Human liposarcoma cell line SW872 as a research tool for differentiation, adipocytokine secretion and pharmacological regulation

Master's Thesis
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Pro Gradu-tutkielma

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Tekijä:	RIMPILÄ, VILLE PAASO OLAVI
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Sivumäärä:	70 s.
Ohjaajat:	Professori Eeva Moilanen, FT Riina Nieminen ja LKT Katriina Vuolteenaho
Tarkastajat:	Professori Markku Kulomaa ja Professori Eeva Moilanen
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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Lihavuuden esiintyvyys on lisääntynyt niin paljon, että voidaan puhua jo epidemiasta. Viimeisen vuosikymmenen aikana on selvinnyt, että rasvakudos on myös endokriininen elin, joka erittää lukuisia eri proteiineja. Näitä proteiineja kutsutaan yleisesti adipokiineiksi tai adiposytokiineiksi. Useita solulinjoja on käytetty adipogeneesin tutkimuksessa menestyksekkäästi ja nykyään tähän prosessiin liittyvät tekijät tunnetaan melko hyvin. Useimmat tutkimuksissa käytetyt solulinjat ovat kuitenkin peräisin hiirestä ja epäselvää on, missä määrin niiden perusteella saatu tieto on sovellettavissa ihmiseen. Tässä tutkimuksessa käytettiin ihmisestä eristettyä liposarkooma solulinjaa SW872 mallina ja näitä soluja erilaistettiin kypsiksi rasvasoluiksi, tarkoituksena tutkia adipokiinien ilmentymistä ja eritystä.

Tutkimusmenetelmät: SW872 soluja tutkittiin soluviljelmässä, useissa eri olosuhteissa. Erilaistumista tutkittiin eri aineyhdistelmillä ja altistamisajoilla. Lipidien kerääntymistä soluihin ja rasvasolujen kehittymistä seurattiin Oil Red O - värjäyksellä. Adiposytokiinien tuottoa ja eritystä kasvatusmediumiin tutkittiin laajasti ELISA-menetelmällä. Adiposytokiinien tuoton farmakologista säätelyä tutkittiin käyttämällä useita eri lääkeaineita (siglitatsoni, ibuprofeeni)

Tutkimustulokset: Pystyimme erilaistamaan SW872 solut kohti kypsän rasvasolun fenotyyppiä. Paras erilaistuminen saavutettiin kun käytettiin yhdistelmää: deksametasoni, insuliini, IBMX, rosiglitatsoni ja BSA:han liitetty rasvahappo. Solut tuottivat kasvatusmediumiin adipokiini adipsiinia (toiselta nimeltään komplementti tekijä D) ja kemokiineihin kuuluvaa monosyytti kemoatraktantti proteiini-1:tä (MCP-1). Näihin tuottotasoihin pystyimme vaikuttamaan eri erilaistamis-olosuhteilla: erilaistuneissa soluissa adipsiinin tuotto lisääntyi selvästi ja MCP-1:n tuotto väheni huomattavasti. Adipokiinien tuottoa pystyttiin myös muuttamaan farmakologisesti. Resveratroli ja ibuprofeeni lisäsivät adipsiinin tuottoa merkitsevästi. MCP-1:n tuotto laski deksametasonilla, siglitatsonilla, kversetiinillä ja kampferolilla, mutta nousi resveratrolilla.

Johtopäätökset: Tutkimuksessa löydettiin olosuhteet, joilla SW872 solut voidaan erilaistaa kohti kypsiä rasvasoluja ja saada aikaan maturaatioon liittyviä muutoksia adiposytokiinien (adipsiini ja MCP-1) tuotossa. Kehitetyllä mallilla voidaan tutkia lääkeaineiden vaikutuksia adipsiinin ja MCP-1:n tuottoon. Tämä tutkimus toimii pohjana jatkotutkimuksille, joissa voidaan selvittää adiposyyttien erilaistumiseen ja adiposytokiinien tuottoon liittyviä säätelymekanismeja, ja sen perusteella selvittää lääkehoitoja lihavuuden ja sen liitännäissairauksien hoitoon.

Master's Thesis

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Abstract

Background and aims: Prevalence of obesity has grown into epidemic proportions. During the past decade it has become clear that adipose tissue is also an endocrine organ secreting myriad of proteins, coined as adipokines or adipocytokines. Number of cell lines are used to study adipogenesis and they have been very successful in determining the factors involved in this process. Most cell lines originate from mice and in this study our aim was to use human derived liposarcoma cell line SW872 as a model and differentiate these cells into mature adipocytes in order to study adipokine expression and secretion.

Methods: SW872 cells were studied in cell culture in various conditions, with different differentiation times and compositions. Lipid accumulation and the adipogenic process were monitored with Oil Red O-staining. Adipokine production and secretion to culture medium was studied extensively with ELISA. Pharmacological regulation of adipokine production was studied with various pharmacological agents, such as ciglitazone and quercetin.

Results: We were able to differentiate the SW872 cells towards a mature adipocyte phenotype. Mixture of dexamethasone, insulin, IBMX, rosiglitazone and fatty acids coupled with BSA were used to achieve differentiation. Adipokine adipsin (also known as complement factor D) and chemokine MCP-1 (monocyte chemoattractant protein-1) were found to be secreted into the culture medium and we were able to alter the production of these substances with different differentiation conditions. Production of these substances was also regulated with different pharmacological agents. Resveratrol and ibuprofen increased the production of adipsin significantly. MCP-1 production was reduced with dexamethasol, ciglitazone, quercetin and kaempferol, but increased with resveratrol.

Conclusions: Culture conditions were found under which the SW872 cells differentiated towards mature adipocytes and showed maturation dependent changes in the production of adipsin and MCP-1. This model can be used to study pharmacological effects on adipsin and MCP-1 production in SW872 cell line. This initial study serves as a stepping stone for more detailed studies in the future, which will be needed in order to determine better culture conditions, more detailed differentiation regime and the overall feasibility of using this cell line to produce and study adipokines. A more detailed understanding on the regulation of adipocyte differentiation and adipocytokine production will open new avenues to develop drugs for the treatment of obesity and its comorbidities

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Abbreviations

ADD1	adipocyte determination and differentiation factor-1
AMPK	adenosine monophosphate-activated protein
AP-1	activating protein-1
AP2	adipocyte protein 2
BAT	brown adipose tissue
BMI	body mass index
bZIP	basic leucine zipper
cAMP	cyclic adenosine monophosphate
C/EBP	CCAAT/enhancer binding protein
CETP	cholesteryl ester transfer protein
CRP	C-reactive protein
GLUT4	glucose transporter type 4
GSK-3	glycogen synthase kinase 3
HIF-1	hypoxia-inducible factor-1
IBMX	isobutylmethylxanthine
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IRS-1	insulin receptor substrate-1
KLF	krüppel-like zinc finger transcription factor
LPS	lipopolysaccharide
LRH-1	liver receptor homolog-1
MAP	mitogen-activated protein
MCP-1	monocyte chemotactic/chemoattractant protein-1
MIX	methylisobutylxanthine
NF-κB	nuclear factor kappa B
PAI-1	plasminogen activator inhibitor-1
PBEF	pre-B-cell colony enhancing factor
PEPCK	phosphoenolpyruvate carboxykinase
PI-3K	phosphatidylinositol-3 kinase
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR-responsive element
RBP-4	retinol binding protein 4
RXR	retinoid X receptor
SREBP	sterol regulatory element-binding protein
SOCS3	suppressor of cytokine signaling 3
STAT	signal transducers and activators of transcription
TNF-α	tumor necrosis factor-α
TZD	thiazolidinedione
UCP	uncoupling protein
VEGF	vascular endothelial growth factor
WAT	white adipose tissue

1. Introduction

Obesity has developed into a major health problem across the world. According to WHO (World Health Organization) there are over 1 billion overweight adults worldwide of which 300 million are clinically obese, defined as body mass index (BMI) greater than 30 (Hotamisligil, 2006; Cao, 2010). In the past, fat was considered to be just a storage depot. However, research in this field during the last few decades has revealed that adipose tissue is actually quite complex tissue type with numerous different functions. Obesity can be characterized by enlarged adipocytes, inflammatory macrophages within the adipose tissue, shift in adipokine production towards pro-inflammatory factors and possibly tissue hypoxia. Overall, obesity is nowadays considered to be a low-grade inflammatory state.

Obesity is a complex metabolic disorder which has been linked to common and severe diseases such as: type 2 diabetes, hypertension, cardiovascular diseases, stroke and number of cancers (colorectal, breast and prostate cancer). It is also common that obese individuals suffer from social and psychological problems such as low self-esteem or depression. (Cao, 2010)

Discovery of leptin and other adipokines, such as adiponectin, has made clear that adipose tissue is actually an endocrine organ and also a part of the immune system. In fact, immune system, adipose tissue and liver have a common ancestral origin. *Drosophila melanocaster* has a “fat body” which is responsible for energy sensing and coordination of pathogen response. (Hotamisligil, 2006) In mammals, these functions are carried out by distinct organs but overlapping of functions is not that surprising: adipocytes can secrete number of inflammatory factors and macrophages can accumulate lipid and become foam cells.

Information about adipocyte differentiation and the transcriptional factors has been derived mostly from cell culture studies. These cell lines (mainly of murine origin) were isolated already in the 1970’s and they are still in use because they replicate the adipogenic events very well. Primary cultures have also been used but not as extensively as cell lines due to their short time window for use. Use of primary cultures is also more labor-intensive compared to cell lines. Number of human derived adipogenic cell lines is scarce, and in this

study the aim was to determine the feasibility of liposarcoma cell line SW872 for adipokine production.

2. Review of the literature

2.1 Adipose tissue

In mammals the adipose tissue can be found in two different types, white adipose tissue (WAT) and brown adipose tissue (BAT). The white adipose tissue is considered to be the storage depot for energy and the brown adipose tissue is used for generating heat in a non-shivering process. (Tilg & Moschen, 2006) Brown adipose tissue in humans can be found almost exclusively in neonates where it surrounds the heart and larger vessels. In adults only a small number of brown adipocytes can be found and these are scattered throughout the white fat depots. (Rosen & Spiegelman, 2006) The main function of adipose tissue is to store energy when nutritional status is good. The energy is stored in the form of triglycerides, which have great property of being able to store without water. (Rangwala & Lazar, 2000) When compared to glycogen, which is stored with water, the triglycerides contain twice the energy when similar masses are compared.(Trayhurn, 2005)

Adipose tissue can be found around the body in various different compartments. In some areas the role of the adipose tissue is considered to be more structural, for example the fat pads in heels, fingers and toes. Another compartment is the subcutaneous adipose tissue which is loosely associated with the skin. Subcutaneous adipose tissue is also the common target for liposuction. The final and perhaps the most relevant compartment to physiology is located within the body cavity. These fat depots can be found around the organs, for example heart. Some of these depots are known as visceral fat and they drain directly to portal circulation. It is these depots that have been connected to obesity related comorbidities. (Rosen & Spiegelman, 2006) Capillary and nervous network within WAT is considerable (Ahima et al., 2006).

In the light of clinical and pathological data, it is conceivable that inflammation, insulin resistance and metabolic syndrome are closely linked with obesity. The big questions are: Why WAT release myriad of pro-inflammatory cytokines and acute-phase proteins and why does the increase in fat mass increase their amount so clearly? Release of inflammatory factors might contribute to inflammation in some specific location or they

might be a response to systemic inflammatory state. The common view is that the inflammation is within the adipose tissue. Elevated inflammation-related products might then represent a spillover from the tissue. If WAT is the primary reason for inflammation what is the local phenomenon that is causing it? (Trayhurn & Wood, 2004)

Hypoxia is considered to be one cause of the inflammation. With increasing obesity the tissue expands and the fat cells become larger and the capacity of existing vasculature is not sufficient enough to maintain normoxia.(Rutkowski et al., 2009) Hypoxia then triggers inflammatory process which increases blood flow and angiogenesis. Process is similar to tumor growth. WAT vasculature is less extensive compared to brown adipose tissue. It has also been shown that adipose tissue is sensitive to angiogenesis inhibitors. Adipocytes also secrete a number of well established angiogenic proteins such as vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1 (PAI-1) and leptin. However, the most important cellular response to hypoxia is hypoxia-inducible factor-1 (HIF-1). HIF-1α has been found both in cultured 3T3-F442A adipocytes and *ob/ob* mice where it is produced by adipocytes and stromal vascular cells. (Trayhurn & Wood, 2004)

Adipose tissue has number of different cell types which contribute considerably to its endocrine function. Approximately 50% of the adipose tissue consists of mature adipocytes and the remaining 50% consist of preadipocytes, macrophages, fibroblasts, endothelial cells, mesenchymal stem cells and neural cells. (Armani et al., 2010; Gustafson, 2010) Figure 1. shows a schematic presentation of adipose tissue.

2.1.1 Pre-adipocytes

Developmental program from fertilized egg to preadipose tissue is unknown. However, some evidence speaks for mesodermal origin; pluripotent fibroblasts can differentiate into bone, cartilage, muscle and committed preadipocytes. (Ntambi & Young-Cheul, 2000) Majority of the adipose tissue differentiation in humans occurs shortly after birth, but this process starts already during late embryonic development (Ntambi & Young-Cheul, 2000). If the need for fat storage capacity is exceeded (individual gains weight), preadipocytes are recruited to differentiate to mature adipocytes so that the storage capacity need is fulfilled. This capacity to differentiate is restored throughout the life span (Gregoire et al., 1998).

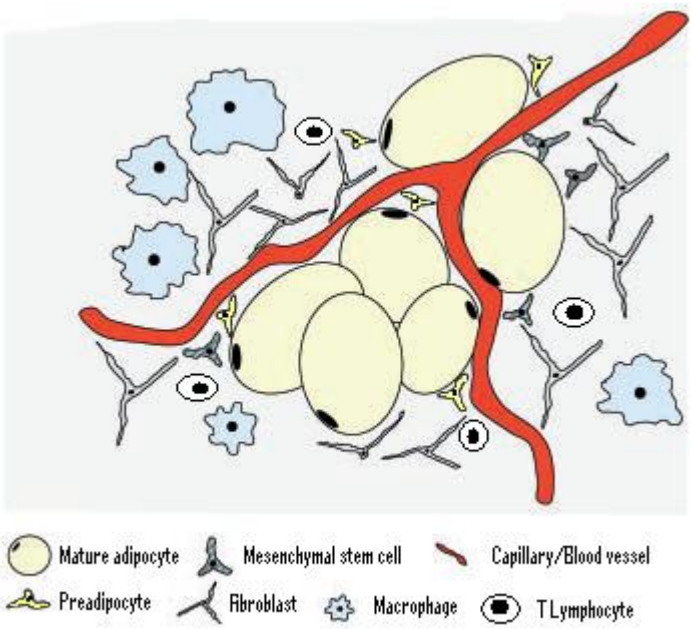

Figure 1. Adipose tissue. Adipose tissue is more than just fat. It is a highly organized network of mature adipocytes and preadipocytes, mesenchymal stem cells, macrophages and fibroblasts. Adipose tissue is also rich in capillaries lined with endothelial cells and pericytes. Figure modified from Armani, 2010.

However, the capacity of preadipocytes to differentiate into mature adipocytes is decreased when individual is obese (Gustafson, 2010).

