UNIVERSITY OF MILAN
Department of Medicine and Surgery

Doctorate of prenatal science, fetal diagnosis and therapy
XXIII Cycle
Settore scientifico-disciplinare MED/40

ADIPOSE TISSUE GROWTH AND MAINTENANCE

Co-ordinator of the doctorate: **Chiar.mo Prof. Enrico Ferrazzi**

Supervisor: **Chiar.ma Prof.ssa Irene Cetin**

Tutor: **Prof. Chad Cowan**

Tesi di Dottorato di
Dott.ssa Alessandra Rigamonti
Matricola R07603

Academic year 2009-2010

SUMMARY

Introduction

Obesity is fast becoming a global health pandemic. Characterized by an increase in adipose tissue to the point where it is associated with adverse health effects, the prevalence of obesity has nearly tripled over the past fifty years. Obesity has an enormous economic burden and is the second leading cause of preventable death. The molecular and cellular processes that regulate fat mass remain almost entirely unknown. White adipose tissue is the only tissue in the body that can markedly change its mass after adult size is reached. Indeed, fat mass can range from 2-3% of body weight to as much as 60-70% of body weight in humans. It is widely believed that the total number of fat cells present in most individuals is set during adolescence and that changes in fat mass generally reflect increased lipid storage in a fixed number of adipocytes. It was recently demonstrated that there is a substantial degree of cellular turnover within the human adipocyte population and the source of new adipocytes during one's lifetime is attributed to the differentiation of new adipocytes from preadipocytes and/or stem cells.

New adipocytes are thought to arise from a pool of progenitors that has recently been described and may reside adjacent to the adipose vascular tissue. A population of cells can also be purified based on cell surface marker expression and transplanted into lipodystrophic mice to reconstitute fat depots. Preadipocytes are generally considered to be replicative until the onset of a transcription factor cascade drives adipogenesis and causes growth arrest. Additionally, primary adipocytes seemingly lack proliferative activity *in vitro* and cell culture lines capable of adipocyte differentiation undergo cell cycle arrest during differentiation. Adipocytes are thought to represent a terminal stage of differentiation and are widely believed to lack proliferative ability.

How adipose tissue is regulated is a central question in the study of obesity. Obesity is commonly considered to be a disorder of energy imbalance in the organism. At the cellular level, however, the development of obesity has been attributed to both an increase in fat cell size, fat cell differentiation and increased adipocyte number. The notion that adipocytes can undergo replication has existed for decades but has not been widely accepted.

There are many important questions that are still open and unresolved: for example how do adipocytes form during development, how is proliferation regulated in adipose tissue, what are the dynamics of progenitors and how they are regulated during development and obesity….

Thus, an understanding of how adipocytes number is regulated postnatally has the potential to expand our knowledge of the pathogenesis of obesity, and may open up possibilities for novel therapeutic approaches that may prove to be of great importance in the treatment of obesity and diseases associated with obesity.

Experimental approach

We sought to investigate the growth and maintenance of adipose tissue and its behavior in the cell cycle through 4 independent experimental approaches:

1) dilution of an inducible histone 2B-green fluorescent protein (H2BGFP) through cell division.
2) DNA-replication dependent incorporation of the nucleotide analog BrdU.
3) labeling with the cell cycle marker Ki67 and PCNA
4) lineage analysis of adipose tissue by using AP2-CreER transgenic mice to investigate whether newly formed fat cells are derived from pre-existing

adipocytes or stem/precursor cell

Materials and methods

Mice

Rosa26-rtTA, tetO-H2BGFP and AP2-CreER mice were generously provided by Rudolf Jaenisch, Elaine Fuchs and Daniel Metzger and Pierre Chambon, respectively. Rosa26-rtTA and tetO-H2BGFP mice were backcrossed to >95% C57BL/6 inbred background. OB/OB, R26R and Rosa26-lacZ mice were obtained from JAX.

Genotyping

Genotyping was performed by adding a tail biopsy to 100µl DirectPCR (Viagen) with 30µg Proteinase K (Roche), incubating overnight at 55°C and denaturing Proteinase K for 20 minutes at 95°C. PCR conditions: 95°C for 5 minutes, then 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and finally 72°C for 5 minutes.

Doxycycline, BrdU and Tamoxifen

Doxycycline (Sigma) was added to drinking water at 1mg/ml and sweetened with sucrose (1%).. Animals were injected with 100 µl of 10mg/ml BrdU (Sigma) or 300µl BrdU Labeling Reagent (RPN201, Amersham). Tamoxifen (Sigma, http://www.sigmaaldrich.com) was resuspended in 90% corn oil, 10% ethanol at 50mg/ml, and heated to 55°C. Mice were injected with 3mg/40g mouse I.P.

Immunohistochemistry

Unless otherwise stated, wild-type mice were sacrificed at either 6- or 14-weeks of age. Abdominal fat tissue was dissected from mice, fixed in paraformaldehyde/PBS. Cryo samples were incubated in 30% sucrose/PBS solution overnight, embedded in OCT (Tissue-Tek) and stored at -80^{0}C. Paraffin samples were dehydrated through an ethanol series, washed three times in xylene, embedded in paraffin. Primary antibodies were applied over night at 4°C ,secondary antibodies were applied at room temperature for 1 hour (see the text for the different antibodies and dilution). To visualize nuclei, slides were stained with 0.5µg/ml DAPI (4',6-diamidino-2-phenylindole). Images were acquired using a Zeiss LSM510 Meta confocal microscope or a NIKON Eclipse TE2000-E. Ki67 and BrdU cell counts were conducted by epifluorescence microscopy or confocal microscopy.

Collagen embedding

Collagen embedding was performed on both floating adipocytes and diluted stromovascular (SV) cells (1:40). In both cases, cells were added to rat collagen type I hydrogel (42% collagen in DMEM), and then, following solidification, fixed in paraformaldehyde/PBS Embedded adipocytes were immunostained and imaged with a Zeiss LSM510 Meta confocal microscope or a NIKON Eclipse TE2000-E T. Embedded SV cells were stained Histomark X-gal Substrate (KPL) at 37°C, stained with DAPI and analyzed at a Leica DM6000B microscope.

For SV fractionation and culture, white abdominal adipose tissue was collected and minced into fine pieces (3 mm^2). To digest, adipose pieces were incubated in adipose isolation buffer containing collagenase II and DNaseI at 37°C. The filtered suspension was then centrifuged. The SV fraction plated in a 6-well culture dish. The cells were cultured in DMEM with FBS, penicillin/streptomycin, and gentamicin for 7 days. SV cultured cells were fixed in formaldehyde/glutaraldehyde. For lacZ staining all samples were incubated in X-gal staining buffer at 37°C for 4 to 16 hours, post-fixed

in formalin for 10 minutes and counterstained with DAPI. For cell counting, stained cells were photographed using a Leica DFC 420 camera (Leica Microsystems Ltd, United Kingdom). The number of β-gal-positive cells and total nuclei were counted. The percentage of β-gal-positive cells was expressed as a proportion to total nuclei.

FACS

For sorting of live, unfixed adipose cells, abdominal fat was dissected from mice, rinsed in PBS, and cut with scissors into small pieces of approximately 3 mm in diameter. The fat was then dissociated for 75 minutes in KRB solution with Collagenase II ,DNAse I. The homogeneous solution was centrifuged , the upper layer was collected and sorted on a BD FACS Calibur (Becton Dickinson).
For sorting of fixed and stained adipose nuclei, the floating fraction is considered to consist primarily of buoyant adipocytes while the pelleted cells are termed stromal/vascular (SV) cells. Both fractions were collected separately..

Nuclei were extracted by incubation 1:20 in cold lysis buffer. Nuclei were fixed in paraformaldehyde, pelleted and finally resuspended in a solution of Tween 20 and paraformaldehyde in PBS for one hour before being subjected to Ki67 (PE conjugated, BD PharMingen) and C/EBPα immunocytochemistry, stained with DAPI and sorted on a BD FACS LSRII (Becton Dickinson).

Results

No label-retaining cells are detected in adipose tissue

To determine whether all the cells in the adipose tissue turnover at the same rate in the adult mouse, the tetracycline-inducible H2BGFP was used as a tool: any H2BGFP label-retaining cell (LRC) is thought to have incorporated the H2BGFP into chromatin after induction and did not replicate (histones are diluted by approximately half each replication cycle); if there is a reduction or total loss of GFP fluorescence this indicates the previously labeled cells have divided or died. No outlying population of LRCs was identified in the adipose tissue. The uniform loss of the H2BGFP label with time suggested homogeneity exists within the adipose pool. Stated otherwise, all the cells within the adipose tissue appear to tunover similarly.

BrdU analysis suggestes 1% of murine cells within the adipose tissue are in S-phase per day

In order to investigate the cellular turnover in the adipose tissue, and verify that the cells in the adipose tissue undergo frequent replication, we next assayed the ability of the cells to incorporate an artificial nucleotide analog, 5-bromo-2'-deoxyuridine (BrdU), via DNA synthesis. Immunohistochemical analysis of BrdU incorporation found that within the Perilipin-expressing cells population, 0.6% of the cells had incorporated BrdU per day and that 1.8% of C/EBPα-positive cells were in S-phase per day. It is important to note that BrdU labeling of Perilipin-expressing adipocytes can also result via BrdU incorporation into replicating preadipocytes immediately prior to adipocyte differentiation.

