would detract from acetyl-CoA

accumulation, was repressed 36 and 2.4 fold in leptin-FF and leptin-NC animals, respectively (Figure 51).

Leptin withdrawal did not regulate the mRNA of genes involved in glycogen synthesis or glycogenolysis suggesting that the induction of glycogen synthesis is not regulated at the level of transcription (Figure 51). As discussed below, these data suggest that the genes required for glycogen synthesis continue to be expressed in adipocytes in the setting of weight loss and thus allow the accumulation of glycogen when food is consumed. Lipid synthesis genes are repressed after a fast and this glycogen then serves as substrate for the biosynthesis of triglyceride when the genes required for lipid synthesis are induced.

Discussion

The cellular and molecular programs responsible for the repletion of adipose tissue mass after weight loss has not been fully elucidated. In this chapter, this issue was addressed using molecular genetics, physiologic and computational tools and show that weight loss after food restriction results in the partial loss of adipose tissue lipid while leptin treatment results in the persistence of adipocytes that are nearly completely depleted of their cellular lipid. It was further shown that the restoration of adipose mass acutely after re-feeding is associated with the production of massive glycogen deposition that temporally precedes the recovery of adipose tissue mass. Finally, it was shown that acute hypoleptinemia activates a novel program of gene expression with the induction

of gene products that convert glucose and other simple sugars into glycerol and acetyl-CoA for triglyceride synthesis.

Leptin expression is markedly reduced after a three day fast and its level of expression is restored nearly to baseline levels within 6 hours after refeeding. This rapid repletion of leptin expression is too rapid to be consistent with the possibility that there is apoptosis of adipose tissue followed by *de-novo* adipogenesis. Rather these data suggested that fat loss after food restriction results the depletion of lipid within adipocytes. This conclusion is supported by data from immunohistochemical staining of adipose tissue with an antibody to aP2, a fat specific gene,(Hunt et al., 1986) which is still expressed at high levels in adipose tissue after a fast, at a time when there are markedly reduced amounts of lipid. Eight days of leptin treatment caused an even more dramatic reduction of adiposity and histological analysis of adipose tissue of leptin-treated animals demonstrated dense nests of aP2 expressing cells that were largely devoid of lipid. Thus, the rapid restoration of fat mass following acute leptin deficiency does not appear to require new fat cell production. Several additional lines of evidence support this conclusion. First, mitoses were not visible in tissue sections from adipose tissue after leptin withdrawal during times when adipose mass is being rapidly repleted. Second, as assessed using the data from the transcriptional profiles, genes known to induce terminal differentiation of adipocytes or that are involved in the clonal expansion phases of adipogenesis *in vitro* (Gutman and Shafrir, 1964) remained unchanged or decreased in abundance during acute leptin deficiency. In addition, previous studies have

reported that adipose tissue from animals treated with leptin contains the same DNA content as control samples (Zhou et al., 1999). Therefore, it was concluded that the restoration of fat mass is the result of the activation of a program of lipid re-accumulation in lipid-depleted adipocytes and does not appear to be associated with *de-novo* adipogenesis.

The re-accumulation of fat mass following weight loss is the result of the activation of a novel gene expression program that is activated by acute hypoleptinemia. The initial phase of this cellular program is marked by an increase in adipocyte glycogen content. The glycogen content of white adipose tissue is normally exceedingly low (Cahill et al., 1959) and previous studies have shown that fasting reduces glycogen levels even further while subsequent refeeding transiently increases adipose tissue glycogen content (Gutman and Shafrir, 1964; Rose and Shapiro, 1955; Tuerkischer and Wertheimer, 1942). The stimulus for this response was not previously known. This data indicates that low leptin levels provide this stimulus, such as those present with refeeding after a period of food restriction or after leptin withdrawal. Furthermore, the studies of animals experiencing leptin withdrawal in the context of restrained food intake (Leptin-NC animals) further indicated that the observed increase in glycogen synthesis in white adipose tissue after refeeding was not the result of hyperinsulinemia (Kaslow and Mayer, 1979).