2.1.2 Adipocytes

Adipocytes possess a number of features ranging from the regulation of the fat mass to blood pressure control (Rosen & Spiegelman, 2006). Under normal conditions, adipocytes are involved in lipid storage and synthesis and secretion of numerous adipokines, which act as transmitters of endocrine and paracrine signals. These signals can have a profound effect on adipocyte function, for example insulin sensitivity. During inflammation adipocytes can secrete various inflammatory factors such as monocyte chemotactic/chemoattractant

protein-1 (MCP-1) and interleukin-6 (IL-6). The amount of fat is regulated both with adipocyte size and number. The number of adipocytes is determined very early, during childhood. Possible changes in fat mass are mainly reflected to changes in adipocyte cell size. Also, it has been recently established that adipocyte turnover is approximately 10% per year (Gustafson, 2010). Adipocyte can store approximately 0.7-0.8 μg of lipids. After this limit is exceeded, there is also an increase in the number of adipocytes. With obesity the number of committed preadipocytes is decreased and this phenomenon is observed in all fat compartments. These findings suggest that most of the adult onset obesity is related to adipocyte hypertrophy (Gustafson, 2010). From the obesity-related factors the enlarged adipocytes correlate best with insulin resistance (Gustafson, 2010). Adipocytes from different compartments possess different characteristics when it comes to replicative potential, developmental features and responsiveness to hormonal signaling. (Rosen & Spiegelman, 2006)

2.1.3 Macrophages

Within the adipose tissue, 10% of the cells are CD14+ CD31+ macrophages and they reside in the stromal-vascular fraction. Adiposity and adipocyte size are directly correlated with the number of macrophages in WAT and no difference can be found between visceral and subcutaneous depots. (Fantuzzi, 2005) Data suggests that macrophages residing in the adipose tissue are bone-marrow derived (Fantuzzi, 2005; Tilg & Moschen, 2006), even though preadipocytes have been shown to be able to differentiate into macrophages in vitro. (Fantuzzi, 2005)

In lean adipose tissue, macrophages show increased expression of *arginase-1* and *Ym-1* genes accompanied with anti-inflammatory cytokine interleukin-10 (IL-10). These macrophages are called M2 macrophages or alternative activated macrophages. (Galic et al., 2010)

Expansion of adipose tissue in obesity has recently been associated with infiltration of the M1 (classically activated) macrophages from the circulation (Galic et al., 2010). WAT produces tumor necrosis factor-α (TNF-α) and majority of it is produced by the macrophages. (Zou & Shao, 2008) Approximately 50% of the IL-6 produced by the WAT

is from the macrophages (Fantuzzi, 2005). Also the expression of inducible nitric oxide synthase (iNOS) is increased (Galic et al., 2010). The exact mechanisms for macrophage recruitment remain unknown. MCP-1 which is produced by the adipocytes and which is over-expressed in obesity might be one piece of this puzzle. Other factors include the increase in free fatty acid production which has been shown to induce inflammatory cytokine production through nuclear factor kappa B (NF-κB) pathway (Galic et al., 2010) and increased adipocyte size which might lead to hypoxia (Trayhurn & Wood, 2004). Adiponectin has been shown to have anti-inflammatory properties and the reduction of this adipokine during obesity might have some effect on this pro-inflammatory state. (Galic et al., 2010)

2.1.4 T lymphocytes

Accumulation of $CD3^+$ T lymphocytes has been described in the adipose tissue of obese mice and human patients. However, detailed information about the activation state or subsets of lymphocytes is scarce. In obese subjects (BMI > 30) the number of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes is significantly higher than in lean subjects. (Duffaut et al., 2009) In obese subjects, the amount of regulatory $CD4^+Foxp3^+$; Treg-cells is decreased (Matarese et al., 2010)

2.1.5 Endothelial Cells

Adipose tissue is filled with relatively dense capillary network. This network provides the nutrients and oxygen to the cells but it also transports the adipokines and free fatty acids from these cells when needed (Rutkowski et al., 2009). Adipose tissue expansion is not different from tumor growth. Rapidly growing adipose tissue becomes hypoxic which stimulates the angiogenesis, which in turn enables the adipose tissue to grow larger. (Rutkowski et al., 2009) Therefore it can be said that angiogenesis and adipogenesis are very tightly connected and they have a substantial role in the remodeling process of the adipose tissue which occurs during obesity. (Lee et al., 2010) HIF-1α (hypoxia-inducible factor 1α) expression leads to up-regulation of IL-6, TNF-α and MCP-1 among others.

Many of these pro-inflammatory products are connected to insulin resistance. Hypoxia can also cause fibrosis within the adipose tissue which leads to further adipose dysfunction. Also preadipocyte differentiation might be blocked by hypoxia but further studies are needed to verify this observation (Rutkowski et al., 2009). It has also been shown recently that mouse pericytes can differentiate into preadipocytes and adipocytes (Cao, 2010).

Currently prescribed anti-diabetic drugs have interesting features related to vasculature. Metformin and thiazolidinediones are successfully used to treat insulin resistance, but they have differential effects on vasculature. Metformin reduces the angiogenesis in the adipose tissue and the use of thiazolidinediones results more vascularized adipose tissue and increased adiponectin secretion. (Rutkowski et al., 2009) Clearly, research on adipose tissue vasculature will be an interesting field to follow. Over-nutrition associated events related to adipose tissue are shown in figure 2.

2.1.6 Fibroblasts and neural cells

Fibroblasts are the most common cell type of the connective tissue and also present in adipose tissue. In certain culture conditions, adipocytes dedifferentiate back to fibroblast-like cells which express markers for osteogenic and chondrogenic development. (Armani et al., 2010) It's interesting to think that adipose tissue fibroblasts could be in some conditions induced to differentiate into adipocytes.

Organization of the adipose tissue is controlled with neuro-hormonal regulation, but the actual mechanisms are still largely unknown. What ever the mechanism may be, neural control of this complex organ is crucial (Armani et al., 2010)

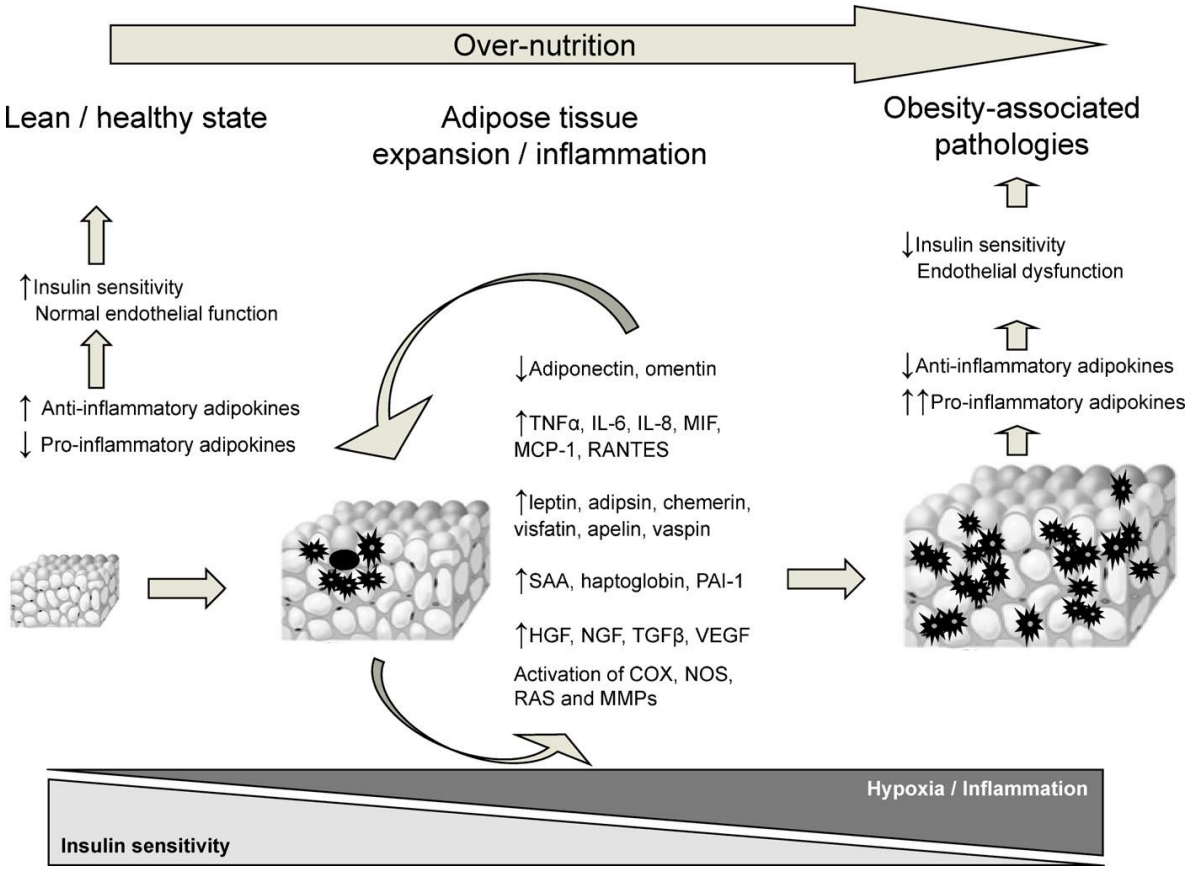

Figure 2. Changes in adipose tissue related to obesity. In the lean/healthy state adipose tissue secretes anti-inflammatory adipokines. As the individual gains weight the adipokine expression profile is shifted towards inflammatory state. In the final pathological stage adipocytes express pro-inflammatory adipokines many of which are connected to decrease in insulin sensitivity and endothelial function. Black oval represents a dead adipocyte and black star represents an infiltrating macrophage. Figure modified from Karastergiou, 2010 (Karastergiou & Mohamed-Ali, 2010)

2.2 Transcription Factors

This section summarizes the transcription factors connected to adipocyte differentiation, growth and other features of these cells. Schematic picture for the timing of the events is shown in figure 3.

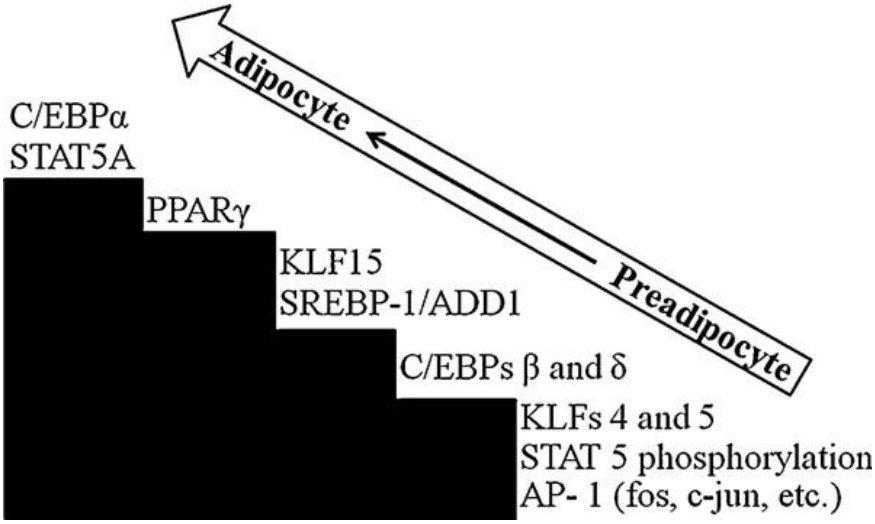

Figure 3. Schematic presentation of transcription factors involved in the differentiation process of adipocytes. Differentiation lasts for a number of days depending on the system studied. Transcription factors in the bottom represent early events and with each step new transcription factors are recruited and the early ones subside. Figure modified from White, 2010.

2.2.1 AP-1, STATs and KLFs

Activating protein-1 (AP-1) is a collective name for a family of dimeric transcription factors that bind to a common site on DNA. These factors are: c-Jun, Jun-B, Jun-D, c-Fos, Fos-B, Fra-1 and Fra-2. As early as in 1987 it was shown that c-Fos modulates the expression of AP2. AP2 is an abundantly expressed adipocyte gene which was discovered in 1984. AP-1 proteins are expressed immediately after the induction of adipocyte differentiation. However, the role of individual AP-1 family members in this process has

not been studied. Data to support an important role of these transcription factors in adipogenesis comes from *in vivo* studies. (U. A. White & Stephens, 2010)

Signal transducers and activators of transcription (STAT) 1, 5A and 5B are highly induced in 3T3-L1 cells. Similar activity has also been shown in human cells. (U. A. White & Stephens, 2010) During differentiation and adipogenesis, the expression of STAT5 proteins doesn't increase until the induction of CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ). However, both of the STAT5 proteins are tyrosine phosphorylated and translocated to the nucleus immediately after the induction of adipogenesis. (U. A. White & Stephens, 2010) Also, one of the PPARγ promoters can be modulated by STAT5. These data suggests an important role for STAT in adipose tissue development. (U. A. White & Stephens, 2010)

Krüppel-like zinc finger transcription factors (KLFs) have diverse roles in the development and cell differentiation in mammals. KLF15 and KLF5 are highly induced during adipogenesis of 3T3-L1 cells (U. A. White & Stephens, 2010). KLFs are involved in the lipid accumulation but they also have a role in the insulin sensitivity of the adipocytes. It has been shown that KLF15 is important in the expression of glucose transporter type 4 (GLUT4), which is expressed in adipose tissue and striated muscle in vivo and transport glucose into cells in response to insulin. Apparently KLF15 has also an effect on PPARγ expression (U. A. White & Stephens, 2010). KLF4 and KLF6 have been reported to have role in the differentiation/adipogenesis. KLF4 can activate C/EBPβ promoter with Krox20 which consequently speeds up the differentiation. KLF6 on the other hand inhibits protein delta homolog 1 (dlk1), an inhibitor of adipocyte differentiation, thus having a favorable effect on adipogenesis. (U. A. White & Stephens, 2010) Negative effects on adipogenesis has been observed with the other members of the KLF family (U. A. White & Stephens, 2010).

2.2.2 PPARs (Peroxisome Proliferator-Activated Receptors)

PPARγ isoforms PPARγ1 and PPARγ2 are members of the nuclear hormone receptor superfamily. PPARγ2 is mainly found in adipocytes and it is 30 amino acids longer than PPARγ1. PPARγ1 is also predominantly expressed in adipocytes but it can also be found in

other tissues such as colon, macrophages and vascular cells. Third human PPARγ, PPARγ3, has also been discovered. This PPARγ is expressed in adipose tissue and small intestine. PPARγ is conserved in mammals and interestingly enough its homolog can be found in *Xenopus laevis* where it is expressed in the fat body of the organism.

Number of adipocyte genes have PPAR response element, including the ones important in lipid metabolism such as: lipoprotein lipase, phosphoenolpyruvate carboxykinase and acyl-CoA synthetase. The potential of PPARγ to differentiate cells into adipocytes is profound, ectopically expressed PPARγ is able to *trans*-differentiate myoblasts towards adipocytic lineage. (Rangwala & Lazar, 2000)

Mechanism of action

The early transactivation assays revealed that fatty acids and their derivatives could activate PPARγ. Prostaglandins PGJ_2 and PGD_2 were found to activate the receptor in transactivation and adipogenesis assays. 15-deoxy-$\Delta^{12,14}$ PGJ_2 was found to be a potent activator of PPARγ. (Rosen et al., 2000) However, this prostaglandin has not been demonstrated to occur naturally in adipose tissue or adipocyte cell lines. Oxidized fatty acids are naturally occurring ligands for PPARγ. (Rangwala & Lazar, 2000)

Heterodimerization with retinoid X receptor α (RXRα) is required for PPARγ to activate a gene. It has been shown to differentiate 3T3-L1 preadipocytes into adipocytes. In the same study, synergistic effect in adipocyte differentiation was observed when PPARγ and RXRα ligands were present. (Rangwala & Lazar, 2000)

Thiazolidinediones (TZDs) are a class of drugs which are used to prevent and treat type 2 diabetes. These compounds are PPAR-agonists (rosiglitazone for PPARγ and pioglitazone for PPARγ and α). It is thought that thiazolidinediones increase insulin sensitivity both in the liver and in adipose tissue, which further has an effect on differentiation. In fact, being PPARγ-agonists, the use of thiazolidinediones increases subcutaneous fat mass as these cells differentiate. Free fatty acid intake into adipocytes is thought to increase with the use of PPARγ-agonists and this might further protect the liver and muscles from the toxic effects of excess free fatty acids, which might lead to insulin resistance. Use of thiazolidinediones alters the adipocytokine production and this might have indirect effect on insulin resistance. Pioglitazone has been shown to increase the levels

of high molecular weigh adiponectin and to lower the levels of TNF-α and retinol binding protein 4 (RBP-4) in type 2 diabetes patients. Rosiglitazone on the other hand, increases the leptin levels, which is considered to be beneficial if leptin resistance has not developed.