Approximately 5% of C/EBPα positive cells in the adipose tissue are in the cell cycle at any time

We next investigated the known marker of the division Ki67 in the adipose tissue of C57/BL6 mice at various ages and we found that 4.8% of C/EBPα-positive cells were

in the cell cycle at any time. FACS analysis of nuclei from dissociated fat tissue of 8 week old wild-type mice showed that the total percentage of Ki67-positive C/EBPα-positive nuclei in the floating fat sample was 2.36%; of this 0.45% represented C/EBPα-high Ki67-positive events. FACS plots of the stromal/vascular nuclei from dissociated fat tissue of wild-type mice, stained for Ki67 and C/EBPα showed that the total percentage of Ki67-positive C/EBPα-positive nuclei was 3.85%; of this 0.06% represented C/EBPα-high Ki67-positive events, and 3.79% represented C/EBPα-low Ki67-positive events. In addition, performing confocal analysis on whole-mount adipose tissue of 3 animals we counted a total of 4.2% of Ki67 positive nuclei, of this 0.8% represented CD31 Ki67 positive cells, 1.8% of Ki67 positive cells were negative for CD31 and overlapped with Perilipin expressing cells, while for 1.5% of Ki67 positive cells we could not determine the nature of the cellular type.

Lineage analysis to investigate whether new adipocytes arise from existing adipocytes
Because the above data suggested adipocytes may give rise to new adipocytes, we directly compared the replication capacity of the adipose cells by performing a lineage analysis of adipose tissue. We examined white adipose tissue from AP2-expressing cell-specific cre excizable LacZ cassette (AP2-CreER;R26R) pulse–chase animals. LacZ would be expressed in the cells of these animals that have expressed AP2, a gene expressed in adipocytes, and we did not observe loss of lacZ label with time.
Therefore, though sufficient turnover of the adipose tissue occurs within two months to dilute the H2BGFP label *in vivo*, our preliminary experiments may suggest that one source of these new adipocytes may be preexisting adipocytes, as evidenced by the permanent, heritable lacZ labeling within fat cells. According to this hypothesis, the adipocyte population would be maintained at least in part by adipocyte replication. However we detected 1.9% of LacZ positive cells in the SV fraction that might represent a population of precursors which proliferate to give rise to a substantial proportion of adipocytes.

Obese mice show increased adipose tissue replication
To ask whether adipose tissue replication is altered in obesity, we determined the percentage of C/EBPα-positive cells in the cell cycle in 14-week-old wildtype and OB/OB mice, all of which were maintained on a C57/BL6 background. Ki67 and C/EBPα co-expressing nuclei were counted for both the wildtype and OB/OB groups, and found to be 4.6% and 7.3%, respectively. This represents a statistically significant increase in adipose tissue replication in OB/OB mice ($p<0.05$). BrdU and C/EBPα co-labeled nuclei were counted for both groups, and BrdU incorporation was found to undergo a significant increase ($p<0.001$), from 2.9% to 9.7%, in OB/OB mice. We then determined the percentage of C/EBPalpha positive cells in the cell cycle in 6-week-old OB/OB and 14-week-old OB/OB mice. We counted the percentage of Ki67, C/EBPα within Perilipin expressing cells and found it to be 2.4% and 10.4%, respectively.

Diet induced obesity (DIO) mice don't show increased adipose tissue replication
We next sought to determine whether adipose tissue replication changes in diet-induced obese (DIO) mice. DIO mice are C57/BL6 mice fed with high fat caloric diet since the day of weaning. We determined the percentage of cells within the adipose tissue in cell cycle in 14-week-old DIO mice. Ki67 and C/EBPα co-expressing cells were counted within Perilipin population and found to be 3.6% while the total number of Ki67 positive cells in the adipose tissue was 4.03%. Our preliminary experiment

didn't show statistically significant increase in adipose tissue replication in 14 weeks old DIO mice compare to the wilde type animals ($p>0.05$).

Murine adipose tissue replication is diminished during the second half of pregnancy.
We sought to determine whether the rate of adipose tissue replication is altered during mice pregnancy. The percentage of BrdU and Perilipin co-expressing cells were counted for the first part of the pregnancy. BrdU incorporation was found to be 0.9% per day compared to 0.6% found in the non-pregnant wilde type mice control ($p<0.05$). During the second part of pregnancy we counted the percentage of BrdU and Perilipin co-expressing cells and found it to be 2% any time. These was a decrease of BrdU incorporation over the last part of the pregnancy. We counted Ki67 positive cells within the Perilipin population and found to be 3.5% compare to 1.8% of the non-pregnant wild type mice.

Increased replication of adipose tissue after partial lipectomy is not statistically significant
We sought to investigate whether the rate of epididymal adipose tissue replication changes after lipectomy. In the lipectomized mice, we found that in the adipose tissue proximal to where fat was removed, 10.9% of the cells incorporated BrdU, while in the adipose depots where fat was not removed, 4.4% of the cells incorporated BrdU. There was not a statistically significant difference in replication between the two fat depots($p=0.07$).

Evidence for replication in human adipose tisssue
To investigate the percentage of adipose tissue replication in human, we assessed a known marker of cell division within the human adipose tissue *in vivo* from human tissue samples. We found 1.5% of this adipose tissue to be Ki67-positive, and, that 0.7% of cells in adipose tissue are positive for Ki67 and C/EBPα within the Perilipin population at any time.

Conclusion and discussion
Our experiments, allowed us to estimate the turnover rate of cells in adipose tissue based on the addition of new cells measured by BrdU incorporation and loss of cells measured by the loss of H2B-GFP in the tissue.
The diminution of H2BGFP intensity in the adipose tissue may be explained by several reasons.
∗ GFP dilution due to high replicative rate of preadipocytes and adipocytes.
One possibility could be a high replicative rate of cell division, suggesting that all fat cells, mature adipocytes and preadipocytes contribute equally to fat growth and maintenance in wild-type mice. However, although our data, including our preliminary lineage tracing experiments may suggest that mature adipocytes are capable of replication, we cannot conclude this hypothesis since we need to provide further evidence of adipocytes replication. It is very important to remember that the analysis of adipose tissue by immunohistochemistry is quite challenging, making it very difficult to determine if a nuclear antigen such as Ki67 is present in the nucleus of an adipocyte. Thus our results cannot be considered definitive proof of mature adipocyte replication due to the absence of a nuclear marker for mature adipocyte. High resolution

histological evidence demostrating fat cells with mitotic figures is critical direct evidence that adipocytes divide. This could be done by staining with markers of mitosis such as PH3, single fat cells after adipose tissue digestion. Another important experiment which would allow us to determine whether mature adipocytes are capable of replication it would be a lineage analysis with a mature marker of fat cells such as Leptin.
In addition, a critical experiment would be to use our inducible CRE system to knockout a protein required for replication in mature adipocytes . This will then allow us to confirm the veracity of our assay but more importantly assess the importance of adipocyte replication for maintenance of adipose tissue mass under normal and obesogenic condition.
Furthermore, we do not know of an example of a mature differentiated cell type that has two populations (one replicative and the other not). We can speculate that when tissues are without an adult stem cell, they are replenished by equal replication of all differentiated cells.
If adipocytes divided, the adipose tissue would represent the first example of tissue that has both stem cells (or progenitors) and mature cells which divide, both maintaining the tissue.
* Dilution of GFP due to apoptosis ,necrosis or macrophages enfulfment.
However, until definitive proof that mature adipocytes can undergo cell division is obtained, other interpretations of the diminution of fluorescence intensity have to be considered. One such alternative explanation for our findings could be that adipocytes are lost due to apoptosis, or macrophage engulfment or necrosis. This support the idea that adipocytes are postmitotic and that adipose tissue is maintained by stem cell/progenitor population. Numerous data show that adipocyte precursors are capable of both maintaining the progenitor pool and producing adipocytes, and indeed, we found evidence of replication in the SV fraction of adipose tissue in all of our experiments

Although obesity is a metabolic disorder ultimately caused by energy imbalance, a greater understanding of adipose tissue growth and maintenance may one day aid in the treatment of obesity. Our data support the notion of a dynamic fat cells turnover, and also that in the case of one of the most clinically relevant models of increased fat mass in mice, the rate of adipose cells replication is significantly increased. If this is the case in the human population we can imagine two timepoints of therapeutic intervention against adipose tissue replication in cases of human obesity. First, it is widely accepted that there is a substantial increase in fat cell number during adolescence and it is believed that an elevated number of adipocytes at the end of this period is a strong predictor of adult obesity. The ability to slow the rate of the adipose tissue replication during this critical period of adolescent and early adult development may therefore prevent both juvenile and adult obesity. Second, it may be that adult obesity itself is caused, or exacerbated, by elevated rates of precursor and/or adipocytes replication. If this is true, targeted intervention to reduce adipocyte replication in adults may facilitate weight loss in obese patients.
There are many important questions that are still open and unresolved: for example how do adipocytes form during development, how is proliferation regulated in adipose tissue, what are the dynamics of progenitors and how they are regulated during development and obesity…. How the turnover of adipose tissue is regulated in term of adipogenesis, differentiation, replication and apoptosis? Thus, the answer to these questions has the potential to expand our knowledge of the pathogenesis of obesity,

and may open up possibilities for novel therapeutic approaches that may prove to be of great importance in the treatment of obesity and diseases associated with obesity.

TABLE OF CONTENTS

SUMMARY…………………………………………………………….... 2

ABSTRACT………………………………………………………… 11

INTRODUCTION…………………………………………………….. 13

1) WHAT IS ADIPOSE TISSUE………………………………………………...21

Adipose as an organ:
energy balance, glucose homeostasis and functions………………..16

Adipocyte and energy balance……………………………………………………….16

Glucose homeostasis and adipose as ogans……………………………………….18

2)WHY STUDY ADIPOSE TISSUE……………………………………………………………21

Link between obesity, insulin resistance and type 2 diabetes….21

Role of obesity in metabolic syndrome and cardiovascular disease.24

Relationship between insulin resistance, visceral adiposity and endothelium dysfunction………………………………………………………25

Obesity and atherosclerosis……………………………………………………..26

Adipose tissue and cancer………………………………………………27

Effect of adipose tissue in human pregnancy………………………28

3) ORIGIN OF ADIPOSE TISSUE…………………………………………….29

Where and when does fat come from?.......................................29.