The increased pool of glycogen in white adipose tissue during fat regeneration appears to provide the substrate for expansion of adipose triglyceride mass. This conclusion is consistent with previous findings showing

that adipose triglyceride stores are primarily synthesized within adipose tissue and rather than being imported into fat cells after being synthesized elsewhere (Rose and Shapiro, 1955). Glycogen-containing adipose tissue from animals refed after a 48 hour fast was previously shown to have a respiratory quotient of greater than 1.0 indicative of a high rate of glucose metabolism and not fatty acids (Tuerkischer and Wertheimer, 1942; Wertheimer, 1945). In re-fed animals, glycogen disappears from adipose tissue at the same time as fatty acid synthesis is at its maximum and PAS positive material and lipid droplets can be easily seen in the same cells during the recovery from weight loss (Rose and Shapiro, 1955). Finally, glycogen is an efficient precursor of both components of adipose tissue triglycerides, the fatty acid and glycerol moieties (Gutman and Shafrir, 1964). In aggregate, these data suggest that the re-accumulation of fat mass following withdrawal of leptin treatment or refeeding following a fast involves 1) the increased glycogen synthesis which is likely to be substrate driven as mRNAs for the genes involved in glycogen synthesis and breakdown do not fall during leptin treatment or subsequent withdrawal, and 2) transcriptional induction of genes required for lipid biosynthesis from monosaccharides.

Lipid stores in the adipocytes may arise from two major routes: de novo lipogenesis from non-lipid precursors or uptake of fatty acids from plasma. In this study, it is suggested that lipid repletion, at least following leptin treatment, may be through a de novo lipogenic pathway involving a glycogen intermediate and upregulation of transcriptional pathways. Adipose tissue, liver and mammary glands (lactation) were before shown to be sites for de novo lipogenesis in

mammals (Blouin et al., 2009; Lafontan, 2008). De novo lipogenesis has been found to be active when small animals are fed on carbohydrate rich and low fat diet (Blouin et al., 2009; Lafontan, 2008). Another major route for triglyceride deposition in adipose tissue is through the uptake of pre-existing fatty acids from circulating triglycerides in form of chylomicrons/VLDL particles. It is still controversial which of the lipogenic mechanisms is the major pathway for adipose tissue lipid accumulation. It is possible that both pathways take place to certain extents in different metabolic conditions. Note, it is also suggested by several studies that the contribution of adipose tissue to the whole body lipogenesis is relatively lower in humans. A comparison of lipogenesis in the adipose tissue of humans confirmed that the capacity of lipogenic pathways of adipose tissue is less in humans, possibly due to the lower abundance of the transcription factors controlling the lipogenic pathways, namely SREBP-1 and CHREBP (Letexier et al., 2003).

The failure of the mRNAs involved in glycogen metabolism to change after fasting or leptin treatment is in contrast to the enzymes of glycolysis and the enzymatic cascade necessary to generate acetyl-CoA from simple sugars, which are clearly induced under these conditions. In previous studies these genes have been reported to be regulated predominantly by post-transcriptional mechanisms (Daran-Lapujade et al., 2004; Daran-Lapujade et al., 2007). Thus, the response to leptin withdrawal or refeeding is not simply an induction of the same genes repressed during leptin treatment and instead constitutes a novel program for adipocyte gene expression. The transcriptional mechanism responsible for the

upregulation of this functionally linked group of genes is not known and future studies of the factors that regulate this program may provide new avenues for limiting lipid re-accumulation in adipose tissue after weight loss and possibly treating obesity.

CHAPTER 8:

Conclusion

Summary of findings

Adipose biology field has long been driven by the *in vitro* models due to the ease and availability of the cell lines that were discovered by Green and colleagues. There is now evidence that the *in vitro* adipogenic processes do in fact reflect *in vivo* events only partially. This urges the need to study adipocytes *in vivo*. Major problems to study adipogenic processes *in vivo* are lack of markers, poorly characterized *in vivo* animal models and unknown identity of the adipogenic cell types. The development of new markers and characterization studies on the adipocyte development *in vivo* will help to solve these problems.