Use of thiazolidinediones is not risk free as both of these drugs are associated with 3-4 kg increase in bodyweight. This effect probably results from fluid retention which further increases the risk for heart failure. Rosiglitazone was actually withdrawn from market in 2010 as it was associated with increased risk of myocardial infarctions. Current research is focused on dual PPARα/γ-agonists (Westerink & Visseren, 2011)

Regulation

PPARγ is regulated post-transcriptionally by mitogen-activated protein (MAP) kinases. A mutated PPARγ2 receptor which lacked the phosphorylation site was found to have an increased adipogenic potential in NIH 3T3 cells. Non-phosphorylated form is also more capable of activating reporter genes with PPAR binding sites and inversively, phosphorylation reduces the transactivation and adipogenesis. MAP kinase pathway activation is thought to block the adipocyte differentiation by phosphorylating PPARγ. It has been shown that phosphorylated form of PPARγ has lower affinity to ligands when compared to non-phosphorylated form. The *in vivo* role of phosphorylation is unclear. In 3T3-L1 cells the levels of phosphorylated and non-phosphorylated receptors remained unchanged during adipogenesis. (Rangwala & Lazar, 2000) Ubiquitin-proteasome system has also been shown to have an important role in the regulation of PPARγ (U. A. White & Stephens, 2010).

2.2.3 C/EBPs (CCAAT/Enhancer-Binding Proteins)

C/EBPs are part of the basic leucine zipper (bZIP) family. Basic DNA-binding region and the leucine zipper, which is responsible for the dimerization, are located in the carboxy terminus of the protein. Adipose tissue expresses C/EBPs α, β, δ and ζ.

Numerous adipocyte-specific genes have a C/EBP-binding site. To mention few, adipocyte protein 2 (AP2), GLUT-4, phosphoenol-pyruvate carboxykinase and uncoupling

protein (UCP) all have such site. C/EBP expression seems to be critical to adipocytes, particularly C/EBPα. (Rangwala & Lazar, 2000)

Mechanism of action

Conditional expression of C/EBPα in fibroblasts induces adipogenesis without the normal hormonal stimulation used in the differentiation of these lineages. C/EBPα protein inhibition leads to decrease in lipid accumulation and blockade of fat-specific marker genes. (Rangwala & Lazar, 2000)

Regulation

C/EBPs activity is regulated with phosphorylation. Glycogen synthase kinase 3 (GSK-3) phosphorylates the C/EBPα. However it is important to note that insulin antagonizes the activity of GSK-3 via phosphatidylinositol 3-kinase pathway. C/EBPβ is serine-phosphorylated by cyclic adenosine monophosphate (cAMP), which increases its ability to activate acetyl-CoA carboxylase. (Rangwala & Lazar, 2000)

C/EBPζ, also known with names CHOP-10 and gadd153, has the ability to bind the other C/EBP proteins. C/EBPζ acts as a dominant-negative inhibitor because the resulting heterodimers cannot bind DNA. Although C/EBPζ was originally described to be induced during adipogenesis it is not part of the adipogenic program. Instead, its activity is dependent on glucose deprivation. Studies are suggesting that C/EBPζ is a mediator of the cellular stress response. (Rangwala & Lazar, 2000)

C/EBPα has a number of translation products resulting from different start sites. Expression levels of these proteins change during the differentiation, which might indicate a role in the regulation of adipogenic process. Protein p42 has the strongest adipogenic and antimitotic protein from C/EBPαs. (Rangwala & Lazar, 2000)

2.2.4 ADD1/SREBP (adipocyte determination and differentiation factor-1/ sterol regulatory element-binding protein)

ADD1 (rat) and SREBP-1 (human) are membrane bound proteins which regulate cholesterol metabolism. Collectively the group is known as SREBPs. ADD1 is a 98-kDa basic helix-loop-helix leucine zipper transcription factor, which binds to E-box (CANNTG) of the fatty acid synthase gene promoter. SREBP-1 exists in two forms, SREBP-1a and -1c, of which 1c predominates in animals (Rosen, 2000) and which result from the use of two alternative start sites in transcription. (Rangwala & Lazar, 2000).

Mechanism of action

In the absence of cholesterol, SREBP1 is proteolytically cleaved from the endoplasmic reticulum and released to cytosol. The protein enters nucleus and increases the transcription of its target genes. ADD1/SREBP-1 regulates the expression of numerous enzymes on both cholesterol biosynthetic pathway and fatty acid synthesis and uptake. (Rangwala & Lazar, 2000) However, the exact mechanism for this event is described in liver cells and it is unknown whether the same mechanism exists in fat cells (Rosen et al., 2000).

2.3 Adipogenesis

In this section the transcription factors from the previous section are brought together and the growth process from pre-adipocyte to mature adipocyte is explained. Also some animal models are introduced.

Adipogenesis, the development of mature adipocytes from preadipocytes, might be one of the most studied models for cellular differentiation. One reason for this is the availability of good *in vitro* models which replicate the *in vivo* process of fat cell formation rather faithfully. These features are: morphological changes, cell growth cessation, lipid

accumulation, cytokine and adipokine production, expression of lipogenic enzymes and sensitivity to hormones. (Rosen & Spiegelman, 2000)

In 1970s Green and colleagues isolated two of the most studied fat cell lines used today. These murine cell lines are 3T3-L1 and 3T3-F442A (Rosen & Spiegelman, 2000). (Detailed description is given in 2.5 Cell lines)

In cell culture the first stage in the differentiation is growth arrest which happens after the cells have reached confluency and become contact inhibited. However, culture studies with low densities have shown that cell-cell contact is not an absolute requirement for growth arrest. In culture models the growth arrest is achieved with prodifferentiative hormonal/chemical mixture. One or two rounds of cell division occur due to these new conditions in a process known as clonal expansion. After initial clonal expansion comes final phase of growth arrest, differentiation process occurs and cells develop into mature adipocytes. (Rosen & Spiegelman, 2000)

C/EBP and PPAR families of transcription factors play a major role in the adipogenesis by inducing the expression of many of the adipocyte-specific genes. These factors are also present in the mature adipocytes. In the early stage of differentiation, C/EBPβ and C/EBPδ seem to have a significant role. When the differentiation is induced, these factors appear and their levels decrease in the terminal phase of the differentiation. Induction of C/EBPβ and C/EBPδ appears to be the result of cAMP and glucocorticoid. C/EBPβ has two isoforms of which the longer seems to be a positive regulator and the shorter a negative regulator. (Hwang et al., 1997) The ratio of these factors favors the positive regulator as the adipogenesis progresses (Rosen et al., 2000). Definitive role of C/EBPβ and C/EBPδ is unclear, but the primary function of these proteins seems to be the induction of C/EBPα (Hwang et al., 1997). Ectopic expression of C/EBPβ is able to force non-adipogenic cell lines to adipocytes (Salma et al., 2006).

C/EBPα is thought to have a central role in the energy homeostasis control. When C/EBPα is expressed ectopically it is capable of inducing differentiation in 3T3-L1 preadipocytes, without any additional hormonal stimuli. On the other hand, expression of antisense C/EBPα RNA in these cells blocks the differentiation program. (Hwang et al., 1997) C/EBPα has different isoforms and the ratio of these isoforms changes during the differentiation to favor the stronger activator p42. (Rosen et al., 2000) C/EBPα is a late

transcription factor as it appears few days after the initiation of the differentiation (U. A. White & Stephens, 2010).

PPARγ is key factor in the adipogenesis. More precisely, PPARγ2 is the fat cell dominant isoform of this transcription factor. PPARγ was found in the early 90s' by several groups and has since then been under intensive research. PPARγ induces the expression of AP2 as well as number of other fat-cell specific genes. For example: the expression of phosphoenolpyruvate carboxykinase (PEPCK) reguires PPARγ binding. The role of PPARγ in adipogenesis is significant because it affects virtually all aspects of this process. The effect can be seen in morphological changes, lipid accumulation and insulin sensitivity. In fact, PPARγ is such a potent transcription factor that it is able to transdifferentiate myocytes to adipocytes. (Rosen et al., 2000) However, in mature adipocytes the expression of PPARγ is reduced as the adipocytes age, suggesting that the maintenance of the phenotype is independent of this factor. In a small number of patients with severe insulin resistance, heterozygous mutations have been found in PPARγ which act in a dominant-negative manner. However, these patients have normal fat content which indicates that the amount of PPARγ needed for normal insulin sensitivity is different from that needed for normal adipose development. (Rosen et al., 2000; U. A. White & Stephens, 2010) Loss-of-function mutations might result a lipodystrophic phenotype in humans (U. A. White & Stephens, 2010). Figure 4 gives a general idea of adipogenesis.

Mature adipocytes are round and filled with lipids which are stored in usually one large vacuole. This vacuole fills most of the cell and the nucleus and cytoplasm are on the edges, in close contact with cell membrane. Over the years it has become clear that adipocytes secrete numerous compounds which have profound effect on energy metabolism. Leptin is a good example of these. In obesity, adipocyte homeostasis is unbalanced which manifests in altered adipokine expression. (Armani et al., 2010)

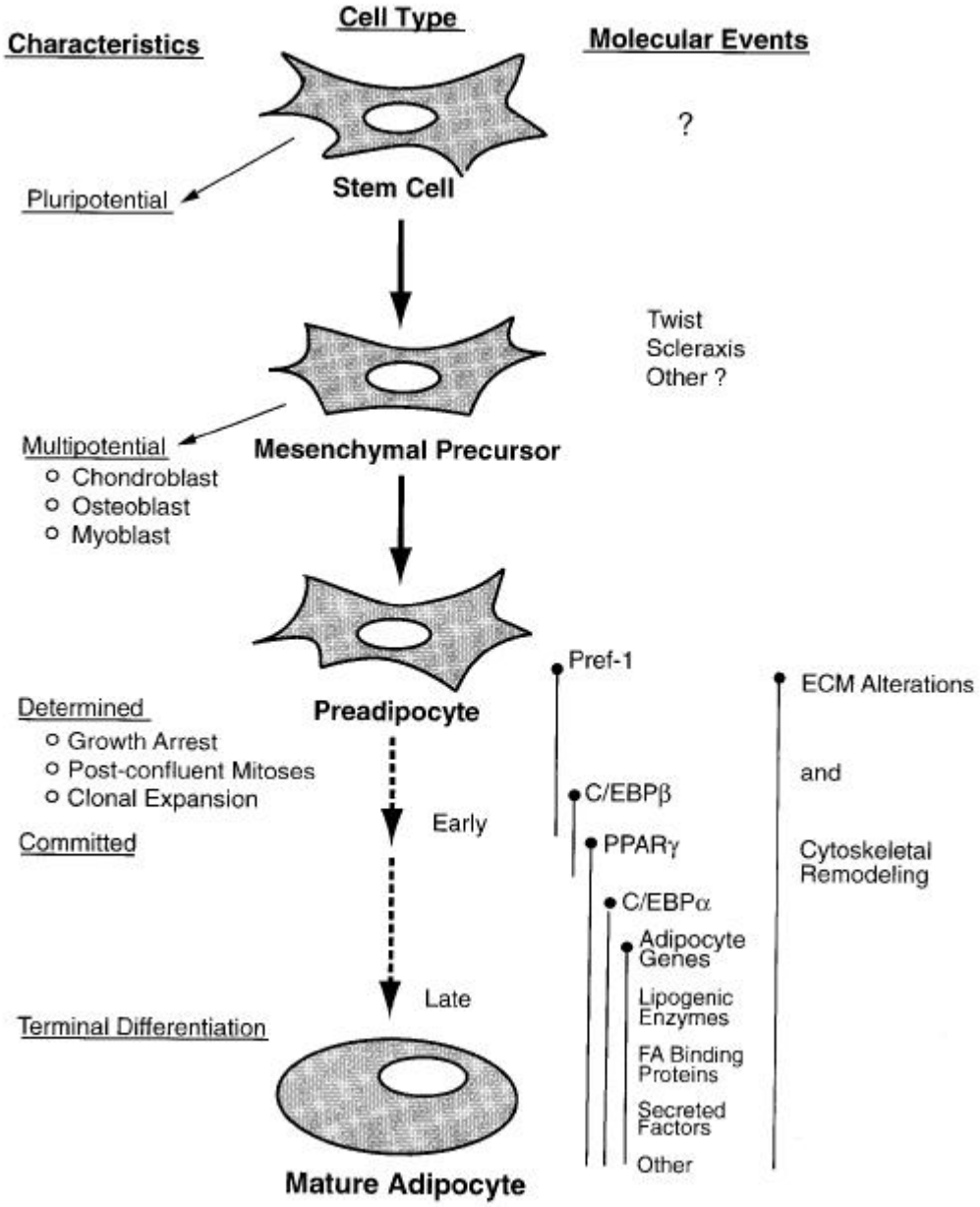

Figure 4. Overall view of adipocyte development from stem cell to mature adipocyte. Initial determination of adipocytic lineage is not known. However, mesenchymal precursor cells can be successfully differentiated into adipocytes. Preadipocyte development into mature adipocyte is characterized by profound changes in morphology along with the upregulation of key adipogenic transcription factors. Pref-1, preadipocyte factor 1; C/EBP, CCAAT/enhancer binding protein; PPARγ, peroxisome proliferator-activated receptor. Figure modified from Gregoire, 1998

2.3.1 *In vivo* models

Few animal models have been developed over the years to study adipocyte development, fat distribution and developmental aspects of these factors. Knockout mice have been developed for most of the transcription factors mentioned here and also to a number of adipokines and their receptors.

Transgenic mice expressing dominant-negative protein for DNA binding of bZIP transcription factors in C/EBP and Jun families have no white adipose tissue throughout life. Transcription factors were controlled with AP2 promoter/enhancer. (U. A. White & Stephens, 2010) STAT5 deletion in mice results considerably reduced fat pads compared to wild-type. Growth hormone stimulated lipolysis was not observed in primary cultures from these animals, suggesting that some growth hormone effects are dependent on STAT5 expression. (U. A. White & Stephens, 2010)

Transgenic mice lacking C/EBPβ and C/EBPδ have defective adipocyte differentiation (U. A. White & Stephens, 2010). C/EBPα knockout mice have some expression of adipocyte-specific genes in BAT, but otherwise they don't accumulate lipids at all. These mice die within 8 hours of birth in hypoglycemia, which further strengthens the role of C/EBPα in energy homeostasis (Hwang et al., 1997). PPARγ knockout mice die in the uterus after 11 days due to placental defect. Different approaches have been used to circumvent this problem. One chimeric approach revealed that PPARγ is required for *in vivo* adipogenesis. The other chimeric model was able to give homozygous mutant that developed to term, but this mouse was lost shortly after birth and it lacked significant brown fat depots. (Rosen et al., 2000)

SREBP-1 deficient mice have almost normal amount of WAT, but the levels of SREBP-2 are increased, suggesting a compensatory mechanism. When these mice are crossed with *ob/ob* mice it is clear that SREBP-1 is not required for the development of the obesity. (U. A. White & Stephens, 2010)

Adiponectin overexpression in *ob/ob* mice results a better overall health despite the further expansion of the subcutaneous WAT. This effect might be the result of highly vascularized WAT in these mice (Rutkowski et al., 2009).

2.4 Adipokines and Adipocytokines

As previously mentioned, adipose tissue has been shown to be a considerable endocrine organ which secretes a myriad of bioactive substances. These substances have been conceptualized as adipokines or adipocytokines. Use of these terms is not coherent and sometimes it is hard to know whether adipocytes or adipose tissue as a whole is referred. This is a minor pitfall, but something one should be aware of.