Early development………………………………………………………………29

White-brown fat and muscle…………………………………………………31

Adipose tissue development during fetal life…………………………………33

Adipogenesis……………………………………………………………………34

Molecular player (transcriptional cascade)……………………………35

4) GROWTH OF ADIPOSE TISSSUE AND MAINTENANCE…………..37

AIM OF THE PROJECT………………………………………….41

MATHERIALS AND METHODS………………………………..43

RESULTS……………………………………………………………. 49

No label retaining cells are detected in adipose tissue……………50.

BrdU analysis suggestes 1% of murine fat cells are in S-phase per day..61

Approximately 5% of C/EBPα positive cells in the adipose tissue are in the cell cycle at any time…………………………………………………………………65

Preliminary lineage analysis suggests that new adipocytes arise from existing adipocytes……………………………………………………………………77

Obese mice show increasd fat cells replication……………………………82

Diet induced obesity (DIO) mice don't show increased fat cells replication……………………………………………………………………….86

In pregnant mice fat cells replication is diminished during the second half of pregnancy……………………………………………………………………….87

Increased replication of adipose tissue after lipectomy is not statistically significant……………………………………………………………………… 91

Evidence for replication in human adipose cells……………………………94

TABLES……………………………………………………………96

CONCLUSION AND DISCUSSION………………………………..115

REFERENCES…………………………………..…………………124

ABSTRACT

Because obesity is fast becoming a global health pandemic, understanding the molecular and cellular processes that regulate fat mass has acquired new importance. Characterized by an increase in adipose tissue to the point where it is associated with adverse health effects, the prevalence of obesity has nearly tripled over the past fifty years. Though for many years adipose tissue was considered to be merely a storage depot for fatty acids, it is now regarded as an important endocrine organ involved in the regulation of energy balance, glucose and lipid homeostasis, blood pressure control, reproduction, inflammation and immune response. Obesity has an enormous economic burden and it is the second leading cause of preventable death. The source of increased fat mass in obesity is currently attributed to two mechanisms: adipocyte hypertrophy, the process by which pre-existing fat cells increase in size due to an accumulation of lipids, and adipocyte differentiation from fat precursor cells.

Stem cells are defined by the ability to self-renew and differentiate into a variety of cell types. While some adult organs, including the intestine (Cheng and Leblond, 1974), skin (Oshima et al., 2001), blood (Spangrude et al., 1988), and parts of the brain (Doetsch et al., 1999; Reynolds and Weiss, 1992), are maintained by stem cells, others, such as the pancreas (Dor et al., 2004), are not. Though it was recently demonstrated that there is a substantial degree of cellular turnover within the human adipocyte population (Spalding et al., 2008), the source of new adipocytes during one's lifetime has been entirely attributed to the differentiation of new adipocytes from preadipocytes and/or stem cells (Avram et al., 2007; Greenwood and Hirsch, 1974; Hausman et al., 2001; Lemonnier, 1972; Salans et al., 1971; Spalding et al., 2008; Tang et al., 2008). Adipocytes are thought to represent a terminal stage of differentiation and are widely believed to lack proliferative ability (Prins and O'Rahilly, 1997). Through four independent experimental approaches —dilution of an inducible histone 2B-green fluorescent protein (H2BGFP) through cell division, incorporation of BrdU, labeling with the cell cycle marker Ki67, and genetic

lineage analysis, we aimed to investigate the adult adipose tissue maintenance and its behavior in the cell cycle.

INTRODUCTION

1) WHAT IS ADIPOSE TISSUE?

Fat tissue is characterized histologically as a special loose connective tissue containing lipid-laden adipocytes, vascular endothelial cells, fibroblasts, macrophages and parenchymal fibers.

There are two principally different types of adipocytes: white and brown. These cells have distinct anatomy and function, different developmental attributes and different responses to hormonal signals. White and brown fat cells are organized in two tissues, the white adipose tissue (WAT) and the brown adipose tissue (BAT) which form a multidepot organ: the adipose organ [Cinti 2005, Rosen and Spiegelman 2006].

In mammalian organisms, the major fat depots are located either subcutaneously, existing in loose association with the skin, or within the body cavity, which include fat associated with the intestinal mesentery, fat in the retroperitoneum and fat surrounding the heart (fig.a).

Brown adipose in rodens resides in interscapular, subscapular, axillo-toracic, superficial and deep cervical regions, whereas in humans it surrounds the heart and the great vessels in infancy but tends to disappear over time residing, in adults, in interscapular, supraclavicular, cervical, axillary and paravertebral regions. Brown fat can also be found in white adipose depots and skeletal muscle in small amount. [Cinti 2005,Rosen and Spiegelman 2006, Nedergaard 2007].

Brown fat cells are multilocular and rich in large mitochondria containing the unique UCP1 protein (uncoupled protein 1) which functions to dissipate the proton motive force, that is normally used to drive the synthesis of cellular ATP, resulting in heat production. Studies in rodents also show that BAT plays an important role in energy balance and influences body weight. [Seale and Lazar 2009,Seale , Spiegelman,2009].

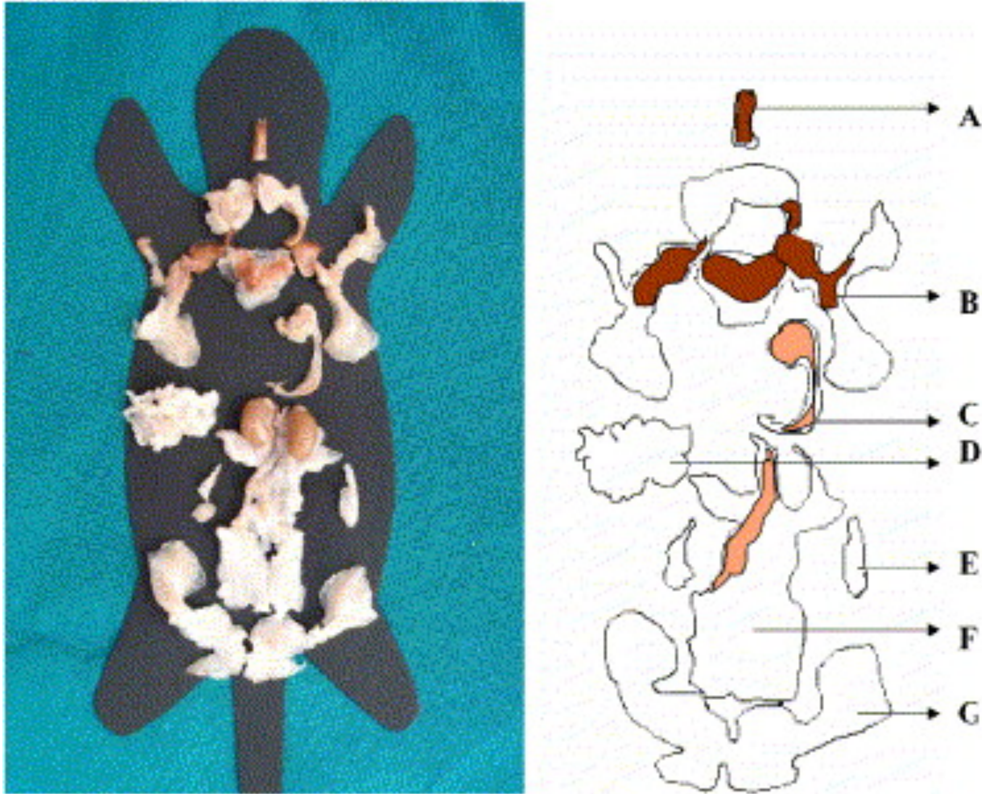


Fig.a The adipose organ of an adult Sv129 mouse maintained at 29 °C for 10 days. The organ has been dissected with the aid of a surgical microscope and each depot has been placed on the mouse profile mimicking its anatomical position. The organ is made up of two subcutaneous and several visceral depots. The most representative visceral depots are shown. Kidneys and testes were dissected together with the depots. Names of the single depot: A=deep cervical B=anterior subcutaneous (interscapular, subscapular, axillo-toracic, superficial cervical) C=visceral mediastinic D=visceral mesenteric E=visceral retroperitoneal F=visceral perirenal, periovaric, parametrial and perivescical G=posterior subcutaneous (dorso-lumbar, inguinal and gluteal). White areas made up of white adipose tissue and brown areas composed of brown adipose tissue are indicated by the scheme. Imagine taken from Cinti S 2005.

Mature white adipocytes are unilocular, contain a single large lipid droplet and posses a spherical form that varies in size. The cytoplasm is thin and stretched to form a sheath that surrounds the lipid drop. Therefore, the nucleus is pushed against the edge of the cell, and resembled a flattened semilunar structure. Few mitochondria can be observed around the nucleus, with roughly arranged membrane cristae. The cytoplasm contains few short profiles of granular endoplasmic reticulum, and the Golgi zone is small. About 90% of the cell volume is a lipid droplet that comprises a mixture of neutral fats, triglycerides,

fatty acids, phospholipids and cholesterol.

Lipids stored in the fat cells are mobilized and renewed through fatty acid esterification and triglyceride hydrolysis which take place to release fatty acids into the circulation when fuel is limited, or to store excess energy [Fruhbeck 2008].

Adipose tissue as an organ: Energy balance, glucose homeostasis and functions

Adipose tissue had long been considered merely a storage depot for fatty acids and of no particular functional relevance. With the discovery of the adipose secreted factor Leptin, and the emergence of obesity as a serious economic burden and public health problem, the biology of adipose tissue has been acquiring scientific and medical interest. Adipose tissue is now broadly accepted as an important endocrine organ involved in the regulation of several physiological pathways, including energy balance, glucose and lipid homeostasis, blood pressure control, reproduction, and inflammatory and immune responses.[Bulcao 2006, Rosen and Spiegelman 2006].