The primary goal of this thesis has been to characterize the molecular and cellular events in white adipose tissue *in vivo*. Firstly, an early transcriptional network has been discovered including KLF4 and Krox20 as essential early regulators of adipogenesis. *Klf4* is expressed in 3T3-L1 cells within 30 min after exposure to a standard adipogenic cocktail of insulin, glucocorticoids, and IBMX. Knockdown of KLF4 inhibits adipogenesis and downregulates C/EBPβ levels. KLF4 binds directly to the C/EBPβ (*C/EBPβ*) promoter as shown by chromatin immunoprecipitation and gel shift assays and, together with Krox20, cooperatively transactivates a C/EBPβ reporter. C/EBPβ knockdown increases levels of KLF4 and Krox20, suggesting that C/EBPβ normally suppresses Krox20 and KLF4 expression via a tightly controlled negative feedback loop. KLF4 is

specifically induced in response to cAMP, which by itself can partially activate adipogenesis. These data suggest that KLF4 functions as an immediate early regulator of adipogenesis to induce C/EBPβ.

Secondly, we characterized an early adipocyte progenitor cell population which can reconstitute functional adipose in lipodystrophic animals. Use and characterization of lipodystrophic animals was crucial for this result, because transplantation studies using wild type animals do not result in any new functional adipocytes, which is probably due to the quiescent state of the wild type adipose as assessed by many other studies. A-ZIP lipodystrophic animals, however, were found to be hyperadipogenic- as evaluated by the upregulation of early transcription factors, KLF4, Krox20 and Pref1 that were previously identified and hyperproliferative- as determined by BrdU incorporation studies. One other model that helped the identification of adipocyte progenitor cells was leptin-luciferase BAC transgenic animals. Leptin-luciferase transgenic mouse expresses the luciferase reporter gene under the control of leptin regulatory sequences, which allows noninvasive imaging of the leptin expression of adipocytes *in vivo*. Cell sorting studies identified a subpopulation of early adipocyte progenitor cells (Lin^{-}:$CD29^{+}$:$CD34^{+}$:$Sca\text{-}1^{+}$:$CD24^{+}$) resident in adult WAT. When injected into the residual fat pads of A-Zip lipodystrophic mice, these cells, sorted from leptin-luciferase animals, reconstituted a normal WAT depot and rescued the diabetic phenotype that developed in these animals. The development of adipose was determined using a CCD camera, since luciferase is expressed only in mature adipocytes. This study provides the identification of an

undifferentiated adipocyte precursor subpopulation resident within the adipose tissue stroma that is capable of proliferating and differentiating into an adipose depot in vivo.

Adipocyte differentiation possibly is most active in the embryonic/postembryonic stages of life, and adipocytes increase their size (hypertrophy) greatly as soon as energy intake starts. However, there is no descriptive study on adipose tissue development in rodents up to this date. This led us to characterize mouse adipose tissue development. One caveat to study adipose tissue development is that the adipocyte markers are thought to be expressed after the accumulation of lipids according to *in vitro* data. However, it is very likely that the adipocyte character and commitment occurs before the accumulation of lipids. In this regard, lipid itself cannot be used to localize where adipose tissue develops. Leptin-luciferase animals proved to be useful in this regard. Even though luciferase expression was extremely low in these animals, it was still detectable, making it possible to dissect and study the developing fat. These studies showed that fat starts to accumulate after birth. More interestingly, many of the adipocyte transcription factors/markers such as ap2, adipsin, adiponectin, PPARɣ and C/EBPα that were thought to be expressed after fat accumulation were actually found to be expressed high levels much earlier than the accumulation of fat. This result was contradictory to 3T3-L1 studies, where these genes are expressed after accumulation of lipid. It implies that adipogenesis *in vitro* does not exactly reflect the *in vivo* processes. This result in a way shows the need for the field to move to *in vivo* systems.