2.4.1. Adiponectin

Adiponectin is a hormone secreted mainly by adipocytes and it is the most abundant transcript of the human fat. In blood, adiponectin can be found in concentrations between 5–20 µg/ml. Mouse homologs for adiponectin are called Acrp30 and AdipoQ. (Iwaki et al., 2003) It regulates the energy homeostasis and both glucose and lipid metabolism. Presense of adiponectin has been connected to healthy, non-inflammatory state of the adipose-tissue and possibly has an insulin-sensitizing effect (Kita et al., 2005; Moriuchi et al., 2007). Therefore it is not surprising that plasma adiponectin levels are lower in diabetics (Iwaki et al., 2003). Studies have shown that plasma adiponectin concentrations decrease in obese mice and human and correlate negatively with insulin resistance (Iwaki et al., 2003; Kita et al., 2005; Moriuchi et al., 2007). In a monkey model for diabetes, low adiponectin levels in plasma precede the development of hyperinsulinemia and hyperglycemia (Iwaki et al., 2003).

Adiponectin has several active forms. Two forms, full-length protein and a proteolytic cleavage fragment which is the globular C-terminal domain, are the predominant entities of adiponectin. C-terminal form is collectively known as globular adiponectin. Full-length form of adiponectin can oligomerize extensively and these forms can be found in circulation, whereas the existence of globular form in circulation has been questioned. Full-length form of adiponectin exists as trimers (low-molecular-weight-adiponectin), hexamers (middle-molecular-weight-adiponectin) and 12- to 18- mers, known as high-molecular-weight- adiponectin. It has been suggested that the ratio of these forms is the key factor in determining insulin sensitivity. Supporting evidence for this follows after

weight reduction: moderate weight loss increases the ratio of high-molecular-weight to middle-molecular-weight and at the same time the amount of low-molecular-weight-adiponectin is reduced in the serum. (Tilg & Moschen, 2006)

Adiponectin has a number of beneficial properties. Antidiabetic properties are manifested through insulin receptor substrate-1 (IRS-1) and phosphatidylinositol-3 kinase (PI-3K) which increase glucose uptake into skeletal muscle. Adiponectin also enhances muscle β-oxidation and suppresses hepatic glucose production. It has antiatherogenic effects in tissue cultures and it also suppresses monocyte adhesion to endothelial cells by reducing NF-κB signalling. Adiponectin also inhibits macrophage foam cell formation and vascular smooth muscle cell proliferation and migration. (Iwaki et al., 2003)

Two receptors for adiponectin have been found. These are expressed mainly in the skeletal muscle (Receptor 1) and in the liver (Receptor 2). Signals are transmitted through adenosine monophosphate-activated protein (AMPK) which functions as an energy sensor and regulates cellular metabolism. (Iwaki et al., 2003)

Insulin resistance and diabetes can be induced with diet in adiponectin deficient mice. Neointimal thickening of the arterial wall is also observed. Adiponectin supplementation reverses the diabetic and atherogenic phenotype in these knockout mice (Iwaki et al., 2003).

It seems that hypoadiponectinemia precedes pathological conditions such as obesity-related metabolic syndrome, accompanied with insulin resistant diabetes and atherosclerosis. A therapy which could increase the endogenic production of adiponectin might be beneficial in treating these complex diseases. Thiazolidinediones, which are used to treat diabetes, have been shown to increase the plasma adiponectin levels in humans (Westerink & Visseren, 2011). Reduction of body mass with diet and enhanced exercise activity are found to increase the plasma adiponectin levels (Moriuchi et al., 2007). PPAR-responsive element (PPRE) upon which PPARγ/RXR complex binds, has been found in adiponectin promoter. Also liver receptor homolog-1 (LRH-1) has been found to enhance adiponectin gene transactivation in adipocytes (Iwaki et al., 2003). TNF-α, which is secreted from the adipocytes, has been shown to decrease the levels of adiponectin expression and secretion (Moriuchi et al., 2007). TNF-α might transmit its effects by modulating the C/EBP activity (Kita et al., 2005). Adiponectin has three binding sites for

C/EBP proteins in its promoter and it has been shown in 3T3-L1 adipocytes that C/EBPβ, δ and α bind to these sites in a temporal manner. First appear β and δ within hours from the beginning of differentiation, and later C/EBPα along with adiponectin, which appears after five days of culturing (Salma et al., 2006).

2.4.2 Leptin

Leptin is the best studied adipokine and the first which was shown to have role in the regulation of adiposity (Rosen & Spiegelman, 2006). Molecular weight of leptin is 16 kDa and it circulates as free and bound forms. It is mainly expressed in the adipocytes, but also at low levels in stomach, mammary gland, placenta and skeletal muscle. (Ahima et al., 2006) Mutations in leptin itself or in its receptors lead to obesity both in humans and animals. Classic animal models for leptin study, *ob/ob* and *db/db* mice, have provided valuable data for understanding the metabolic pathways related to obesity. The *ob/ob* mice have a mutation on the coding region of the *obese* gene (leptin), which leads to truncated nonfunctional leptin protein. As a consequence these mice are obese, have severe insulin resistance, steatosis (adipose degeneration), hyperglycemia, lowered fertility and immunosuppression. (Hwang et al., 1997; Ahima et al., 2006) The *db/db* mice have mutation on the gene which codes for leptin receptor. This mutation leads to alternative splicing and an intermediate form of the receptor which lacks most of the signaling capacity. These mice have similar phenotype as *ob/ob* mice (Hwang et al., 1997). Therefore, it is not surprising that plasma and WAT leptin levels correlate positively with WAT mass, adipocyte size and triacylglycerol content (Ahima et al., 2006). Leptin levels are higher when individual is obese. Also women have higher leptin levels, even after adjustment with body mass. Sexual dimorphism is probably due to a higher amount of subcutaneous WAT in women. (Ahima et al., 2006) Receptor activation induces the anorexigenic pathways and represses the orexigenic ones (Rosen & Spiegelman, 2006). Cold exposure and adrenergic stimulation decrease leptin levels whereas insulin, cytokines and glucocorticoids increase leptin levels (Ahima et al., 2006). Concept “leptin resistance” has been introduced since obese individuals express high levels of leptin but the suppression of appetite is not functional. Number of explanations has been proposed to

contribute this phenomenon. It has been suggested that high leptin levels themselves are inducing the resistance through suppressor of cytokine signaling (SOCS3), which blocks the leptin receptor signaling. (Ahima et al., 2006; Gualillo et al., 2007)

Leptin levels peak during the night in humans, but the mechanism and possible functional significance is unknown. Fasting decreases leptin levels and eating increases them. These changes happen with a few hour delay. (Ahima et al., 2006) Leptin receptor has at least six different splice variants (Tilg & Moschen, 2006). LRa, the short form of leptin receptor, lacks cytoplasmic domain required for signaling. The long form of leptin receptor (LRb) mediates intracellular signaling. This receptor is expressed in the hypothalamus and brainstem where it participates in the control of feeding, metabolism and neuroendocrine function. (Ahima et al., 2006) Leptin signaling is classically mediated through JAK2 and STAT3 (Ahima et al., 2006). Leptin promoter has at least one functional C/EBP binding site and in 3T3-L1 adipocytes the C/EBP transcription factors behave similarly as in adiponectin: C/EBPs β and δ appear within hours after differentiation is initiated and C/EBPα appears only after few days of differentiation along with the leptin, which appears in 5-7 days. (Salma et al., 2006) TZDs inhibit leptin gene expression in adipocytes (Rangwala & Lazar, 2000)

2.4.3. Adipsin/Complement factor D

Adipsin is part of the alternative pathway of the complement activation. It is the rate-limiting enzyme of this pathway and its enzymatic activity is targeted to complement factor B, which is cleaved when it forms a complex with complement factor C3 (R. T. White et al., 1992; Fantuzzi, 2005) Adipsin was also one of the first proteins found to be secreted from the white fat adipocytes. It was discovered in cell culture studies and found to be expressed in a differentiation-dependent manner (Trayhurn & Beattie, 2001). The cell line was mouse 3T3-F442A (R. T. White et al., 1992). Studies with animal models for obesity showed that the expression of the adipsin gene was reduced considerably in this condition. This led to the original view that adipsin might serve as lipostatic signal (Trayhurn & Beattie, 2001). However, it was later discovered that adipsin levels are not reduced in obese humans but rather correlate with the fat mass and increase with obesity (Esterbauer et al.,

1999). The signaling role of this protein for energy balance was therefore abandoned (Trayhurn & Beattie, 2001). One study concentrated on fat depot specific gene expression in adipocytes and it was found that adipsin is expressed at similar levels both in omental and subcutaneous adipose tissue (Montague et al., 1998)

2.4.4 Resistin

Resistin, also known with the name FIZZ3, is an adipocyte-derived peptide hormone which was first described in 2001 in rodents. The name resistin comes from insulin resistance which was observed when mice were injected with resistin. In humans, resistin is also expressed in macrophages. Production of resistin is increased in obesity and decreased by PPARγ ligands. Animal studies have shown a causal role between serum resistin levels and glucose homeostasis. Increase in resistin (infusion, over-expression) leads to hyperglycemia and decrease from the normal levels (gene knockout, antibody infusion) protects from the effects of obesity-induced hyperglycemia.

In humans the role of resistin is not as clear as in rodents since not all of the studies have been able to show the increase of serum resistin in obese type 2 diabetics. *In vivo* studies consistently show that resistin suppresses muscle and liver AMPK but in general, very little is known about the intracellular signaling pathways. (Galic et al., 2010)

2.4.5 MCP-1

Monocyte chemotactic protein-1 (MCP-1) is a chemokine expressed by adipocytes and number of other cell types including macrophages, smooth muscle cells and endothelial cells. MCP-1 is also known by the name chemokine ligand 2 (CCL2). MCP-1 is a member of small inducible cytokine family and it has an important role in recruiting monocytes and T lymphocytes. (Sell et al., 2006; Sell et al., 2006; Gualillo et al., 2007) In diabetic patients the plasma levels of MCP-1 are significantly higher than in lean subjects (Sell et al., 2006). MCP-1 has been shown to up-regulate the leptin synthesis in cultured adipocytes (Gualillo et al., 2007). MCP-1 polymorphism has been linked to high risk of coronary atherosclerosis. Epicardial adipose tissue has been shown to produce higher amounts of this

protein than subcutaneous white adipose tissue. Thus, the link between cardiovascular dysfunction and this chemokine/adipokine is easy to draw and it might be that this factor acts via paracrine or juxtacrine mechanism and the effect is local rather than systemic (Gualillo et al., 2007)

2.4.6 TNF-α

Tumor necrosis factor- α (TNF-α) is a soluble pro-inflammatory protein. 17-kDa biologically active form is released after cleavage. Originally found in macrophages and lymphocytes this pro-inflammatory cytokine was later demonstrated to be produced also by adipocytes. However, the main source of TNF-α in adipose tissue is macrophages. Obesity induces significantly the expression of TNF-α in adipose tissue and this effect has been seen in both animal and human studies.

TNF-α has been linked to insulin resistance and there is a strong inverse correlation between TNF-α secretion and insulin stimulated glucose metabolism. TNF-α has the ability to decrease the tyrosine kinase activity of the insulin receptor and consequently attenuate the downstream signaling. Proteins in the insulin signaling pathway such as GLUT4 and PPARγ are down-regulated by TNF-α in adipocytes. Use of TNF-α antibodies in obese patients with T2D did not improve their insulin sensitivity. (Zou & Shao, 2008)

2.4.7 Other adipokines

Adipose tissue secretes several proteins and at the moment the number is closer to one hundred (Gnacinska et al., 2009), but not all of them are adipocyte derived. The following list is limited to those adipokines known to be secreted by the adipocytes.

Vaspin, visceral adipose tissue-derived serine protease inhibitor, was identified in obese and hyperinsulinemic rats (Rabe et al., 2008). *In vivo* administration of vaspin improved glucose tolerance and insulin sensitivity in mice (Lago et al., 2007). Vaspin serum levels are also correlated with human obesity but abrogated in type 2 diabetes (Rabe et al., 2008). Levels of leptin, resistin and TNF-α have been shown to decrease by vaspin (Wozniak et al., 2009).

Visfatin was found to be an adipokine in 2005. It was previously known with the name PBEF (pre-B-cell colony enhancing factor) (Fantuzzi, 2005; Rabe et al., 2008). Visfatin levels are high in visceral fat and the plasma protein levels increase with obesity (Zou & Shao, 2008). Insulin-mimetic effects were shown in initial studies with cultured cells and mice but further studies have failed to show the connection between visfatin levels and parameters of insulin sensitivity (Rabe et al., 2008). Visfatin binds the insulin receptor, but different site than insulin (Zou & Shao, 2008) Also nicotinamide phosphoribosyltransferase enzymatic activity has been shown in this adipokine (Rosen & Spiegelman, 2006)

Apelin is secreted by adipocytes but also by number of other cell types, including the cells of cardiovascular system (Gualillo et al., 2007) Increase in the insulin levels increases the apelin levels, which leads to potential link between obesity-related insulin sensitivity (Trayhurn, 2005). Apelin has also been shown to have positive hemodynamic and inotropic activities (Guzik et al., 2006; Wozniak et al., 2009). Interestingly, apelin has been proposed to have an effect on adiponectin levels and to brown adipose tissue uncoupling proteins (Gualillo et al., 2007; Wozniak et al., 2009)

IL-6, interleukin 6, is a cytokine of which approximately 25% is secreted by adipocytes (Gualillo et al., 2007). Its plasma concentrations correlate with metabolic syndrome and type 2 diabetes. In fact, IL-6 has been shown to impair insulin signaling and up-regulate the MCP-1 expression, along with the down-regulation of adiponectin and visfatin. (Gualillo et al., 2007) High levels of IL-6 might be due to increase in the acute-phase proteins observed in obese patients, such as C-reactive protein (CRP) (Fantuzzi, 2005)

RBP4, retinol-binding protein 4, was previously thought to have a role only in retinol transportation, but it has now been characterized as adipocytokine. RBP4 serum levels are increased with type 2 diabetes. (Tilg & Moschen, 2006) Transgenic expression of RBP4 in mice causes insulin resistance (Tilg & Moschen, 2006; Rabe et al., 2008). However, the detrimental role of RBP4 is not confirmed in all studies and it might be limited to rodents (Rabe et al., 2008) RBP4 is regulated by the GLUT4 transporter and the overexpression of RBP4 impairs the insulin action is muscle and liver. (Rosen & Spiegelman, 2006)

2.5 Cell Lines and differentiation protocols

2.5.1 SW872 cell line

SW872 is a human liposarcoma cell line which was isolated in 1974 by A. Leipovitz from a surgical specimen of fibrosarcoma at Scott and White Clinic in Temple, Texas (hence the name SW). Histopathological analysis graded the tissue as undifferentiated malignant tumor consistent with liposarcoma. The subject was 36 year old caucasian male. SW872 cells are available for purchase from ATCC (American Type Culture Collection) under the name HTB-92^{TM}. (www.atcc.org)

The earliest publications in which this cell line was used are from 1996 and they focus on vitamin D and cholesteryl ester transfer protein (CETP) (Richardson et al., 1996). Since then the research has been focused mainly on lipoproteins. Also some cancer research has been performed with SW872 cell line (Guo et al., 2008). However, none of the studies have focused specifically on adipokine production. At the moment (May, 2011), the number of published studies with this particular cell line is rather scarce. Literature search in PubMed gives only 33 publications (keywords: SW872, HTB-92). Only in 2 publications the cells have been grown more than five days, 15 and 17 days respectively, and both papers are from the same research group (Wassef et al., 2004; Carmel et al., 2009). This research group has their focus on apolipoprotein E. These two studies show clear expression patterns for PPARγ and C/EBP-α at mRNA level. In the two studies the initial expression of PPARγ (2-4 days in culture) is higher than in the middle of the protocol (8-10 days). In the end however (15-17 days in culture) the expression of PPARγ reaches its peak value. The magnitude of PPARγ expression is within a narrow range but changes are consistent. (Wassef et al., 2004; Carmel et al., 2009) The expression of C/EBP-α is available from only one study (Carmel et al., 2009), but the results clearly show that the expression of C/EBP-α rises considerably between days 2 and 15 in culture. Interestingly, the over expression of ApoE leads to increased proliferation and differentiation is blunted (Carmel et al., 2009). Other experimental setups found in the literature are much shorter and generally the approach is to grow the cells near-confluent or confluent and then perform an experiment with maximum duration of 2 days. (Izem & Morton, 2001)

Adipokine expression in this cell line hasn't been studied very extensively, but some results are available. Adipsin expression was reported in 2001 by Vassiliou (Vassiliou et al., 2001). However, that data was not shown. Leptin and adiponectin expression has been shown at mRNA level by the previously mentioned study group (Tarnus et al., 2009), but none of the papers show expression on protein level.