Adipocytes and energy balance

The First Law of Thermodynamics is an expression of the principle of conservation of energy and states that energy can be transformed (changed from one form to another), but cannot be created or destroyed. Between any two equilibrium states, the change in internal energy (E) is equal to the differences of the heat transfer (Q) into the system and the work (W) done by the system.

$$E2-E1= Q-W \quad (1)$$

The First Law of Thermodynamic governs energy balance in animals, and can be expressed by this simple equation:

Energy intake = energy burned + energy stored

Several factors impact energy balance, including hormones and neural input, in addition to physiological and cultural factors [Abizaid 2006,Rosen and Spiegelman 2006]. It is widely believed that energy homeostasis depends upon the balance between caloric intake and energy expenditure, encompassing basal metabolism, physical activity and adaptive thermogenesis (energy spent to maintain body temperature and energy spent in absorbing and processing the diet).

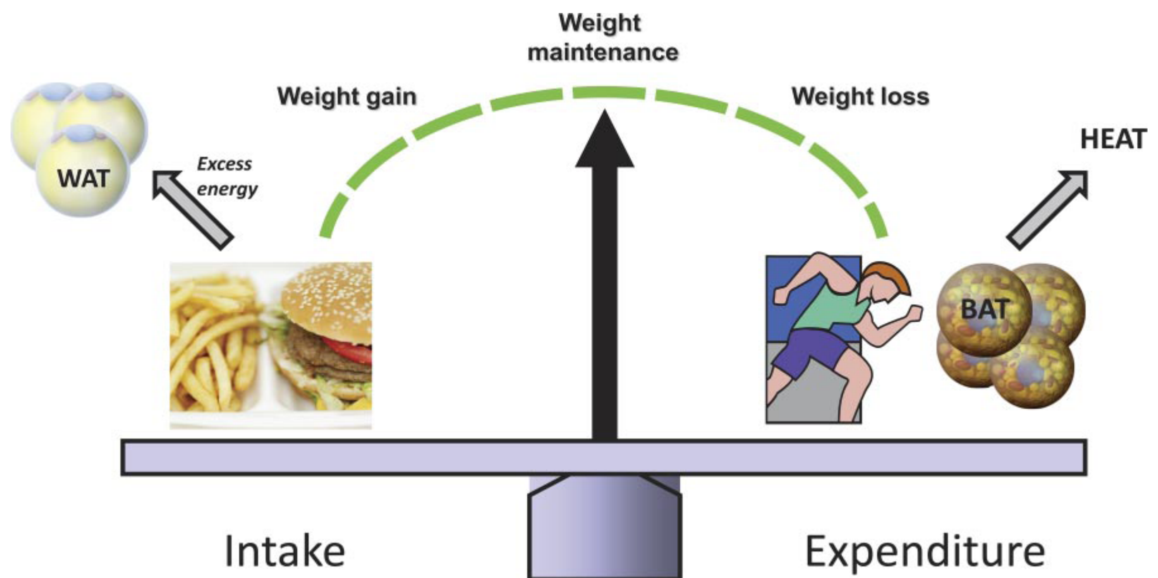


Fig.b Weight gain is caused by a fundamental energy imbalance when energy intake from food chronically exceeds energy expended by physical activity and metabolic processes. Several studies suggested that BAT activity could impact daily energy expenditure Imagine taken from Seale P, Lazar M.A. Diabetes 2009.

Lipid storage in fat cells is the result of excess energy consumption relative to energy expenditure (fig.b). Thus adipose tissue, through both endocrine and non-endocrine effects, plays a critical role in energy balance.

To achieve a balanced energy homeostasis, fatty acids are released through lipolysis when energy expenditure increases, whereas less energy expenditure and increased food intake results in enhanced fat storage. The central and autonomic nervous systems (CNS, ANS) are integrated to regulate energy expenditure depending on physiological needs. Fat pads are richly innervated

by sympathetic fibers that enhance lipolysis. Conversely, the parasympathetic nervous system positively influences lipid storage [Redinger 2009. Rosen and Spiegelman 2006].

The hypothalamic arcuate nucleus (ARC) houses two kinds of neurons: NPY/AgRP and POMC/CART neurons that are regulated by circulating hormones. During fasting, the gastric hormone ghrelin is released into the circulation and stimulates the ARC to release neuropeptide Y (NPY) and Agouti related protein (AgRP) which stimulate food intake and decrease energy expenditure.

Postprandial fasting for 6 or more hours also stimulates the lateral hypothalamic nucleus to release the orexigenic neuropeptides orexin A and B, which increase caloric intake. In contrast, after a meal, insulin and adipocyte secretion of leptin, which increase in proportion to body adipose stores, inhibit NPY/AgRP neurons and stimulate adjacent POMC/CART neurons, resulting in decreased food intake and increased energy expenditure. Furthermore, both leptin and insulin positively facilitates mitochondrial beta oxidation of released fatty acid, resulting in increased peripheral energy expenditure [Cumming and Schwartz 2003,Redinger 2009].

Glucose homeostasis and adipose as organs

In healthy mammals, serum glucose levels are relatively steady throughout the day, despite intermittent intake of dietary carbohydrates. Glucose homeostasis requires the concerted actions of various organs. For instance, increased glucose levels after eating leads to the release of insulin by β pancreatic cells, resulting in glucose disposal in adipose tissue and muscle and suppression of glycogenolysis and gluconeogenesis in liver. During fasting, low insulin levels together with elevated levels of hormones such as glucagon, adrenaline and corticosteroids promote hepatic glucose production. [Tirone 2001, Herman 2006,Rosen and Spiegelman 2006].

Recent evidences suggest that the CNS can also coordinate many of these

effects and affect systemic glycemia regulating gluconeogenesis. [Herman 2006].

Adipose tissue has recently been recognized as having important effects on glucose balance through several different mechanisms, endocrine as well as non-endocrine. Adipocytes and other cells which reside within the adipose tissue secrete proteins with varied effects: leptin and adiponectin (discussed below), visfatin, and omentin have anti-diabetic action whereas other factors including resistin TNF-α, IL-6, and RBP4 have pro-hyperglycaemic action tending to raise blood glucose.

- Leptin

The multifunctional protein leptin, secreted by adipocytes, plays an important role in the regulation of whole-body metabolism by inhibiting food intake, stimulating energy expenditure and by its anti-hyperglycaemic action. Serum leptin levels manifest a diurnal rhythm with the highest levels between 23:00 and 01:00 h, after which plasma leptin declines until early afternoon. During the day leptin levels vary based on meal timing.[Galic 2009, Sinha 1996,Saladin 1995] Furthermore, it has been reported that there is a positive linear correlation between circulating levels of leptin and total body fat mass [Galic 2009, Lonnqvist 1997]. The leptin receptor (Ob-R) is found in high concentration (30-40% of tot Ob-R) in the brain, in areas that regulate feeding such as the arcuate, dorsomedial, and ventromedial hypothalamoc nuclei [Tartaglia 1997]. It is also found in lower concentrations (5-8% of tot Ob-R) in several tissues including adipose tissue, pancreatic β-cells, liver, heart and skeletal muscle, lung, peripheral blood mononuclear cells, ovaries, testis, placenta, adrenal medulla, and articular chondrocytes [Galic 2009].

The anti-hyperglycaemic actions of leptin are mediated by direct and indirect effects on several target organs. Leptin determines increased insulin sensitivity in liver and muscle. Through the activation of AMP protein kinase and an indirect CNS pathway, leptin determines a reduction in intra-myocellular lipids,

improving insulin sensitivity. [Rosen and Spiegelman 2006,Minokoshi 2002, Kamohara 1997]. It is also hypothesized an 'adipo-insular axis', with insulin promoting leptin release and leptin inhibiting insulin secretion. [Kieffer 2000, Rosen and Spiegelman 2006].

The role of leptin in energy balance and glucose homeostasis has been ascribed to genetic mutation in mammals. Ob/Ob mice ,where the leptin gene is mutated due to a C-T substitution leading to a non-functional gene product, and db/db mice, carrying a loss-of-function mutation in the Ob-R gene, are obese and hyperglycaemic. In keeping with this, it has been reported that the administration of leptin can reverse hyperglycaemia in Ob/Ob mice but not in db/db mice. Leptin also ameliorates glucose homeostasis in lipodystrophic mice and in humans with congenital leptin deficiency and lipodystrophy. [Schwartz 1996,Shimomura 1999,Oral 2002]

- *Adiponectin*

The hormone adiponectin is an abundant plasma protein secreted by the adipose tissue. This protein contains an amino-terminal collagen-like domain and a carboxy-terminal globular domain that mediates multimerization. Adiponectin circulates in serum at extraordinary high concentrations and can exist as a trimer, hexamer or at highermolecular-weight dodecamers [Waki 2003,Pajvani 2003, Barre 2006]. It has been reported that unlike other adipokines, plasma adiponectin concentration is negative correlated with body fat mass, with obesity reducing adiponectin levels, while weigh reduction increases it.[Hu 1996, Yatagai 2003, Galic 2009].

Like leptin, adiponectin improves fatty acid oxidation and glucose uptake in skeletal muscle and adipose tissue and suppress hepatic glucose production and release in an AMPK-dependent manner [Tomas 2002,Wu 2003,Yamauchi 2002, Combs 2001]. Through activation of AMPK in the hypothalamus, adiponectin regulates also energy expenditure stimulating appetite.

2) WHY STUDY ADIPOSE TISSUE?

Obesity has become a global health concern, surpassing famine and infectious diseases. Rapidly increasing over recent decades, obesity is characterized by an increase in fat mass to the extent that it causes adverse effects on health and longevity. Obesity is a tremendous economic burden and the second leading cause of preventable death in US (Flegal 2002, Ogden 2006, Kuczmarsky 2002, Kopelman 2000 Mokdad et al 2004).