As explained above, *in vivo* and *in vitro* adipogenic processes are probably different. One other example to this phenomenon is the expression of leptin gene. Leptin expression is ~100 times lower in *in vitro* differentiated adipocyte cell lines compared to tissue levels (Hwang et al., 1997; Mandrup and Lane, 1997; Mandrup et al., 1997a). Surprisingly, these adipocyte cell lines abundantly express the master regulator PPARγ, C/EBPs and their targets (Hwang et al., 1997). This supports that the adipocyte-specific expression of leptin is controlled by a different transcriptional mechanism than the conventional adipocyte regulators. Studying leptin gene expression will give more insights about adipocyte specific transcriptional networks. Moreover, leptin is secreted in proportion to adipose tissue mass and in turn regulates food intake and energy expenditure. Adipocytes secrete leptin in proportion to their size meaning that as they accumulate more lipids, they secrete more leptin. This suggests the possibility that cellular lipid content is sensed and that a fuller understanding of the mechanisms controlling leptin gene expression could also lead to the delineation of a lipid sensing mechanism in fat cells and possibly other cell types. To discover this mechanism, we searched for the region in the leptin gene promoter that controls the quantitative expression of the leptin gene; ie the elements that confer increased leptin expression with obesity and decreased expression after weight loss. Since leptin is expressed at much higher levels in fat tissue *in vivo* than *in vitro* where it is not regulated in proportion to cellular lipid content, these studies require that gene expression be monitored *in vivo*. We thus generated a deletion series of BAC transgenic mouse lines that express

luciferase under the control of overlapping leptin regulatory sequences. We already have evidence that the cis element that confers qualitative and quantitative control of the leptin gene is located between – 762bp and +8kb relative to the transcription start site. This result suggests that leptin expression is more complex than it was thought before.

As explained above, leptin mRNA levels are highly correlated with adipose tissue mass, and leptin expression can thus be used as a surrogate for changes in the amount of adipose tissue. Since luciferase is expressed by leptin regulatory sequences in leptin-luciferase animals, which allows noninvasive imaging of the leptin expression of mice *in vivo*, luciferase signal can be used to track the lipid accumulation. Here leptin-luciferase mice were used to show that weight loss induced by fasting or leptin treatment results in the retention of lipid-depleted adipocytes in adipose depots. To further study the cellular response to weight regain after leptin treatment, a leptin withdrawal protocol was used to induce a state of acute leptin deficiency in wild type mice. Acute leptin deficiency led to the transient deposition of large amounts of glycogen within pre-existing, lipid-depleted adipocytes. This was followed by rapid reaccumulation of lipid. Transcriptional profiling revealed that this cellular response was associated with induction of mRNAs for the entire pathway of enzymes necessary to convert glucose into acetyl-CoA and glycerol, key substrates for the synthesis of triglycerides.

Future Directions and Concluding Remarks

The advent of DNA chip technology made it possible to analyze a plethora of genes across different experimental conditions or follow gene expression patterns through a time course. In this thesis, KLF4 and Krox20 were discovered through a comparison between *in vitro* differentiated adipocytes versus *in vivo* adipose tissue. The fact that there are earlier factors in 3T3-L1 differentiation suggests that there may actually be more genes upregulated very early in adipogenesis. These earlier genes may be discovered by an earlier time course microarray analysis of 3T3-L1 differentiation. Identification of these factors may give rise to broader analysis of early regulation of adipogenesis.

One caveat of studying earlier factors in adipogenesis is that the role of several genes expressed early during development such as Krox20, KLF4 and C/EBPβ cannot be studied in fat specific knockout mice because ap2-cre and other drivers such adiponectin are expressed late in the course of differentiation and would thus knock out these early genes at a point in time after their expression is required. Furthermore, often times germ line and adipocyte specific knockouts are uninterpretable when adipocyte genes are expressed in other tissues in adults or during development. For this reason, either earlier markers should be identified or another technique should be devised. Identification of earlier markers is relatively difficult, because many genes that have roles in differentiation of adipogenesis do also have roles in other differentiation processes. To address this, an ES complementation approach can be developed. This approach depends on the ability of ES cells to reconstitute the