SW872 cells differentiate without the differentiation cocktail and they constitutively express genes important to adipocyte development (Wassef et al., 2004). It has also been stated by Izem and Morton that SW872 is a fully mature, lipid-poor adipocytic cell line (Izem & Morton, 2007). SW872 cells proliferate continuously to reach confluency (Carmel et al., 2009). Stained SW872 cells can be seen in Figure 5.

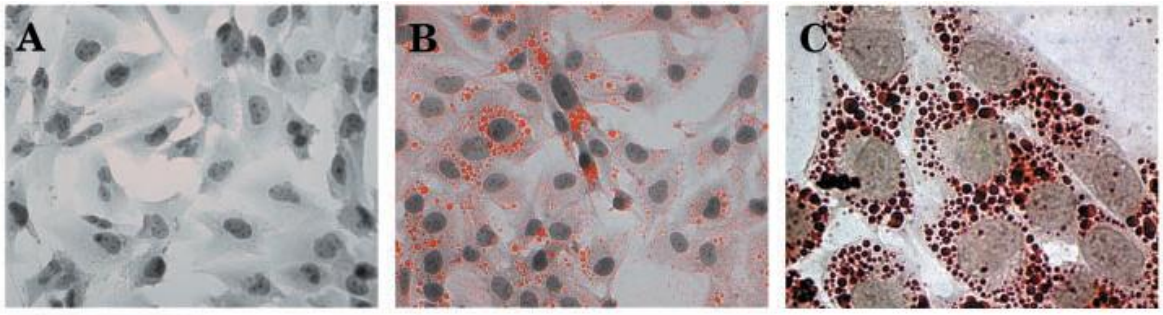

Figure 5. Oil Red O and hematoxylin staining of SW872 cells. A and B has been grown without or with oleate, respectively. C is a 2.5 x magnification of B. Picture modified from Izem, 2001.

2.5.2 3T3-L1, 3T3-F442A and other cell lines used

3T3-L1 and 3T3-F442A cell lines are still perhaps the most used models for adipocyte behavior (Hwang et al., 1997; Gregoire et al., 1998; Ntambi & Young-Cheul, 2000; Armani et al., 2010). These cell lines were clonally isolated from Swiss 3T3 cells, which are derived from disaggregated 17- to 19-day mouse embryos (Gregoire et al., 1998; Armani et al., 2010). 3T3-L1 and 3T3-F442A are unipotent preadipocytes committed to adipocyte lineage. Other cell lines used are: Ob1771 and Ob17, which are from *ob/ob* mice; TA1 and 30A5, which are from multipotent cell line C3H-10T1/2, and 1246 which is from mouse

teratocarcinoma (Hwang et al., 1997). Literature search in Pubmed for these cell lines gives over 8000 publications.

In contrast to SW872, differentiation protocols for these adipocytic lineages are well established. These lines display morphological and biochemical characteristics of the mature adipocytes (Hwang et al., 1997). In fact, when 3T3-L1 preadipocytes are injected into mice, they differentiate and, form fat pads which are indistinguishable from normal adipose tissue (Ntambi & Young-Cheul, 2000). Most of the current knowledge of adipocyte transcription factors and differentiation is from these cell lines (Armani et al., 2010).

Upon confluence these cells become growth arrested and that serves as a signal to differentiate. After the cells have become confluent the differentiation is initiated with appropriate stimuli (described in detail later). The temporal features of transcription factors in 3T3 cells have been determined in detail. After the hormonal stimulus is given to the cells they express *c-fos, c-jun, junB* and *c-myc* within one hour after the induction. C/EBPβ and C/EBPδ appear also shortly after the induction and their expression lasts approximately 2 days. During the expression of C/EBPβ and C/EBPδ, the expression of master regulators of adipocyte phenotype increase, namely PPARγ, C/EBPα and SREBP. (Ntambi & Young-Cheul, 2000; Rangwala & Lazar, 2000) Article by Salma and others gives a nice view to these events (Salma et al., 2006)

2.5.3 Primary cultures from human and animal sources

Along with the use of established cell lines, also primary cultures have been used. These cells have the advantage of being diploid, which is not usually the case with cell lines. Another advantage of the primary cultures is that they can be taken from different fat pads and it has been shown in many occasions that fat from different anatomical locations has different molecular and biochemical features (Gregoire et al., 1998). Also the age of the donor has considerable reductive effect on the replicative potential of the adipocytes (Armani et al., 2010). On the downside however, the hormonal regime for differentiation varies according to the cellular context and the cells cannot be grown many passages as they retain the original diploid genotype (Armani et al., 2010) Primary cultures have been initiated from many species; human, pig, rabbit, rat and mouse and usually subcutaneous fat

is used. With rat, epididymal and retroperitoneal fat has also been used. Perirenal fat from rabbit and pig has also been used (Gregoire et al., 1998).

2.5.4 Differentiation protocols

At least 3T3-L1 adipocytes differentiate spontaneously into fat-cell clusters after several weeks in culture with fetal calf serum. However, with differentiation procedures this process can be greatly enhanced and also synchronized and therefore the conclusions made from transcription timing and all other time related issues are more valid. (Gregoire et al., 1998)

Since 3T3-L1 is the most frequently used cell line in adipocyte research, the differentiation is also fairly standardized. Usually the differentiation medium includes insulin, dexamethasone (or some other glucocorticoid) and phosphodiesterase inhibitor methylisobutylxanthine (MIX, IBMX). Insulin is used to stimulate the IGF-1 (insulin-like growth factor-1) receptor. IGF-1 itself binds the receptor 100-fold more efficiently compared to insulin. Preadipocytes have large number of IGF-1 receptors but only few insulin receptors. During the differentiation the number of insulin receptors increases approximately 20-fold and the adipocytes become sensitive to insulin. (Hwang et al., 1997)

Glucocorticoids (like dexamethasone) have been shown to stimulate the adipocyte differentiation. Direct activation of the transcription can happen upon ligand binding since glucocorticoid receptor is a member of nuclear hormone receptor family. Also, prostaglandins PGE_2 and $PGF_{2\alpha}$ and prostacyclin have been shown to inhibit the differentiation process. As a result, inhibition of prostaglandin synthesis from arachidonate in the cyclooxygenase pathway has a stimulatory effect on differentiation. Dexamethasone inhibits the phospholipase A2 catalyzed release of endogenous arachidonate. Cyclooxygenase inhibitors, such as indomethacin and ibuprofen, have also been shown to promote the differentiation (Hwang et al., 1997). Methylisobutylxanthine (MIX) or isobutylmethylxanthine (IBMX), same compound with two different names, has been found to accelerate the differentiation process and is routinely used in the differentiation of the most common cell line 3T3-L1. Increase in the expression of C/EBPβ has been shown with the use of IBMX. Mechanisms behind IBMX are not totally resolved but it is known

to block A1 adenosine receptor and inhibit phosphodiesterases and to stimulate adenylate cyclase activity. The suggested mechanism of action for IBMX might be the increase in cAMP, but probably this is not the whole story since other compounds that increase the amount of cAMP have given contradictory results with the differentiation. (Gregoire et al., 1998) General timing of the events in the differentiation program of 3T3-L1 is shown in Figure 6.

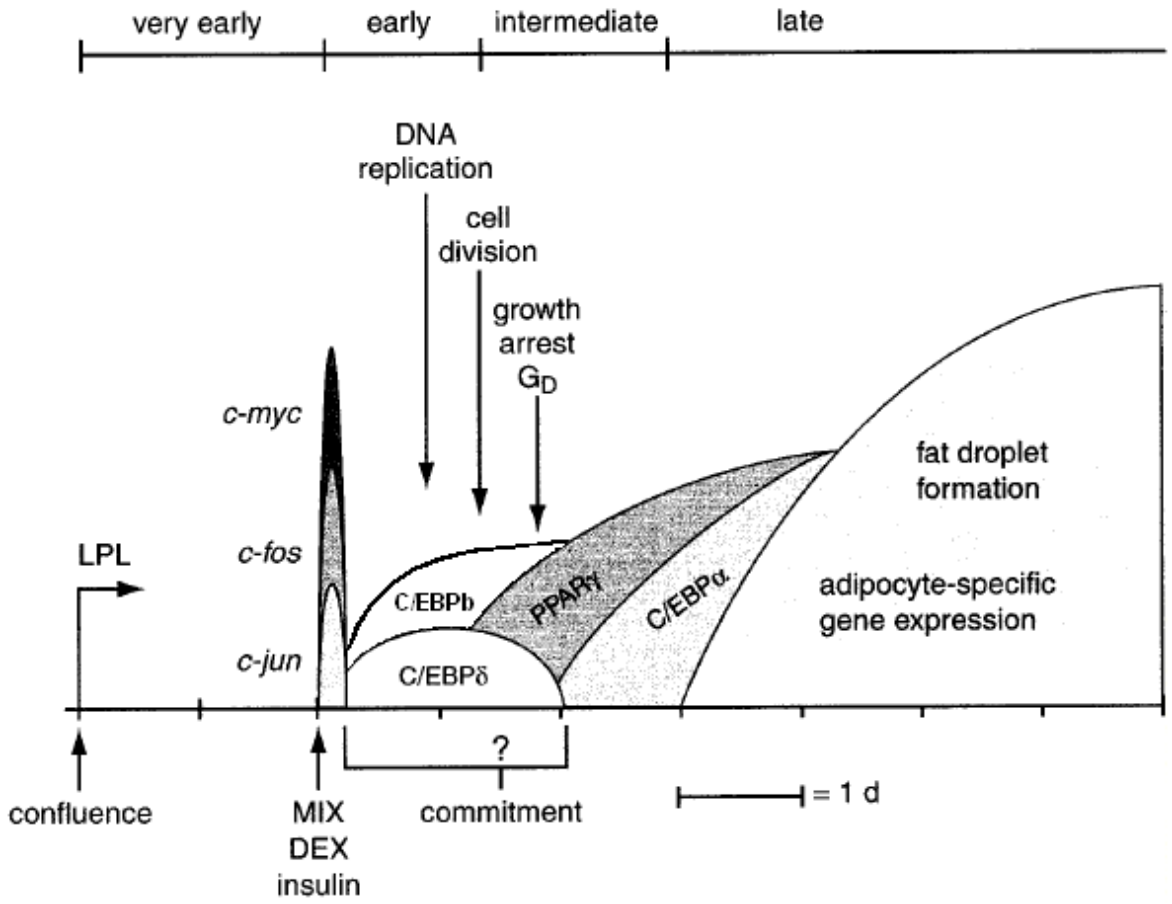

Figure 6. 3T3-L1 preadipocyte differentiation. Major events of the differentiation are presented on a timeline. Areas labeled with gene names represent the periods of expression during the differentiation program. LPL: lipoprotein lipase; MIX: methylisobutylxanthine; DEX: dexamethasone; C/EBP: CCAAT/enhancer binding protein; PPAR: peroxisome proliferator-activated receptor. Picture modified from Ntambi 2000.

In one SW872 study, insulin, dexamethasone, triiodothyronine (T3), biotin and pantothenate were used to stimulate the maturation in serum-free conditions (Wassef et al., 2004). The other study used 500 µM oleate and 2 µM rosiglitazone for four days to accelerate the growth and maturation (Carmel et al., 2009).

Primary cultures are usually differentiated in serum free conditions with insulin and a glucocorticoid (usually dexamethasone), with some exceptions (Gregoire et al., 1998).

3. Aims of the study

The aim of the present study was to investigate the feasibility of a human cell line for pharmacological studies on the regulation of adipocytokine production in adipocytes. Human liposarcoma cell line SW872 was selected based on a literature search and used as a research model. The detailed aims of the study were:

1) Establish the cell culture conditions for SW872 cell line

2) Establish the differentiation procedures after which the cell line shows adipocyte phenotype and shows maturation dependent changes in adipocytokine production

3) Study the effect of selected pharmacological substances upon adipocytokine production after the established differentiation regime.

4. Methods

4.1 Cell culture

4.1.1 Materials

Human SW872 liposarcoma cells were bought from the ATCC (American Type Culture Collection.) DMEM (Dulbecco's modified Eagle medium), FBS (fetal bovine serum) and Preadipocyte Growth Medium-2™ were bought from Lonza. Antibiotic/antimycotic solution was bought from GIBCO. The following reagents were purchased from Sigma: linoleic acid (L9530), oleic acid (O3008), 3-isobutyl-1-methylxanthine (I7018), human insulin (I9278), dexamethasone (D4902), DMSO (D8418). Rosiglitazone was bought from Cayman Chemical.

Pharmacological experiments were carried out using following substances: ibuprofen (550-249-G001, Alexis), dexamethasone (D4902, Sigma), rofecoxib (Merck & Co), paracetamol (UC448, Sigma), glucosamine (Sigma), iodoacetate (I9148, Sigma), ciglitazone (1307, Tocris), 15d-PGJ2 (538927, Calbiochem), resveratrol (1418, Tocris), quercetin (337951, Sigma), kaempferol (1124S, Extrasynthese). See table 2.

4.1.2 Cell culturing

Human SW872 liposarcoma cell line was used in the studies and it was purchased from ATCC (American Type Culture Collection) ATCC's brand name for this cell line is HTB-92.

Four different types of media were used in the course of this study and three of them in the actual differentiation and pharmacological experiments. These media were: Leipovitz L-15 Medium supplemented with L-glutamine and Phenol Red (Gibco®, Invitrogen), DMEM with 4,5 g/l glucose and L-glutamine (BioWhittaker®, LONZA), Ham's F-12 with L-glutamine (BioWhittaker®, LONZA) and Preadipocyte Growth Medium-2; PGM™-2

(Poietics®, LONZA). (Table 1.) In addition, all media were supplemented with 10% fetal bovine serum (FBS) and Antibiotic-Antimycotic solution (15240-062, Invitrogen).

Basal Media	Media used in experiments	Manufacturer, Brand
Leipovitz L - 15 with L-glutamine	-	Invitrogen, GIBCO
DMEM 4,5 g/l glucose with L-glutamine	DMEM 4,5 g/l glucose with L-glutamine	LONZA, BioWhittaker®
-	DMEM 4,5 g/l glucose with L-glutamine, Ham's F-12 with L-glutamine (1:1)	LONZA, BioWhittaker®
-	PGM™-2	LONZA, Poietics®

Table 1. Media used in the study

Leipovitz L-15 medium was used in the beginning of the study according to manufacturer's instructions but was later replaced with DMEM due to practical reasons; Leipovitz L-15 medium requires atmosphere with 100% oxygen and all the other media are kept in atmosphere with 95% oxygen and 5% carbon dioxide. According to the literature DMEM has been succesfully used in SW872 studies. (Izem & Morton, 2001; Wassef et al., 2004; Carmel et al., 2009; Tarnus et al., 2009)

4.1.3 Differentiation

Pre-adipocytes can be differentiated to adipocytes in several ways. The protocol shown here is an amalgamation from different sources. (Hwang et al., 1997; Wassef et al., 2004; Izem & Morton, 2007) In general, adipogenic mixture of insulin, dexamethasone and IBMX has often been used in differentiation. In addition, PPARγ-agonists can be used to enhance the differentiation. Also fatty acids are used.

Human insulin (10 µg/ml), dexamethasone (1µM), IBMX (0.1 mM and 0.5 mM), oleic and linoleic acid (3 µM and 30 µM) were used. Also rosiglitazone (0.5 µM and 1.0 µM) was

used. When plated, cells were allowed to grow for 2-3 days in order to reach confluence and become growth arrested. Confluency also provides the signal for the cells to start the differentiation. Two different time periods were used, 3 days and 10 days. During these periods the cells were incubated with different types of differentiation media. Detailed explanation of individual growth conditions is given within the Results.