The etiology of obesity is multifactorial; a combination of genetic factors [Barsh et al 2000, Kopelman 2000] interacting with environmental factors such as diet, physical activity and sleep, influence the individual susceptibility to develop obesity. The ultimate cause of increased fat mass, however, is thought to be due to an energy imbalance, in which food intake exceeds energy expenditure.

Chronic energy imbalance results in an increase in triglyceride stores in adipocytes, leading to adipocyte hypertrophy and hyperplasia. These changes in adipose tissue provoke endoplasmic reticulum stress and mitochondrial dysfunction, which in turn lead to the release of adipokines, fatty acids and inflammatory mediators in the blood stream. These alterations in circulating factors have adverse effects on skeletal muscle, heart, liver, vascular endothelium and pancreatic β-cells. (DeFerranti 2008, Kahn 2006, Guilherme 2008). Consequently, obesity is associated with a number of diseases, including type 2 diabetes, insulin resistance, cardiovascular disease, and cancer.

Link between obesity, insulin resistance and type 2 diabetes

It is believed that obesity plays a critical role in the development of insulin resistance and metabolic diseases. Insulin resistance may occur during puberty, pregnancy and with aging, while increased physical activity and a carbohydrate enriched diet are associated with enhanced insulin sensitivity. [Moran 1999, Buchana 1990, DeFronzo 1979, Goodyear 1998, Kahn 2006]. The mechanisms linking obesity to insulin resistance and type 2 diabetes are not well understood; though a defect in insulin release by β cells could be crucial for the pathogenesis of these diseases.

In obese individuals, adipose tissue secretes increased amounts of non-esterified fatty acids (NEFAs), glycerol, hormones and pro inflammatory factors that have all been suggested to contribute to the development of insulin resistance. Under normal conditions, the pancreatic β cells increase insulin release sufficiently to overcome the reduced efficiency of insulin action, thereby maintaining normal glucose tolerance [Perley 1966, Polonsky 1988, Kahn 1993, 2006]. When insulin resistance becomes associated with type 2 diabetes, β-cells are incapable to compensate fully for decreased insulin sensitivity. Even when glucose levels are still normal, β-cells dysfunction could exist and lead to a high risk of developing the diseases. NEFAs are believed to be one of the major culprits as they have been found to induce both insulin resistance and beta cells dysfunction which often result in type 2 diabetes [Reaven 1988,Boden 1997]. It has been reported that when intracellular NEFAs increase, a competition with glucose for substrate oxidation occurs, leading to the serial inhibition of pyruvate dehydrogenase, phosphofructokinase and hexokinase II activity [Randle 1963, Kahn 2006]. Moreover, a decrease in fatty acids metabolism results in an increase of metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme A and ceramides which cause the diminish of a series of events downstream the insulin receptor signaling, and affect insulin sensitivity [Shulman 2000].

The distribution of body fat may play a role in insulin sensitivity. Peripheral distribution of fat is mostly associated with insulin sensitivity whereas

abdominal and chest fat is linked to insulin resistance and metabolic disorders [Kahn 2006]. Abdominal and subcutaneous fat express different genes encoding secretory proteins and proteins responsible for energy production.[Kadowaki, 2006,Maeda 1997,Kahn 2006]. Intra-abdominal adipose tissue is also more lipolytic and less sensitive to the anti-lipolytic effect of insulin compared to subcutaneous fat [Montague 2000, Kahn 2006].

Furthermore, nearness of the liver to the intra-abdominal fat may result in a greater exposure of this organ to NEFAs .This could be one of the reason of why the liver can be insulin resistant at a time when other distal organs are not. [Kahn 2006,Kim 2003].

In obesity, other factors released in greater quantity by the adipose tissue, are believed to have an important impact on the metabolism. In muscle, retinol-binding protein-4(RBP4) induces insulin resistance through the reduction of PI(3)K signaling, while in liver a retinol-dependent mechanism induces the increase of the gluconeogenic enzyme phosphoenolpyruvatecarboxykinase, which contributes to enhance insulin resistance [Wellen 2005, Yang 2005, Kahn 2006].

In addition, macrophages and other cells that populate the adipose tissue are entailed in developing insulin resistance. Proinflammatory proteins such as chemoattractant protein-1 (MCP-1), secreted by adipocytes, endothelial cells and monocyte, increase macrophages recruitment that can lead to insulin resistance [Kahn 2006].

Increased release of tumor necrosis factor-α (TNFα) and interleukin-6(IL-6) stimulate the c-Jun amino-terminal kinase (JNK) and the IkB-β(IKK-β)/nuclear factor-kβ (NF-kβ) pathway, determining upregulation of inflammatory mediators and thereby contribute to a feedforward process.[Kahn 2006].

Genes and environmental factors together are believed to be largely responsible for the development of obesity and insulin resistance. So far several genes have been identified to be susceptible for developing obesity, including mutations in the melanocortin-4 receptor genes, leptin and leptin receptor, prohormone

convertase 1 (PC1) and pro-opiomelanocortin (POMC). Other genes are associated with insulin sensitivity like P12A polymorphism in PPARγ and several other genes are known to be associated with βcells dysfunction [Barroso, 2005, Kahn 2006].
It is thought that genes accountable for insulin resistance and obesity interact with environmental factors such as decreased physical activity and increased fat and caloric intake determining secretory demand on β cells. In case β cells are healthy, they increase their function and mass in response to the increased secretory demand, leading to compensatory hyperinsulinemia and maintenance of normal glucose tolerance. Whereas, susceptible β cells which have a genetically determined risk, are incapable of responding to the increased secretory demand. This, together with a nocuous environment leads to β cells dysfunction and decreased β cells mass, resulting in progression to impaired glucose intolerance followed, ultimately, by the development of type 2 diabetes [Kahn 2006].

Kahn and colleagues [Kahn 2006] summarize a possible model that link the role of impaired insulin release with obesity, insulin resistance and type 2 diabetes.
Beta cells dysfunction results in impaired insulin secretion with decreased insulin levels and decreased signaling in the hypothalamus which leads to increased food intake and weight gain, increased hepatic glucose production, reduced efficiency of glucose uptake in muscle and increased lipolysis in the adipocyte, resulting in increased plasma NEFA levels. The increase in body weight and NEFAs contributes to insulin resistance. The increased glucose and NEFA levels together may have additional adverse effect on beta cells health and insulin action, causing glucolipotoxicity [Kahn 2006].

Role of obesity in metabolic syndrome and cardiovascular disease

Central obesity, insulin resistance and type 2 diabetes together with hypertension and dyslipidemia are mutually related are suggested to form a cluster of cardiovascular risk factors which define the metabolic syndrome.

Insulin resistance results in metabolic derangements leading to hypertension, dyslipidemia and an increased cardiovascular disease (CVD) risk. Adipokines secreted by visceral fat can have vascular consequences that increase CVD risk.

A common item of many cardiovascular risk factors is endothelial dysfunction [Huang,2009]. The endothelium comprises the inner lining of blood vessels and not only serve as a mechanical barrier but also responds to physiological and pathologic stimuli producing vasoactive substances such as nitric oxide (NO), prostacyclin and endothelins [Huang, 2009, Gimbrone 1995, 2000]. In normal circumstance endothelium protects against atherogenic processes which involve oxidative stress, abnormalities in vascular signaling, inflammatory cells and thrombosis, whereas endothelial dysfunction is central to the pathogenesis of atherosclerotic lesion development [Ross, 1999,Steimberg 2006, Huang 2009].

Type 3 nitric oxide synthase (eNOS) enzyme produces NO which serves several functions such as the suppression on vascular smooth muscle cell proliferation, the

regulation of vascular tone and regional blood flow, modulation of leukocyte-endothelial interactions and modulation of thrombosis. When the endothelium is incapable of serving its normal protective and physiologic mechanism, endothelium dysfunction occurs. The endothelium can be damaged due to metabolic toxins such as free fatty acids (FFAs) and inflammatory cytokines, including IL-6 and TNFα. Reactive oxygen species (NOS), including superoxide, could prevent NO from inducing vascular smooth muscle relaxation. Moreover, hyperglycemia along with other metabolic abnormalities may alter endothelial intracellular signaling pathway such the PI3K-Akt pathway.

Overall, there are multiple mechanisms that can lead to endothelium dysfunction, however, the endpoint is thought to be the reduction in the amount

of bioavailable NO which normally serves to protect the vessel from the molecular events that lead to atherosclerosis [Huang 2005].

Relationship between insulin resistance, visceral adiposity and endothelium dysfunction

In normal condition insulin signaling culminates in glucose uptake by fat and skeletal muscle, vasodilatation from increased eNOS activity and suppression of hepatic gluconeogenesis.[Kim 2006,Semenkovich,2006, Huang 2009].

After insulin binds the insulin receptor, two separate and parallel pathways are believed to occur: PI3KAkt and Ras/Raf/MAP kinase.

In muscle and adipose tissue, PI3K-Akt pathways results in translocation of GLUT4 and glucose uptake, whereas in endothelial cells causes increased NO production and vasodilation.

The MAP kinase pathways results in growth and mitogenesis in vascular smooth muscle and determines endothelin-1 production and vasoconstriction in endothelial
cells [Nystom 1999, Saltiel 2001, Huang2009].

The relationship between endothelial dysfunction, insulin resistance and visceral
adiposity is thought to be intricate.

Insulin resistance alters the balance between the two pathways downstreams insulin
signaling and can cause endothelial dysfunction. In turn, endothelial dysfunction can lead to insulin resistance due to diminished blood flow and capillary recruitment which cause decreased substrate and insulin delivery.