germ line when injected into blastocysts A/ZIP transgenic animals. Since A/ZIP lipodystrophic animals do not have any fat tissue, all the fat in the chimeric animals comes from ES cells. This approach can be employed to study the role of specific genes during adipocyte development or on adipocyte function. For example, the function of adipose genes by introducing siRNA plasmids to knock-down the expression of specific genes into ES cells can be used to validate the role of three major early genes described above C/EBPβ, Krox20 and Klf4. Preliminary experiments demonstrated that microinjection of wild-type embryonic stem (ES) cells into lipodystrophic blastocysts produces chimeras that are positive for the A/ZIP transgene but which have a normal adipose tissue mass and normal blood glucose. This is demonstrated directly by the fact that in adipose tissue from these chimaeras, all of the adipocytes express YFP while in other tissues there is a variable contribution from the ES cells such that fat tissue is 100% YFP positive while liver and other tissues are not. Since the original lipodystrophic background cells are incapable of making ES cells due to the expression of dominant negative transcription factor against B-ZIPs, this shows that all of the adipocytes are derived from the wild type ES cells. These preliminary results indicate that indeed an ES cell complementation strategy may provide the tool to study adipogenesis *in vivo*.

Adipocyte progenitor cells can also be useful to study adipocyte development *in vivo*. Since it was shown that CD24+CD34+ cell population can differentiate into adipocytes *in vivo*, these cells can now be modified by lentiviruses carrying siRNA/plasmids and transplanted into A/ZIP fat pads. This

system will be extremely fast and cost–effective. It should be noted that it is still not known how many different cell populations or differentiation stages there are in adipogenesis. It is possible that adipocyte differentiation occurs through different stages as it is for hematopoiesis. It is also not known if there are several different cell types for forming different adipocytes such as the ones in subcutaneous/ visceral or bone marrow adipocytes. Heterogeneity in adipose tissue is an area that is poorly studied. It can be speculated that there can even be different progenitor cells for different adipose types. If this is true, it is possible to find different progenitors using same scheme but different cell sorting markers.

Leptin gene expression studies in this thesis sets up a starting point for further studies. Up to this date, sequences regulating fat specificity and high expression were confined between -762bp and +8kb, which spans the first intron. Surprisingly, 762bp upstream sequence itself is not enough to confer fat specificity and 22kb upstream sequence is enough to confer fat specificity. This result suggests a redundancy for leptin expression. It is also possible that 762 bp fragment is enough to confer fat specificity but is only effective with the enhancer from either upstream or downstream. In either case, these studies will help for further studies to identify the cis-elements that regulate leptin.Once the promoter region(s) is localized to less than 1kB region, gel shift experiments can be performed with nested ~ 50-100 bp fragments across the region using nuclear extracts from ob, fed and fasted adipose tissue. With this information in hand, it is straightforward to perform DNAse foot printing to map the precise binding site. This sequence can then be used as an affinity probe to facilitate the biochemical

purification of the differentially expressed transcription factor. This could potentially allow working back to a putative lipid sensing mechanism.

One other approach to find possible trans factors and mechanisms that regulate leptin expression is by using microarray analysis. Leptin expression is upregulated in obese/high fat fed animals and downregulated in fasted animals. Furthermore, leptin is adipocyte-specific such that when the adipose tissue is dissociated by collagenase digestion, the pelleted stromavascular fraction does not have any detectable leptin mRNA compared to adipocytes. In addition, leptin treatment of obese animals causes a time-dependent decrease in leptin gene expression. During development, leptin is reported to be upregulated postnatal day 8-10 -also known as the leptin surge (Ahima and Flier, 2000). In addition, time course microarray analysis of adipose tissue development show that leptin expression increase as lipid accumulates after P0. All these different conditions can provide as tools to find transcription factors that correlate with leptin gene expression.

The identification of molecular and cellular pathways regulating adipocyte development and regulation *in vivo* will have implications for basic and clinical understanding of obesity. Furthermore, the availability of systems such as the *leptin-luciferase* mice and hyperadipogenic A-Zip lipodystrophic animals will help to identify new cell populations and give the way to characterize *in vivo* adipogenesis. Overall, these approaches will provide a means to study the potential of cells from various sources to form WAT and creates new possibilities for the determination of the roles that individual factors play in the control and

maintenance of WAT mass *in vivo* in a variety of nutritional states, including obesity.

CHAPTER 9:

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