4.1.4 Pharmacological experiments

The effects of selected pharmacological agents on adipokine production were also assessed. Tested pharmacological agents included substances from different compound families including: anti-inflammatory drugs, medicines for osteoarthritis (OA), type 2 diabetes medicines (PPARγ-agonists) and polyphenols and flavonoids. (Table 2.)

Molecule	Class	Concentration	Manufacturer
Dexamethasone	Anti-inflammatory steroid	10 µM	Sigma
Ibuprofen	Non-selective COX inhibitor	10 µM	Alexis
Rofecoxib	COX-2 inhibitor	10 µM	Merck & Co
Paracetamol	Analgesic, used in OA	100 µM	Sigma
Glukosamine	Amino sugar, used in OA	100 µM	Sigma
Iodoacetate	Peptidase inhibitor	100 µM	Sigma
Ciglitazone	Thiazolidinedione	100 µM	Tocris
15d-PGJ2	PPARγ agonist	10 µM	Calbiochem
Resveratrol	Stilbenoid	10 µM	Tocris
Quercetin	Flavonoid	100 µM	Sigma
Kaempferol	Flavonoid	100 µM	Extrasynthese

Table 2. Substances used in the pharmacological experiments

4.2 Oil Red O staining

Oil Red O - staining is an easy and fast method to illustrate lipids inside of the cells. The method is not quantitative but it still gives a reasonable measure for lipid content of the

cell. Given its nature, the method is not usually described in detail in publications. (Okada et al., 2008)

Stock solution (0.35%) was prepared by mixing 0.7 grams of Oil Red O powder (Sigma O0625) with 200 ml of 100% isopropanol. The solution was mixed overnight with magnetic mixer (Variomag) and filtered through 22 µm filter on the following day. The stock solution was stored in + 4° until used.

New staining solution was prepared for each cell staining as the staining solution is usable for only 2 hours. Staining solution was prepared by mixing 6 parts of the stock solution and 4 parts of distilled water and allowed to stand for 20 minutes, and thereafter filtered through a 22 µm filter. (Salma et al., 2004; Salma et al., 2006)

Medium was gently removed from the wells with water suction and 500 µl of 10% formalin was immediately added on cells and incubated for 5 minutes. Formalin was then replaced with a new one and incubated for 1 hour covered with foil and parafilm. At this stage the staining solution was usually prepared. In the next step the formalin was removed and the cells were washed with 500 µl of 60% isopropanol. 200 µl of Oil Red O staining solution was then added gently in the middle of the well and incubated for 10 minutes. Care was taken not to touch the walls of well as this would impede the staining. After incubation, the Oil Red O staining solution was removed and the wells were immediately washed with distilled water several times in order to remove all of the uncoupled Oil Red O. Since absorbance measurements were included to the study, the protocol was also done with empty wells. After staining, the cells were photographed under inverted microscope with different magnifications.

The amount of Oil Red O incorporated into the cells was quantified by measuring its absorbance after photographing the cells. First, water was removed from the wells and the stained cells were allowed to dry completely. Wells were then incubated for 10 minutes with 750µl of 100% isopropanol in order to elute the Oil Red O from the cells. After 10 minute incubation, 200µl of the solution was moved to 96-well plate as duplicates and its absorbance was measured at 540 nm. 100% isopropanol was used as blank and stained empty well was used as control.

4.3 ELISA

4.3.1 Materials

Two different ELISA kits were used in the measurement of adipokines/chemokines. Duoset® ELISA Development System kits from R&D Systems were used for adiponectin, leptin, adipsin, resistin, MCP-1 and TNF-α. PeliPair™ ELISA kit from Sanquin was used for measuring IL-6. All samples, standards and quality controls were measured as duplicates. Samples supernatants were kept at -20ºC until analyzed. Quality controls were used to monitor the working accuracy within individual plates and across the plates.

4.3.2 R&D ELISA

Duoset® ELISAs are based on sandwich ELISA and it is used to detect the presence of target protein in a sample. PBS used contains NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8.1 mM) and KH_2PO_4 (1.5 mM). pH of the solution was adjusted between 7.2 - 7.4 and filtered through a 0.2 µm syringe filter. Wash buffer contained the aforementioned PBS and 0.05% Tween® 20. Reagent diluent contained 1% BSA (Sigma, A6003) and PBS. After mixing the solution was filtered through a 0.2 µm syringe filter. Substrate solution was TMB (3,5,3',5'-tetramethylbenzidine, TMB Conductivity 1 Component HRP Microwell Substrate, Bio FX Laboratories). Sulfuric acid (1M H_2SO_4) was used as stop solution. Duoset® ELISAs were used for measuring adiponectin, adipsin, leptin, resistin, MCP-1 and TNF-α (Table 3.)

96-well plates (EIA plates, Costar) were used in all measurements. Day before the measurement plates were coated with 100 µl of capture antibody diluted in PBS. Plates were sealed and kept at 4°C overnight. On the following morning the plates were washed 3 times with autowasher (hydroflex, TECAN) and the remaining wash buffer was removed from the wells by forcefully blotting the plate against paper towels. Unspecific binding sites were then blocked with 275 µl of reagent diluent, sealed with adhesive strip and incubated for 1 hour in room temperature. After incubation the plate was again washed as previously

mentioned and 100 µl of samples, standards and quality controls were added to the wells. Plates were sealed and incubated for 1.5 hours. After incubation the plates were washed and 100 µl of detection antibody diluted in reagent diluent was added to the wells. Plates were again sealed and incubated for additional 1.5 hours. After incubation the plates were washed and streptavidin-HRP conjugate was added to the wells where it binds to biotin conjugated detection antibody and forms extremely tight bond. Wells were incubated 15 minutes in dark at this stage. After incubation the plate was washed again and 100 µl of substrate solution (TMB) was added to the wells initiating the blue color formation. Enzymatic color reaction was allowed continue for 15 minutes in dark after which it was stopped with 50 µl of H_2SO_4 and yellow color was formed.

After adding the stop solution absorbance were measured immediately with Victor^3 1420 Multilabel Counter (Wallac, PerkinElmer) at 450 nm and wavelength correction was measured with 540 nm. The results were calculated with MultiCalc-software which was in conjunction with the measuring device. Results were put in excel and corrected if samples were diluted.

4.3.3 Sanquin ELISA

Like R&D ELISA this PeliPair ELISA is also based on sandwich-ELISA. Coating buffer was a mixture of sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) in distilled water. Solution was prepared by adding bicarbonate solution to carbonate solution until pH of 9.6 was reached. PBS stock solution was prepared my mixing monosodium (NaH_2PO_4), and disodium phosphate (Na_2HPO_4) and sodiumchloride (NaCL) in distilled water. pH of the working solution was adjusted to 7.3 with HCl. Blocking buffer contained PBS and 1% BSA (Sigma) Wash buffer contained PBS and 0.005% of Tween® 20. High performance ELISA buffer (HPE) was used as a dilution buffer. Samples, standards and strepavidin-HRP conjugate (1:10000) were diluted to dilution buffer. Substrate solution was TMB (3,5,3',5'-tetramethylbenzidine, TMB Conductivity 1 Component HRP Microwell Substrate, Bio FX Laboratories). Sulfuric acid (1M H_2SO_4) was used as stop solution. Sanquin ELISA was used to measure the amount of IL-6 (Table 3.)

96-well plates (Nunc Maxisorp) were used in all experiments. Day before measurements the plates were coated with primary antibody diluted in carbonate/bicarbonate (coating) buffer (pH 9.6). The plates were kept overnight at 4ºC. After incubation the plates were washed 5 times with PBS. Autowasher (hydroflex, TECAN) was used. After each wash the plates were forcefully blotted against paper towels in order to remove the remaining wash buffer. Unspecific binding was prevented by blocking the wells with blocking buffer (1% BSA in PBS) for one hour. After blocking the plates were washed with PBS applied with 0.01% Tween® 20. Standards, samples and quality controls were added to the plate and incubated for one hour. After the incubation the plates were again washed and secondary antibody (conjugated with biotin) was added to the wells and incubated for 30 minutes. In the next step the wells were washed and streptavidin-HRP conjugate was pipetted to the plate and incubated for 15 minutes in room temperature. After HRP incubation the plates were washed and 100 µl of TMB was added followed by 15 minute incubation in dark. The color reaction was stopped with 100 µl of 1 M H_2SO_4, and the absorbance was measured and the results were calculated as explained above.

Protein measured	Kit/Manufacturer	Sensitivity (pg/ml)
Adiponectin	Duoset® ELISA Development System human Adiponectin/Acrp30 (R&D Systems)	15.6 - 1000
Leptin	Duoset® ELISA Development System human Leptin (R&D Systems)	31.3 - 2000
Adipsin	Duoset® ELISA Development System human Complement factor D (R&D Systems)	31.3 - 2000
Resistin	Duoset® ELISA Development System human Resistin (R&D Systems)	31.3 - 2000
MCP-1	Duoset® ELISA Development System human CCL2/MCP-1 (R&D Systems)	15.6 - 1000
TNF-α	Duoset® ELISA Development System human TNF-α (R&D Systems)	15.6 - 1000
IL-6	PeliPair™ human cytokine ELISA reagent set IL-6 (Sanquin)	0.6 - 150

Table 3. ELISA kits used to measure adipokines and cytokines.

5. Results

5.1 Differentiation of the SW872 liposarcoma cell line

After initial testing, series of experiments were carried out in order to assess the effect of different treatments on adipocyte differentiation. Two different fatty acids (oleic acid and linoleic acid) were used to enhance the differentiation process and lipid accumulation. Fatty acids were conjugated with BSA to achieve better solubility and to avoid oxidation.

The effect of fatty acids, rosiglitazone and lipopolysaccharide (LPS) was examined in the first experiment. Cells were plated with density of 0.3 million/ml on 24-well plates in DMEM: Ham's (1:1). This medium was used also in the differentiation. Cells were grown for 13 days which was divided into growth period and differentiation period. The growth period lasted for 3 days during which cells reached confluency. Growth medium was switched to differentiation medium and the cells were kept in this mixture for the following 10 days with medium change twice a week. Two time points were selected upon which the effect of the differentiation was studied. Time points were 6 and 13 days of growth, corresponding to 3 and 10 days of differentiation, respectively. Similar plates were plated for Oil Red O - staining. Undifferentiated cells served as controls.

Differentiation mixture contained the following compounds: dexamethasone (1 µM), insulin (10 µg/ml) and IBMX (0.1 mM), which was the stardard differentiation medium. Standard differentiation medium was also supplemented with rosiglitazone (1 µM) or LPS (10 ng/ml). (Figure 7.) All treatments were carried out with or without fatty acids: oleic acid (30 µM) and linoleic acid (30 µM). Rosiglitazone and IBMX were dissolved in DMSO and therefore DMSO was used into the controls also. Media were collected for ELISA measurements and duplicate plates were used for the Oil Red O-staining. (Figure 7.)

When SW872-cells were plated their appearance was similar to fibroblasts: larger nucleus and fiber-like threads pointing away from the nucleus. Upon differentiation the appearance of the cells changed rather dramatically. The cells became more rounded and small lipid

vacuoles started to appear. In the course of the differentiation, the vacuoles grew and their lipid content increased. However, these lipid vacuoles did not merge so the actual appearance was different from that of mature adipocytes. Contact inhibition of these cells can also be argued as they had the tendency to grow on top of each other, but no more than one layer was formed although there was a partial overlap.

The differentiation of the cells was evaluated with Oil Red O – staining, which stains triglycerides and other lipids. Longer differentiation time increased the lipid content of the cells noticeably. Use of fatty acids: oleic acid and linoleic acid in the medium increased the amount and size of the lipid droplets within the cells. (Figure 7.)

Addition of fatty acids in the culture medium increased the lipid content of the cells over 50%. Differentiation of the cells increased the lipid content, but not as much as the use of fatty acids. When 3 and 10 days of differentiation were compared, the effect of differentiation and fatty acids became more evident along longer differentiation time. After 10 days of differentiation, the lipid content of the cells, which were differentiated and treated with fatty acids, was more than 100% higher than in controls without the fatty acids. (Figure 8.)

PPARγ agonist and insulin sensitizer, rosiglitazone, increased the lipid content of the cells when added to the differentiation mixture. Rosiglitazone had no effect on differentiation after 3 days, but after 10 days the effect was noticeable. Use of fatty acids increased the lipid content more than the use of rosiglitazone. (figure 9.)

Differentiation with LPS was also tested. When differentiation mixture was supplemented with LPS the lipid content of cells slightly increased, both with or without the fatty acids. Use of fatty acids increased the lipid content of cells more than the use LPS. The effect of LPS is noticeable after 10 days of differentiation. (Figure 10.)

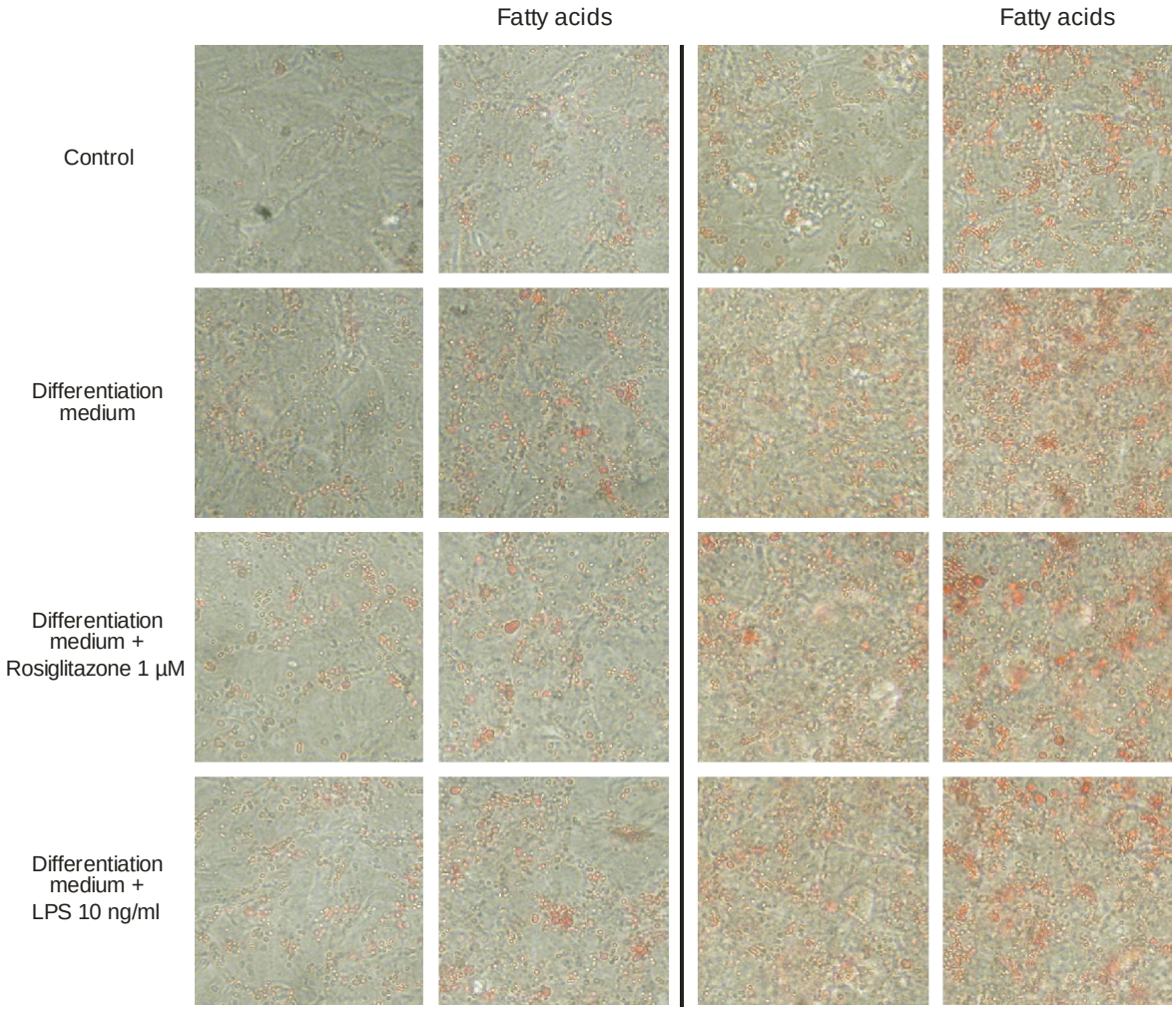

Figure 7. Oil Red O - staining of the SW872 cells. Cells on the left side have been grown for 3 days and differentiated for 3 days (excluding the control cells) and cells on the right side have been grown for 3 days and differentiated for 10 days. Each row represents different growth conditions which are: controls, differentiation with insulin, dexamethasone and IBMX (standard differentiation medium), standard differentiation medium supplemented with rosiglitazone and standard differentiation medium with LPS. See text for details. Cells which have been given an additional fatty acid treatment are shown on columns 2 and 4, respectively. Endogenous lipid droplet formation is detectable when controls are compared to differentiated ones, especially after 10 days. With fatty acids the staining of the lipids was more profound. 40 x magnification.