Endothelial dysfunction is also believed to lead to both insulin resistance and visceral adiposity by impaired mitochondrial biogenesis. A reduction of eNOS-derived NO may lead to flaw in energy homeostasis, resulting in insufficient generation of energy from stored fat in white adipose tissue and insufficient

generation of heat from brown fat. These effects might lead to increased fat storage.

Visceral fat secrete adipokines and FFAs which cause insulin resistance and endothelium dysfunction. Resistin, IL-6 and TNFα may decrease eNOS activity causing less NO production. TNFα stimulates lipolysis resulting in more FFA release which contribute to endothelial dysfunction by a combination of diminished PI3K-Akt signaling, increased ROS and increased endothelin-1 generation.

Finally, it has also been reported that insulin resistance might contribute to atherogenic dyslipidemia which can lead to visceral obesity.[Huang 2009].

Obesity and atherosclerosis

In summary, it is suggested that visceral obesity together with insulin resistance increase cardiovascular risk by several mechanism such as glucose dysmetabolism, dyslipidemia and hypertension. Additionally, factors produced by adipocytes and macrophages such as adipokines, proinflammatory cytokines and procoagulant protein, may lead to increased oxidative stress and endothelial dysfunction, promoting atherosclerosis. [Van Gaal 2006]. Procoagulant proteins such as PAI-I,secreted by visceral fat, enhances thrombosis within atherosclerotic vessels. Visceral adipocytes also releases renin and angiotensinogen which contribute to hypertension by enhancing the rennin-angiotensinogen system.

Atherosclerosis can be accelerated due to proinflammatory and procoagulant adipokines secreted by white adipose fat which surrounds in excess major blood vessels[Trayhurn 2001,Redinger 2009].

The insulin resistant state of obesity, which is also characterized by increased levels of NEFAs, might cause lipotoxicity, impair endothelium-dependent vasodilation, increase oxidative stress and have cardiotoxic effect. Left ventricular remodeling and contractile dysfunction may results due to several mechanism linking central obesity and insulin resistance.

Adipose tissue and cancer

Nowdays it is suggested that the pathway of tumor formation requires several steps including at least six aberrations in cell physiology which lead to altered cell growth and the expansion of transformed cell clones. [Hanahan 2000]. These alterations responsible for tumor progression depend on multifactors including genetic susceptibility and environmental factors.

When malignant cells, under the influence of external signals such as chemokines and neurotransmitters, are capable of cell migration, cancer cell invasion and metastasis occur. [Entschladen 2004]

Obesity is associated to a state of low chronic inflammation characterized by increased production of inflammatory cytokines which has the potential to affect tumor initiation and progression.[Ratke 2009]. Furthermore, recent studies supported the idea that leptin and adiponectin, two important adipokines secreted by adipose tissue and that influence energy homeostasis as well as metabolism and immune system, participates in the process of carcinogenesis, including tumor cell growth, migration and invasion and immune response [Lang and Ratke 2009].

Effect of adipose tissue in human pregnancy

It has been reported that during pregnancy maternal metabolism undergos important changes due to the growing fetus and placenta formation. (Valsamakis et al.2010, Cetin I. et al. 2009). The first trimester of gestation is characterized by an anabolic state in which the mother increases her adipose tissue deposition in order to support the development and growth of the fetus. It is believed that during the second part of the pregnancy fat metabolism switched to a catabolic condition to provide sufficient nutrients to the fetus

which increases its nutritional demands (Cetin et al. 2009). It is known that from the second to the third trimester of gestation, there is an increase of insulin resistance due to the increased metabolic needs of the growing fetus and placenta (Valsamakis G. et al 2010). The human placental lactogen, human placental growth hormone, prolactin, cortisol and progesterone act together in order to support adeguate transfer of glucose to the fetus; these hormones antagonize the action of insulin leading to a state of insulin resistance over the course of the pregnancy. (Valsamakis et al. 2010). The adipose tissue deposition during pregnancy has important consequence on fetal growth. Obesity contributes to develop a pro-diabetogenic environment affecting the normal growth of the fetus (Callaway L.K et al. 2006). It has been reported that increased BMI before pregnancy and increased weight during gestation have been associated with increased incidence of gestational diabetes mellitus, preeclampsia, high blood pressure and other several conditions which may occur during pregnancy (Catalano et al 2009, Valsamaki 2010). At cellular level it is known that during starvation stored fat in adipose depots undergo lipolysis in order to supply tisssues with fatty acids as energy substrates. Dysfanctional lipolysis may affect energy homeostasis and contribute to obesity and insulin resistance. Thus it would be important to study maternal metabolism , adipose tissue deposition and behaviour and their effect on the fetal growth.

3) ORIGINS OF ADIPOSE TISSUE

Where and when does fat come from?

The development of adipose tissue during embryogenesis is regulated by the complex interaction of cell intrinsic mechanisms, such as transcription factor expression, with external stimuli including adipokines, nutrients, paracrine, endocrine, and maternal factors [Gesta 2007, Kiess 2008]. Remarkably, early events in adipogenesis in the fetus have been connected to diseases manifesting up to several decades later. For example, maternal nutrition and change in glucose homeostasis during gestation may play an important role in the development of insulin resistance in postnatal life. [Catalano 1998, McMillen 2006, Kiess 2008].

Thus, it is important to elucidate the regulation of energy homeostasis and adipose tissue development during embryonic, fetal, and neonatal life.

The adipose lineage is thought to originate from mesenchymal stem cells (MSC), a stem cell population with rather broad developmental potential. MSCs develop into adipoblasts, a further restricted progenitor cell committed to give rise to adipocyte progenitor cells or preadipocytes, that subsequently differentiate into mature, lipid filled adipocytes. Although the molecular mechanisms directing preadipocyte differentiation into mature adipocytes have been characterized to some extent, the upstream events controlling MSC differentiation to adipoblasts and preadiopocytes are poorly understood [Ailhud 1992, Hausman 2001].

Because there are no specific markers that can be used to identify adipoblasts and preadipocytes, very little is known regarding the origin and the development
of the adipose tissue during embryogenesis [Rosen 2006,Gesta 2007].

Early development

It had long been postulated that adipose tissue is mesodermal in origin. During gastrulation, mesoderm formation is induced by the migration of a layer of cells between the primitive endoderm and ectoderm. This layer of cells, the primitive mesoderm, gives rise to various kind of muscle including cardiac, all connective tissue, blood vessels and blood, and lymph tissue. The embryonic mesoderm can be divided into paraxial, intermediate and lateral mesoderm. The paraxial mesoderm forms the bone, skeletal muscle, dermis and subcutaneous tissue as well as cartilage, and the ligament of the spine and base of the skull. The intermediate mesoderm gives rise to part of the urogenital system and the lateral mesoderm develops into tissues that form the pericardial, pleural and peritoneal cavities [Clinical anatomy by system, Richard S. Snell]. Adipose tissue is thought to derive from each of these mesodermal regions.

Interestingly, recent studies have shown that the mesoderm is not the only germ-layer source of mesenchymal cells. The neural crest (NC), composed of cells located dorsally in the neural tube after its closure and consequently of ectodermal origin, undergo an epithelial-to-mesenchymal transition and migrate to different regions in the developing embryo, where they differentiate into various cell types, including pigment cells, neuron and glia cells of PNS and endocrine cells. In the head and the neck, the NC also give rise to tissue of mesenchymal origin such as connective tissue, vascular smooth muscle cells, tendons, dermis, odontoblast, cartilagines and bones [Billon 2008, Dupin 2006, Le Douarin 2004, Kalcheim 1999]. Recent studies have revealed that NC also is able to differentiate into adipocytes [Billon 2007, Gesta 2007]. *In vitro* experiments, conducted in embryonic stem (ES) cells, showed that pluripotent cells differentiated into NC cells could be further differentiated into adipocytes. Additional support came from lineage-tracing experiments in mice, where it was shown that adipocytes around the salivary gland and ears are derived from neural crest progenitors. Other connective tissues and bone in the head and neck have been shown to be of neural crest lineage as well [Billon 2007]. In contrast,

other anatomically distinct units of adipose tissue, including intrascapular, inguinal and abdominal fat pads, have been shown to not be of neural crest lineage [Wrage, 2008]. These finding open new questions regarding the developmental ontogeny of adipose tissue, leaving us with the fascinating concept that adipose tissue and other connective tissues may arise from multiple germ layers.

White-brown fat and muscle

White and brown fat cells, having strikingly different histology and functions, were previously believed to be derived from the same precursor cell. However, a recently published *in vivo* fate mapping experiment in mice has provided evidence that brown adipocytes arise from a different and distinct population of progenitors expressing myf5, a marker of the myoblastic lineage [Seale, 2008]. Furthermore this progenitor population gives rise to both brown adipocytes and myocytes but fails to develop into white fat cells. (Fig.c)

Several studies suggested that brown adipose tissue (BAT) activity could impact daily energy expenditure. [Seale and Lazar 2009]. The last several years have seen an explosion of information related the transcriptional control of brown fat cell development, differentiation and function. Recently, *in vivo* and *in vitro* experiments performed by Kajmura and colleagues have shown that PRDM16 forms a transcriptional complex with the active form of C/EBPβ, controlling the cell fate switch from myoblastic precursor to brown fat cells and suggested that forced expression of the PRDM16-C/EBPβ complex is sufficient to induce a fully functional brown fat program in naïve fibroblast cells. Furthermore they showed that transplantation of fibroblasts expressing these factors into mice gives rise to an ectopic fat pad with morphological and biochemical characteristics of brown fat, and acts as a sink for glucose uptake [Kajmura 2009]. It is believed that these data, indicating that the PRDM16-C/EBPβ complex initiates brown fat formation from myoblastic precursor cells,

may provide new opportunities to develop new therapies aimed at treating metabolic disease such as obesity and type 2 diabetes.

Fig.c. Adipocyte and myocyte differentiation from mesenchymal stem cells. Mesenchymal stem cells can enter several cell lineages, resulting in the formation of bone, muscle, and adipose tissue, among others. (a) The precursor cells destined to become white adipocytes first differentiate into adipoblasts and then preadipocytes through carefully timed exposure to key regulators. The determination phase results in the conversion of the stem cell to a preadipocyte, which still shares some morphological features with its precursor cell but has lost the potential to differentiate into other cell types. In the subsequent phases of terminal differentiation, the committed preadipocyte takes on the characteristics of the mature adipocyte by acquiring all the machinery needed for lipid transport, synthesis and mobilization, hormonal responsiveness and the secretion of adipocyte-specific proteins. Two members of the family of bone morphogenetic proteins (BMPs), specifically BMP2 and BMP4, as well as PPARγ and C/EBPs are pivotal to drive these different phases. (b) Myf5-expressing precursors give rise to skeletal muscle and brown adipose tissue, but not white adipose tissue. Whereas BMP2 and BMP4 promote the differentiation of the white adipocyte lineage, BMP7 singularly drives the brown fat cell fate in both mesenchymal progenitor cells and committed brown preadipocytes suppressing early adipogenic inhibitors, such as necdin, Pref-1 and WNTs (red arrow) [30], at the same time as inducing the key molecular determinant PRDM16, which triggers the activation of the complete brown adipogenesis programme and blocks the induction of myotube-specific genes such as Myf5 and MyoD. Thus, PRDM16 operates as the key regulator in these myogenic progenitors inducing the commitment of the precursor to a brown preadipocyte at the same time as repressing skeletal muscle differentiation. Brown adipogenesis is stimulated by the transcriptional factor PRDM16, at least in part, via binding and coactivating PPARg with subsequent induction (green arrow) of key features to specify a brown fat fate (i.e. increased mitochondrial biogenesis and expression of p38 MAPK, UCP1, Cidea, and Elovl3, among others, which lead to a mature brown adipocyte phenotype). Simultaneously, PRDM16 suppresses myogenesis, thereby decreasing the expression of myogenic genes

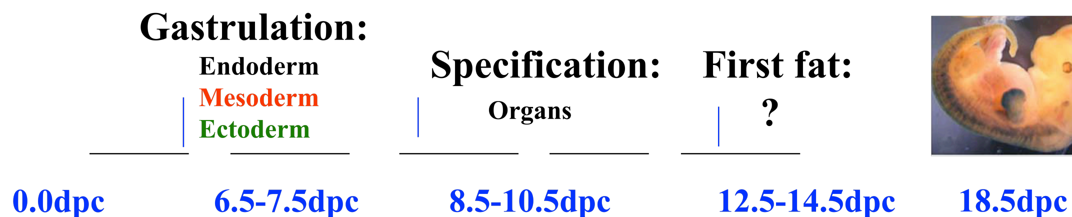
such as MyoD, myogenin, myocyte enhancer factor 2 (MEF2) and myogenic related factor 4 (MRF4). Some aspects of the differentiation pathways still remain uncertain. Because the differentiation programme is complex and orchestrated by multiple factors intervening at diverse steps, the figure only presents a tentative placement of some of them. Chronic cold exposure or prolonged catecholaminergic stimulation are followed by the emergence of brown adipocytes in white adipose tissue. Interestingly, these brown fat cells seem to have an independent origin to that of the myogenic-derived lineage. The question marks represent some plausible steps that still need to be elucidated, such as the possibility of emergence of brown adipocytes from a common adipoblast-derived precursor, and the potential transdifferentiation between the developmentally distinct adipocytes.
Picture and legend are taken from *Fruhbeck et al. Cell press 2009*

Adipose tissue development during fetal life

Very little is known regarding adipose tissue during embryonic development in mice. There is as of yet no evidence of white adipose tissue (WAT) being present during fetal life while interscapular BAT can be detected.

The only specific marker for adipose tissue during both fetal and postnatal life is adipocyte protein 2 (aP2). aP2 also called Fatty Acid Binding Protein 4 (FABP4), is a cytosolic fatty acid chaperone protein found predominantly in mature adipocytes, where it facilitates the utilization of lipids in metabolic pathways (Maeda et al. 2005; Makowski et al.). However, its role during fetal development is not clear.

Sumithra and collagues investigated the expression of Cre recombinase under the control of the promoter for the aP2 gene in a lineage tracing experiment [He et al. 2003], and they demonstrated that the aP2-Cre transgene is expressed and consistently localized within the embryo from mid-gestation stage 9.5 dpc (day post coition), and by 15 dpc transgene expression was detected primarily in BAT, trigeminal ganglia, dorsal root ganglia, cartilage primordial and vertebrae [Urs 2006].(fig.d)



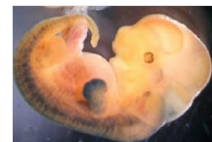


Fig.d Timeline of mouse embryonic development: gastrulation , the process which give rise to the 3 germ layers, occur around stage 6.5 dpc; specification of organs happens around 8.5-

10.5dpc. aP2-Cre transgene has been shown to localized withing the embryo from midgestation stage 9.5dpc

In humans, adipose tissue can be detected during embryonic development and at birth [Kiess 2008]. Fat cells are first noticeable between the 14th and 16th weeks of gestation, suggesting the second trimester of gestation as the critical period of adipogenesis [Aihaud 1998, Poissonet 1984, Niemela 2007, Kiess 2008]. Around the third semester, relatively small adipocytes are already detectable in the principal sites of fat deposition. During early development no differences in WAT localization between male and female fetuses has been described, suggesting that such differences develop gradually later in life, presumably due to differential effects of sex hormones [Kiess 2006, Aihaud 1998, Enzi 1980].
During the late gestation there is an increase in BAT specific expression of UCP1, which is activated at birth and determines heat production. Then, during postnatal life BAT, and UCP1 expression, gradually disappears [Niemela 2007, Enzi 1981, Kiess 2008].

Adipogenesis

The physical events determing the transition from preadipocytes to mature adipocytes have been studied *in vitro* in mouse 3T3-L1 and 3T3-F442A cell lines and immortalized brown preadipocytes cell lines [Rosen and Spiegelman 2000]. The process of terminal adipocyte differentiation can be divided into several distinct stages. After a brief proliferative burst, adipocytes undergo growth arrest in G0-G1 of the cell cycle. Upon exposure to mitogens, differentiating agents and hormonal induction, growth-arrested preadipocytes re-enter the cell cycle and undergo several rounds of cell division, known as mitotic clonal expansion. Subsequently, terminal differentiation is associated with the expression of the nuclear receptor PPARγ, C/EBPα, and lipid droplet formation [Gesta 2007, Hausman 2001, Lefterova 2009].

It is noteworthy that there are significant differences between *in vivo* adipose tissue and cell lines cultured *in vitro*. The expression levels of the adipogenic transcriptional regulator KLF4, leptin, and TNFα are higher *in vivo* than *in vitro.* [Gesta 2007, Rosen and Spiegelman 2000, Soukas 2001] Furthermore, adipocyte cell lines are often aneuploid, which may influence the adipogenic transcriptional program during differentiation [Rosen and Spiegelman 2000]. Additionally, primary cultures of stroma vascular cells can differentiate without undergoing the clonal expansion stage, [Entenmann 1996, Rosen and Spiegelman 2000], which is contraty to the current model of adipocyte differentiations. Furthermore, the most studied preadipocytes cell line (3T3-l1) is derived from a mouse embryonic fibroblast cell line, not adipose tissue (Stembook : Cook, Cowan 2009). Thus, it is likely that *in vivo* experiments may provide additional insights into the transcriptional cascade of events leading to mature adipocyte differentiation.

The molecular cascades controlling adipogenesis

The transcriptional cascade controlling adipocyte differentiation has been investigated intensely. Peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT /enhancing binding protein (C/EBPs) are considered the master regulators of adipogenesis. PPARγ and C/EBPα constitute a self-regulatory feedback loop and activate the expression of genes controlling terminal differentiation of adipocytes.

PPARγ is a member of the nuclear hormone receptor superfamily and is required for the development of adipocytes. There are 3 isoforms, with PPARγ2 being specific to adipocytes. Deletion of PPARγ in mice results in placental dysfunction and embryonic lethality [White and Stephens 2009, Barak 1999, Rosen 1999]. Furthermore, PPARγ has been described to be necessary and sufficient for adipogenesis. [Tontonoz and Spiegelman 2008]. PPARγ binds DNA as a heterodimer with retinoid X receptor (RXR), thereby promoting the

expression of its target genes. The molecular basis for how the PPARγ-RXR heterodimer binds to DNA has recently been elucidated [See Chandra 2009 for details].

C/EBPα,β, and δ belong to a family of highly conserved basic-leucine zipper proteins and they were the first family of transcription factors shown to play a critical role in the differentiation of adipocytes *in vitro*. Additionally, *in vivo* studies demonstrated that transgenic mice lacking these factors have defective fat cell differentiation [White and Stephen 2009, Tanaka 1997, Wang 1995].

C/EBPα is expressed late in adipogenesis and is most abundant in mature adipocytes, where it is crucial for insulin-dependent glucose uptake [Wu 1999, White and Stephens 2009]. However, while PPARγ has shown to be necessary for adipogeneis, elegant *in vivo* studies have shown that C/EBPα is dispensable in adipogenesis [Rosen 2002].

Although PPARγ and C/EBPα are considered the two primary transcription factors that mediate adipogenesis, and PPARγ is critical for fat cell differentiation, these transcription factors are under control of several other key transcriptional modulators. (White and Stephens 2009).fig., For example, members of KLF family, STAT5 and SREP-1c have been shown to play a role in promoting adipogenesis. Additional factors affect this chain of events. For example, KLF2, E2F, CHOP, members of the Wnt ligand family, delta-interacting protein A, ETO/MTG8 and members of the GATA transcriptional factor family have all been shown to negatively regulate adipogenesis [Lefterova 2009, White and Stephans 2009, Farmer 2006].