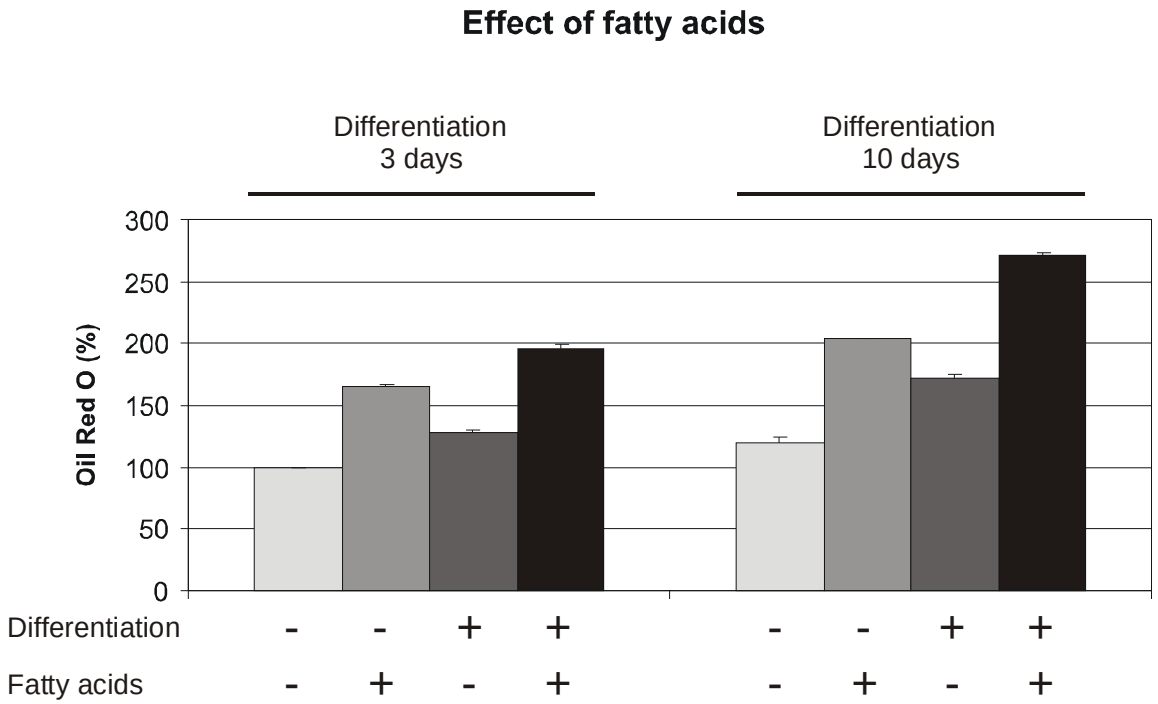

Figure 8. The effect of differentiation mixture and fatty acids on SW872 cells lipid content measured by Oil Red O - staining. Adipocytes were grown with or without fatty acids. Controls were not differentiated (light grey bars on far left). After 10 days in the differentiation conditions, lipid content of the cells has increased almost 50% compared to undifferentiated cells. Fatty acids increase the lipid content of the cells over 50%. Longer incubation time increases the effect of differentiation mixture and also the effect of fatty acids. Mean + SEM.

Commercial differentiation protocol with PGM™-2 –medium from Lonza was tested in the SW872 cells. This protocol had to be adjusted to fit 24-plates as it was initially designed to be performed with 96-well plates. Differentiation medium was different from what we used but only slightly. Indomethacin was used in differentiation mixture whereas we used rosiglitazone in our own protocol. Commercial differentiation medium was tested along with our protocol but the results were not convincing enough so that we would have continued with the commercial medium. We detected adipsin on both cultures but the levels were higher with our own protocol. Also the manufacturer did not share the information about concentrations used so we proceed to perfect our own protocol.

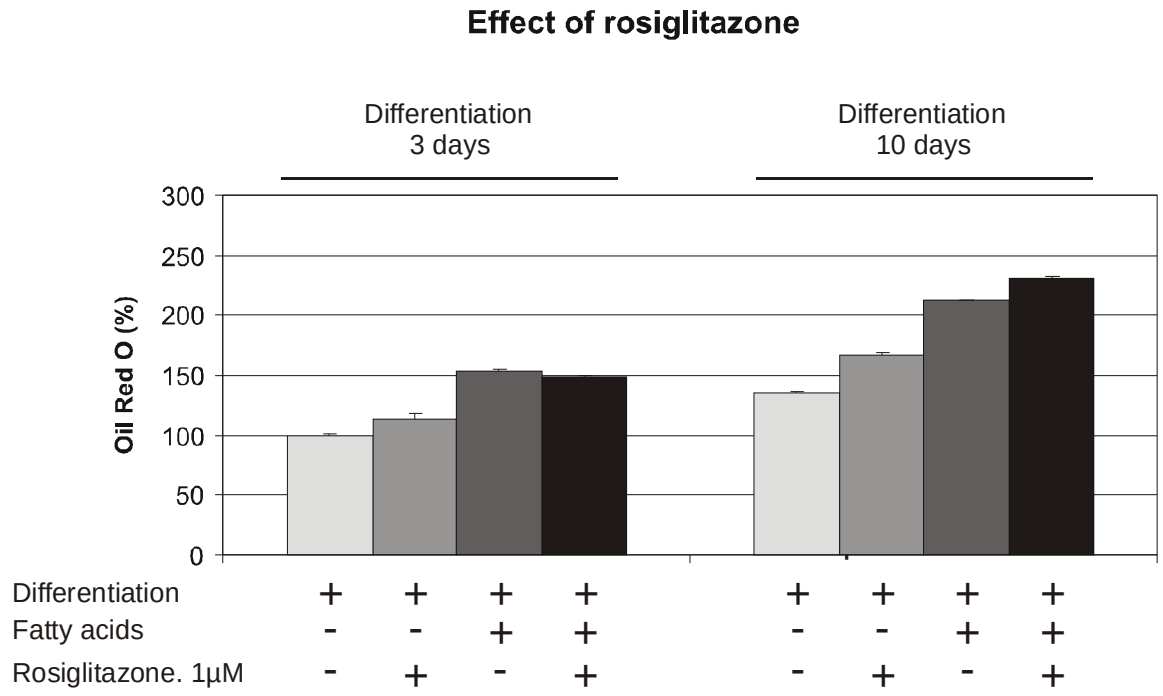

Figure 9. The effect of rosiglitazone on differentiation of SW872 cells measured by Oil Red O-staining. Differentiation with dexamethasone, insulin and IBMX is used as control (light grey bars on far left). The effect of rosiglitazone on lipid content of cells is noticeable after 10 days of differentiation. Supplementation of the differentiation mixture with fatty acids increased the lipid content of the cells more than the use rosiglitazone. Use of rosiglitazone and fatty acids in differentiation mixture increased the lipid content of the cells by 70% after 10 days. Mean + SEM.

5.2 The effect of differentiation on adipokine production in SW872 cells

The media was collected after 3 and 10 days of standard differentiation (dexamethasone (1 µM), human insulin (10 µg/ml) and IBMX (0.1 mM)) and supplemented substances (rosiglitazone (1 µM) or LPS (10 ng/ml)). Chemokine MCP-1 and adipokines leptin, adiponectin, resistin and adipsin were measured by ELISA. Measurements were carried out in media colected after 3 and 10 days of differentiation and the data was normalized to give daily amounts of the protein produced.

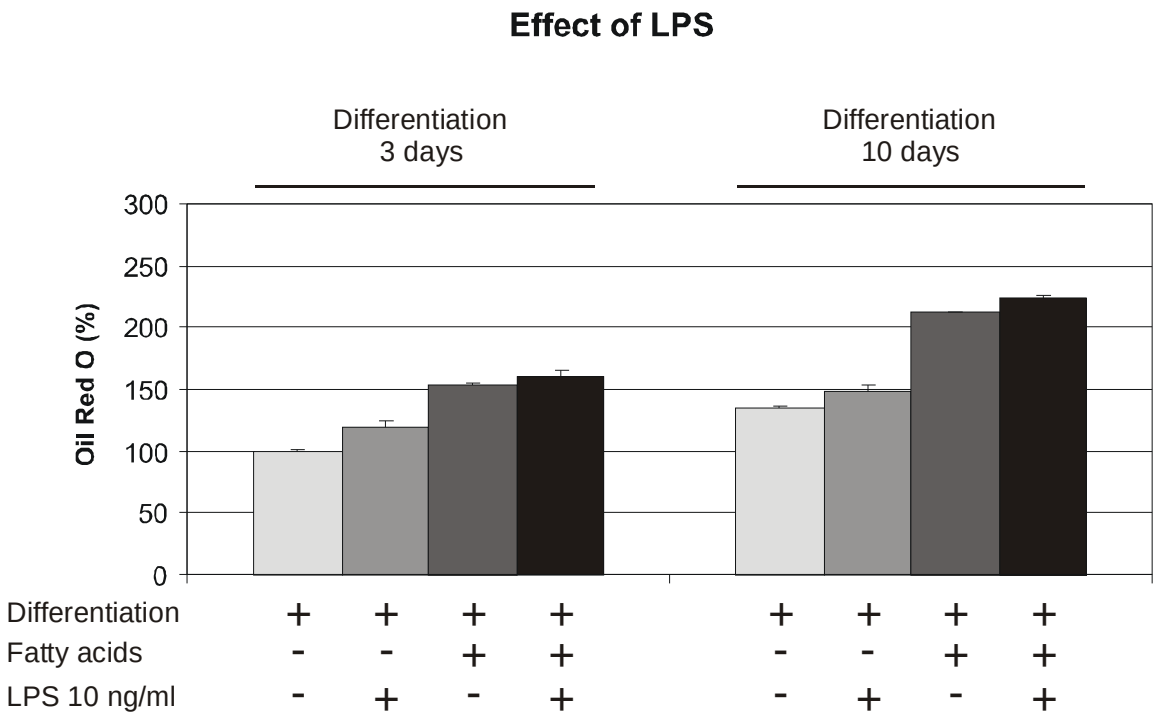

Figure 10. The effect of LPS on differentiation on SW872 cells measured by Oil Red O-staining. Differentiation with dexamethasone, insulin and IBMX is used as control (light grey bars on far left). Use of LPS in the differentiation mixture slightly increased the lipid content of the cells. The effect of fatty acids on lipid content is greater than the effect of LPS. After 10 days of differentiation the fatty acids increase the amount of lipids more than 50%. Mean + SEM.

From the tested proteins, adipsin and MCP-1 were detectable. During the differentiation, the production of adipsin increased considerably. (Figure 11.) After 10 days of differentiation the adipsin production had increased more than 300% when compared to production in undifferentiated cells. Supplementation with rosiglitazone further increased the amount adipsin. After 10 days of differentiation the levels of adipsin production between undifferentiated cells and the cells which had been differentiated with the standard differentiation medium and supplemented with rosiglitazone were 700% higher. Supplementing the differentiation mixture with rosiglitazone increased the adipsin production by 100% when compared to standard differentiation. LPS treatment didn't seem to have any effect on adipsin production and was almost similar with normal differentiation.

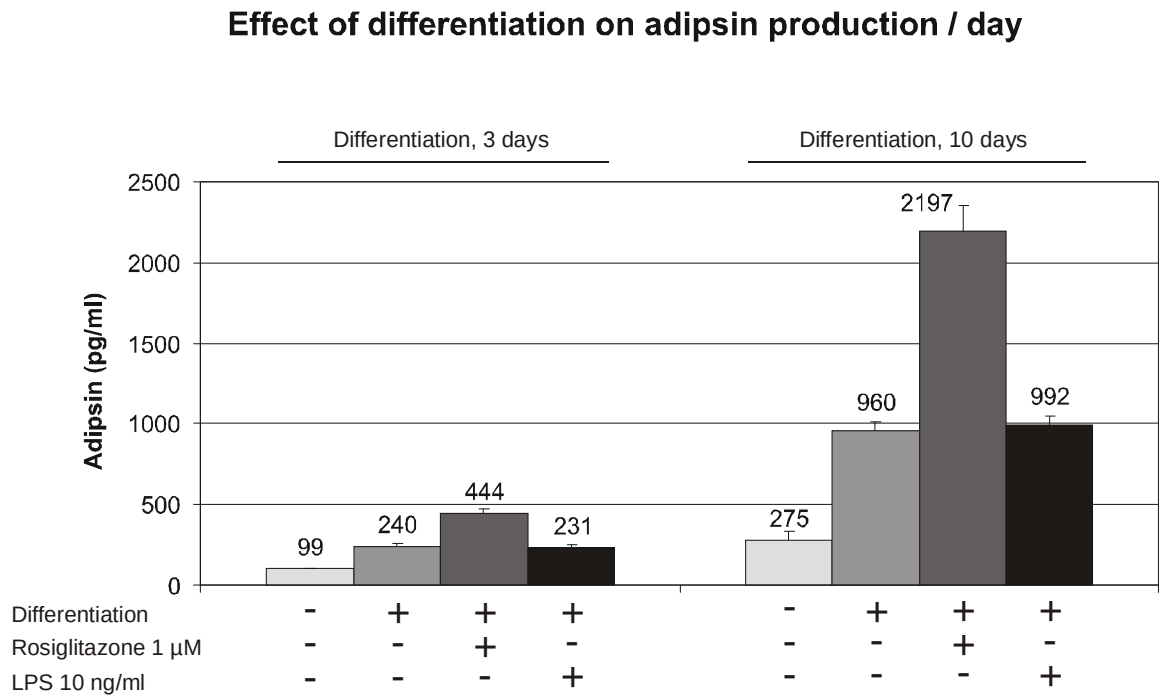

Figure 11. The effect of differentiation on adipsin production measured with ELISA. Undifferentiated cells are used as controls (light grey bars on far left). Differentiation markedly increased the adipsin production. Mean + SEM.

SW872 cells produced considerable amounts of MCP-1. The amount of MCP-1 in the media increased during the experiment in undifferentiated cells. Use of differentiation mixture decreased the MCP-1 production dramatically. After 3 days of differentiation the production of MCP-1 had decreased by over 85% and after 10 days of differentiation the production had dropped by over 95% when undifferentiated cells were compared to cells which underwent the standard differentiation. Supplementation of differentiation mixture with rosiglitazone further decreased the MCP-1 production and abolished it almost completely. 3 days of differentiation with this mixture decreased the MCP-1 production by 91% and after 10 days by over 98%. After 3 days of differentiation, LPS increased the MCP-1 production by almost 400%, but after 10 days the increase was only 100% when compared to normal differentiation and the MCP-1 levels were considerably lower than in undifferentiated cells. (Figure 12.)