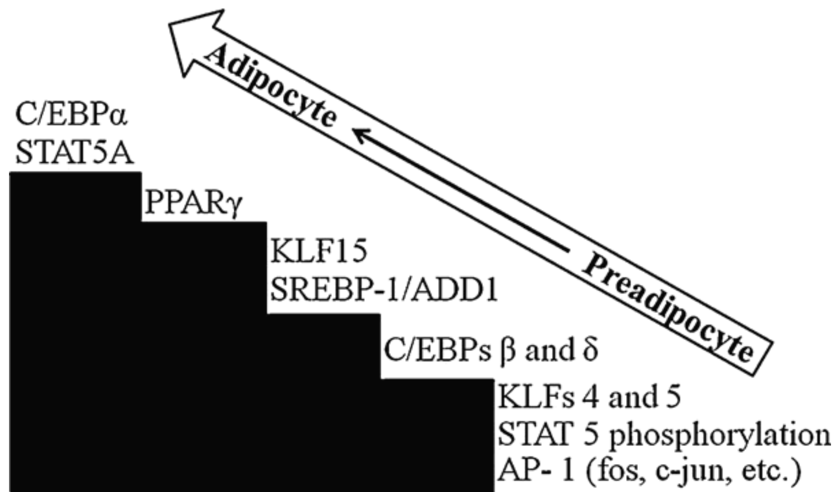


Fig.e Trancription factor activation and expression during adipogenesis (White and Stephens,2009).

4) GROWTH AND MAINTENANCE OF ADIPOSE TISSUE

It is widely believed that the total number of fat cells present in most individuals is set during adolescence and that changes in fat mass generally reflect increased lipid storage in a fixed number of adipocytes [Hirsch, J. & Batchelor, B 1976, Bjorntorp, P 1974, Spalding K.L, 2008]. Though it was recently demonstrated that there is a substantial degree of cellular turnover within the human adipocyte population [Spalding 2008], the source of new adipocytes during one's lifetime is attributed to the differentiation of new adipocytes from preadipocytes and/or stem cells [Hirsch, J. & Batchelor, B 1976, Spalding K.L, 2008, Tang, W 2008].

Preadipocytes are generally considered to be replicative [Miller, W. H., Jr., Faust, I. M. & Hirsch, J. 1984, Staszkiewicz, J. et al. 2008] until the onset of a transcription factor cascade driving adipogenesis [Tontonoz, P. & Spiegelman, B. M. 2008, Rosen, E. D., Walkey, C. J., Puigserver, P. & Spiegelman, B. M. 2000] causes growth arrest [Altiok, S., Xu, M. & Spiegelman, B. M. 1997]. Additionally, primary adipocytes seemingly lack proliferative activity *in vitro*

[Hiragun, A. 1985] and cell culture lines capable of adipocyte differentiation undergo cell cycle arrest during differentiation. [Gregoire, F. M., Smas, C. M. & Sul, H. S. 1998] Adipocytes are thought to represent a terminal stage of differentiation and are believed to lack proliferative ability [Prins, J. B. & O'Rahilly, S 1997]. Thus, data from *in vitro* experiments all suggest that one or several of stem and/or progenitor cell populations give rise to new adipocytes during postnatal life.

However, it is important to remember that *in vitro* models have their limitations although they can recapitulate some of the characteristic of adipocyte differentiation *in vivo*. For example, 3T3-L1 cells, when injected into mice, differentiated and formed fat pads that were indistinguishable from normal adipose tissue [Green and Kehinde, 1979], and a population of progenitor cells capable of differentiating into adipocytes can also be purified based on cell surface marker expression and transplanted into lipodystrophic mice to reconstitute fat depots [Rodeheffer, M. S., Birsoy, K. & Friedman, J. M. 2008]. Despite this and other examples of successful transfer of cell culture models to the *in vivo* system [Farmer, 2006, Ross et al. 2000, Lazar, 2005], *in vitro* culture models can still not be considered to perfectly mimic the *in vivo* situation. They can rather be viewed as simplified systems of adipogenesis that offer opportunities to more conveniently modulate expression and activity of key molecules controlling the genetic program of adipogenesis.[MacDougald and Mandrup, 2002]. However, it is important to verify findings from *in vitro* experiments *in vivo* as there are numerous examples of differences when comparing development of fat in tissue culture and in the living organism. For instance, cell culture medium and conditions are far from resembling *in vivo* conditions, and some genes show differential effect and expression *in vitro* and *in vivo* [Tanaka et al, 1997]. Furthermore, the growth arrest due to contact inhibition and the subsequent clonal expansion might be an event that occur in cell culture system and not *in vivo*.

An additional limitation of *in vitro* experiments is that they require that stem and progenitor cells are removed from their anatomical location in the body,

thereby disrupting the microenvironment where they normally reside. It has been shown that this microenvironment, or niche, provides factors and signals that contribute to maintain the stem-like capacity of progenitor cells. Well-documented stem cell niches have been described for the hematopoietic, epithelial, neural and intestinal systems [Xie and Li, 2007]. In fat tissue, progenitor cells with the capacity to differentiate into adipocytes were suggested to reside in the adipose stromal-vascular (SV) compartment two decades ago [Ailhaud et al 1992]; however, the adipose SV progenitors had been studied after enzymatic digestion and density separation from adipocytes, resulting in disruption of any existing structure and loss of anatomical context of adipose progenitors [Hausman et al, 1980]. Furthermore, the lack of markers for adipocyte progenitor cells made it difficult to definitively identify progenitor structures.

Adipose tissue is highly vascualarized and every adipocyte is juxtaposed with multiple capillaries [Crandall et al., 1997]. Hausman and Richardson proposed that adipose development temporally and spatially correlates with vascular development (Hausman and Richardson, 1994). However, experimental evidence for direct involvement of vascular componets in adipogenesis was not presented. Several interacting cell types, including endothelial cells and mural cells, form the blood vessels. Mural cells, mainly vascular smooth muscle cells and pericytes, are important regulators of blood flow, vascular permeability and blood vessel development. It has been shown that cultured mural cells display remarkable plasticity and can undergo adipogenesis, chondrogenesis, myogenesis and osteogenesis [Farrington-Rock et al., 2004, Dellavalle et al., 2007, Doherty et al., 1998], similar to mesenchymal stem cells [Pittenger et al.,1999]. Thus, mural cells had been suggested to be a potential progenitor cell reservoir *in vivo*.

As evident from the above, although several hypotheses had been put forth, surprisingly little has been known about adipose tissue *in vivo* because of the lack of genetic tools.

However, in a recent elegant study Tang and colleagues were able to genetically mark adipose progenitors with GFP, allowing direct visualization of progenitors cells with the capacity to differentiate into adipocytes. Furthermore, this pool of progenitors was suggested to reside adjacent to the adipose vascular tissue [Tang W. et all, 2008].

As discussed above, peroxisome proliferator-activated receptor gamma (PPARγ), a central regulator of fat formation, is necessary and sufficient for adipogenesis [Lazar M. 2005, Farmer S. 2006]. By generating PPARγ–tet transactivator (tTA) [Kistner A, 1996] knock-in mice placing tTA under the control of the PPARγ locus, and leading to Cre expression, Tang and colleagues were able to capture the expansion of the adipose lineage that, according to several previously published reports [G. Ailhaud, P. Grimaldi, R. Negrel,1992, J. R. Cook, L. P. Kozak,1982], occurs during the first postnatal month. After they Dox-treated PPARγ-R26R mice, starting at different days during this crucial window, they found that adipose lineage cells, already instructed to express PPARγ prenatally, proliferate and are the major source of the spurt of adipocyte development observed in the first month of life. They also found that these progenitors reside in the mural cell compartment of the adipose vasculature, but not in the vasculature of other tissues. Thus, the experimental evidence that strongly supports the notion that the adipose vasculature appears to function as a progenitor niche and may provide signals for adipocyte development. [Tang W et all, 2008]. However, it is currently not known if there are other populations of progenitors with adipogenic potential postnatally, nor has it been definitively determined if adipocytes can replicate *in vivo*.

How adipose tissue is regulated is a central question in the study of obesity. Obesity is commonly considered to be a disorder of energy imbalance in the organism. At the cellular level, however, the development of obesity has been attributed to both an increase in fat cell size, fat cell differentiation and increased adipocyte number [Bjorntorp, P. et al. 1995, Sjostrom, L. &

Bjorntorp, P. 1974, Faust et al, 1978, Wise 1975]. The notion that adipocytes can undergo replication has existed for decades [Zhang, H. H., Kumar, S., Barnett, A. H. & Eggo, M. C. 2000; Klyde, B. J. & Hirsch,1979, Klyde, B. J. & Hirsch, 1979] but has not been widely accepted.

There are many important questions that are still open and unresolved: for example how do adipocytes form during development, how is proliferation regulated in adipose tissue, what are the dynamics of progenitors and how they are regulated during development and obesity….

Thus, an understanding of how adipocyte number is regulated postnatally has the potential to expand our knowledge of the pathogenesis of obesity, and may open up possibilities for novel therapeutic approaches that may prove to be of great importance in the treatment of obesity and diseases associated with obesity.

AIM OF THE PROJECT

We sought to investigate the growth and maintenance of the adipose tissue and its behaviour in the cell cycle through 4 independent experimental approaches:

1. dilution of an inducible histone 2B-green fluorescent protein (H2BGFP) through cell division.
2. DNA-replication dependent incorporation of the nucleotide analog BrdU.
3. labeling with the cell cycle marker Ki67

4. lineage analysis of adipose tissue by using AP2-CreER transgenic mice to investigate whether newly formed fat cells are derived from pre-existing adipocytes or stem/precursor cell