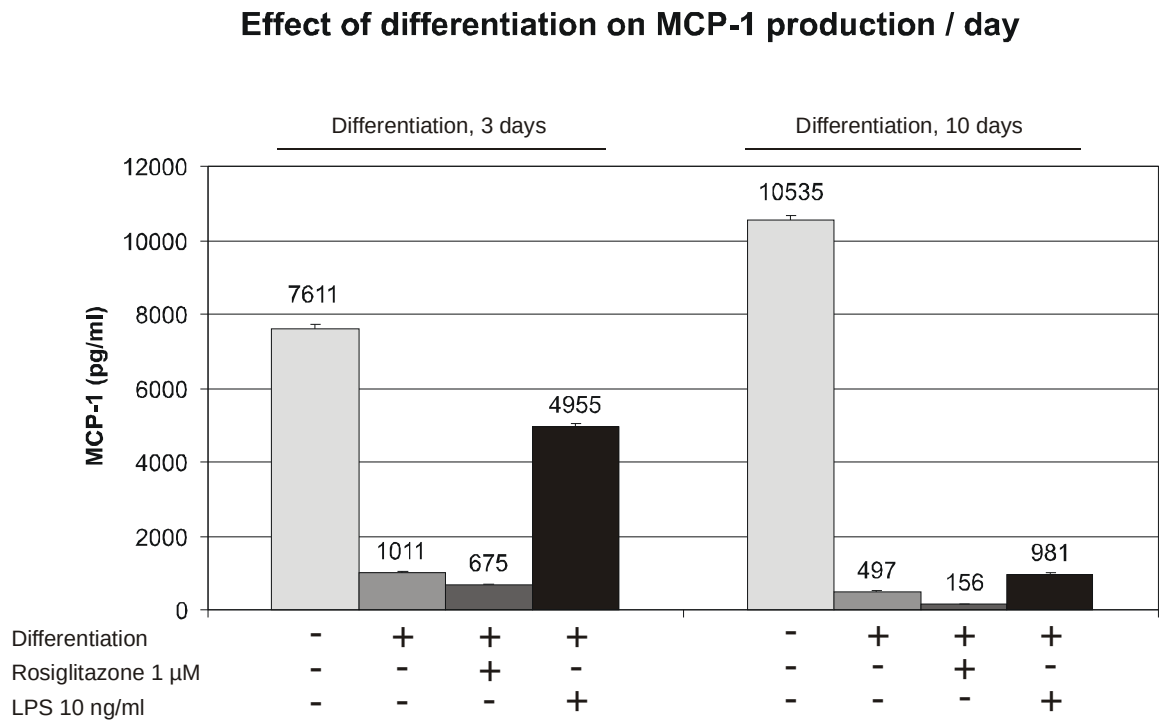

Figure 12. The effect of differentiation on MCP-1 production measured by ELISA. Undifferentiated cells are used as controls (light grey bars on far left). Differentiation considerably decreased the MCP-1 production. Differentiation with LPS increased the amount of MCP-1. Mean + SEM.

5.3 Pharmacological regulation of adipsin and MCP-1 production in SW872 cells

Based on differentiation experiments the following conditions were used in adipokine regulation studies. Medium used was DMEM:Ham's (1:1) throughout the experiment and the differentiation mixture added was dexamethasone (1 µM), insulin (10 µg/ml), IBMX (0.1 mM) rosiglitazone (1 µM) and linoleic acid (30µM). The cells were plated, grown and differentiated as before (3 day growth period and 10 day differentiation period). After 10 days of differentiation, 1 day wash-out period was performed without differentiation mixture and with linoleic acid. After 1 day wash-out period the medium was changed and the cells were incubated for 1 day with linoleic acid and selected substances. (Table 2, Methods).

Medium was collected after the experiment and the secreted protein levels were analyzed by ELISA. Adipokines leptin, resistin, adipsin, chemokine MCP-1 and cytokines

IL-6 and TNF-α were measured and out of these, adipsin and MCP-1 were detectable. Differentiated cells which were not treated after wash-out were used as controls. One-way Analysis of Variance (ANOVA) was performed along with Bonferroni post-test and P-values smaller than 0.05 were considered as significant.

Ibuprofen and resveratrol increased adipsin production significantly ($P<0.01$ and $P<0.001$). In addition, kaempferol and dexamethasone seemed to increase, and 15d-PGJ_2 and paracetamol to decrease adipsin production but these effects were not statistically significant when Bonferroni post-test was applied. (Figure 13.) Dexamethasone, ciglitazone, quercetin and kaempferol decreased the MCP-1 production significantly ($p<0.001$) while resveratrol increased MCP-1 production ($P<0.01$). (Figure 14.)

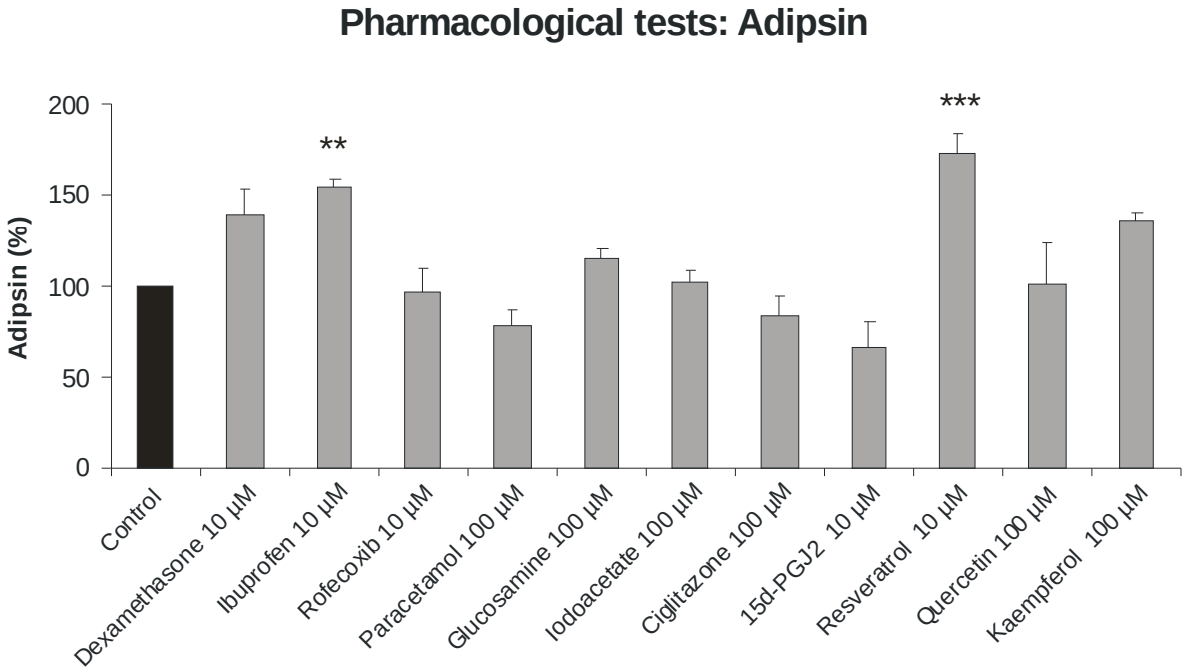

Figure 13. The effect of pharmacological substances on adipsin production. Untreated cells were used as control, and adipsin produced by the control cells was set as 100% and the other values were raleted to that. Resveratrol and ibuprofen increased adipsin production significantly. Mean + SEM, n = 5-6, *** = P<0.001, ** = P<0.01.

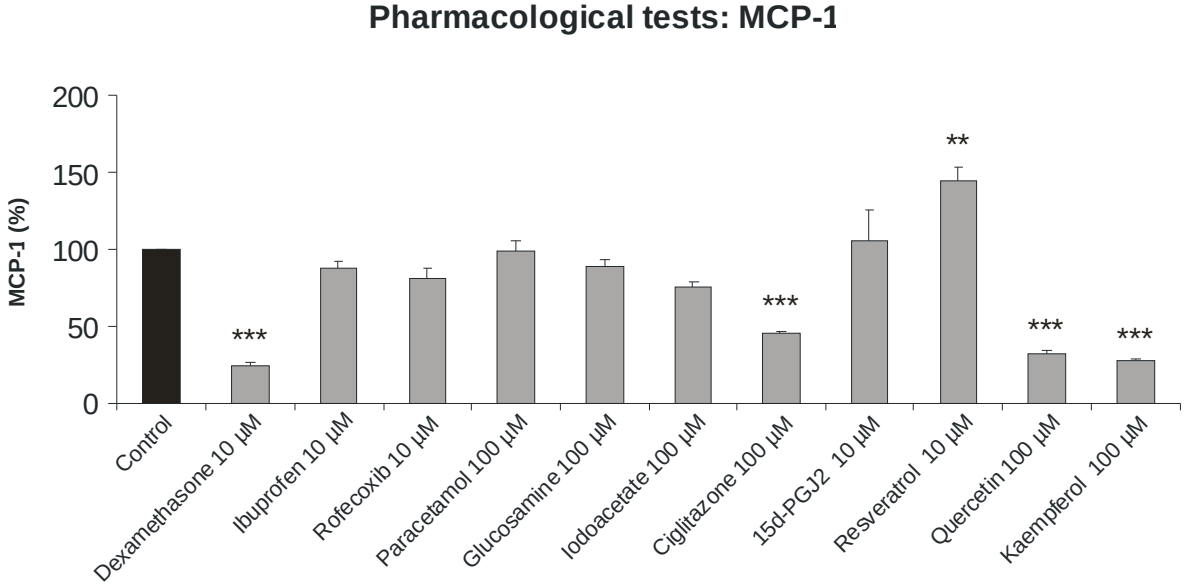

Figure 14. The effect of pharmacological substances on MCP-1 production. Untreated cells were used as control, and adipsin produced by the control cells was set as 100% and the other values were raleted to that. Dexamethasone, ciglitazone, quercetin and kaempferol decreased MCP-1 production significantly and resveratrol increased the MCP-1 production significantly. Mean + SEM, n = 5-6, *** = P<0.001, ** = P<0.01.

6. Discussion

Cultured adipocytes have been used in research for decades now. Two of the most used cell lines were isolated already in the mid-70s but it was the discovery of leptin in 1994 which really took this field forward. It was realized that fat is not just a simple storage organ but it also has an endocrine function. It is now clear that the adipose tissue synthesizes close to hundred different substances from different cell types (Gnacinska et al., 2009). Also the worldwide incidence of obesity has increased into epidemic proportions and this has also accelerated the research. It has been estimated that 1 billion individuals worldwide are overweight (Hotamisligil, 2006).

Initial events that steer the cells towards adipogenic development are still unknown. However, the events after preadipocyte-stage are relatively well-defined in terms of transcription factor expression. The used models have been very successful because they replicate the events of the differentiation and the features of the mature adipocyte very well. Downside of these cell lines is that they are derived from mice. The lack of decent human cell line is clearly an obstacle. There is however at least one human cell line that is isolated from fat and resembles some features of the adipocytes, the SW872. Primary cultures have also been used alongside with cell lines, but research with these systems is challenging as they only last for only a few cell cycles. Yet these primary cultures have given an important lesson. Adipose tissue is not homogenous throughout the fat pads of the body, but reacts differently to hormonal stimulus depending upon source. (Rosen & Spiegelman, 2006)

6.1 Early events and the differentiation

As our primary target was to investigate adipokine production in SW872 cells, the early events in the growth of the cells were given less attention. Our goal was to differentiate the SW872 cells into mature adipocytes. However, the observed initial events were similar to those depict in the literature: when plated, the cells had the shape of a fibroblast but as they grew, they became more rounded and started to accumulate lipids.

It has been previously shown that the use of fatty acid and rosiglitazone increases the triglyceride and cholesterol content of the cultured SW872 cells (Carmel et al., 2009). In our studies we were able to confirm this finding with another method, the Oil Red O -staining (Figures 7., 8. and 9.) We also showed that LPS slightly increased the lipid content of the cells. The change was rather modest, but consistent. (Figure 10.) Lipid accumulation could also be seen without adding exogenous fatty acids, suggesting that the used differentiation mixture *per se* was having an impact. (Figures 7. and 8.) This effect was clearest after 10 days of differentiation as the cells were further in their adipogenic development process. Fatty acids had the largest effect on Oil Red O-staining. Higher external lipid content might stimulate endocytosis of the lipids and triacylglycerol synthesis which would then lead to higher lipid content within the cells. Figure 1 gives an idea of lipid accumulation into these cells. Staining of the nuclei and ECM components would have given more information about the organization of the cells.

Important aspect of the whole process presented here is differentiation status of the cells. The process is expected to be more complex in these cells than in murine cell lines and it is an important question whether the cells were fully differentiated at the end of the differentiation procedure or not. Fat accumulation was not as extensive as has been published in murine adipocyte cell line. But given the sarcoma background of the SW872 cells used in the present study an identical phenotype with mature human adipocytes will probably remain fantasy. So, we are faced with two scenarios: 1) The SW872 is fully differentiated with the current differentiation regime and it expresses all factors which are natural to this stage. Or, 2) the differentiation process is still in action in the experiments carried out after the differentiation and the results are an examples from the differentiation continuum. Further experimentation is needed to address the full potential of this cell line both with adipokine expression profile and maturity.

6.2 Adipokine secretion

Secretion of adipsin into growth medium increased both with time and with differentiating agents. Interestingly enough, 10 ng/ml of LPS seems to have no effect on adipsin production (Figure 11.) Adipsin secretion was increased after 10 days of differentiation.

This might be due to at least two reasons: 1) the substances themselves activate the transcription of this gene or 2) the differentiation process and the maturation of the adipocytes also activate the adipsin gene. The use of 1 μM rosiglitazone, a PPAR agonist, doubled the secretion of adipsin into the growth medium. It has been elegantly shown by Hamza and others that adipsin production is completely abolished when PPARγ is inhibited with siRNA. (Hamza et al., 2009)

The levels of adipsin in differentiated cells with and without LPS treatment were are virtually the same. It is unlikely that the amount of LPS was too low as we were able to see a profound response in the MCP-1 production to LPS in the same cells.

Regulation of and changes in MCP-1 production were in many ways opposite to those of adipsin. (Figure 12.) Undifferentiated cells produced very high amounts of MCP-1. On the other hand, the differentiation procedure is effectively down-regulated the secretion of this protein. LPS gave a suspected response with MCP-1. High expression levels in controls might be a result of more than one reason. First, SW872 is a liposarcoma cell line and it might be that high MCP-1 expression is an intrinsic property. Another explanation might be that the growth conditions were not optimal which might then lead to high expression of MCP-1. However, MCP-1 was down-regulated with the use of differentiation cocktail. It might also be possible, that low MCP-1 production is an intrinsic property of the differentiated cells. Further studies are needed to dissect out the molecular mechanism of the profound decrease, but the compound responsible is possibly dexamethasone as it is a powerful anti-inflammatory drug and immunodepressant. MCP-1 promoter contains the binding sites for NF-κB and AP-1 (Hacke et al., 2010) both of which can be down-regulated by dexamethasone (Ma et al., 2004). Further reduction of MCP-1 production with rosiglitazone is also an interesting feature and the mechanism behind this is something that should be looked into.

6.3 Pharmacological regulation of adipokine production

After the 10 day differentiation and 24 hour wash-out period the cells were exposed to pharmacological agents for 24 hours. Adipsin and MCP-1 were again detected. (Figures 13. and 14.)

Ibuprofen and resveratrol increased the adipsin production into the growth medium significantly. Dexamethasone increased the adipsin production as expected because it is part of the differentiation mixture which we had already shown to increase the adipsin production. However, increase with dexamethasone was not statistically significant. It is interesting that ibuprofen had a more powerful effect on adipsin production and it reached statistical significance. There are reports that cyclooxygenase inhibitors, such as ibuprofen and indomethacin, can be used as substitutes for glucocorticoids in promoting the differentiation (Hwang et al., 1997) and therefore it would be very interesting to test the differentiation efficiency with indomethacin and ibuprofen.

Resveratrol, polyphenol stilbene, has been shown to be an antioxidant and also to possess anti-inflammatory properties (Gullett et al., 2010). Majority of these properties are probably mediated through sirtuins which have an effect on gluconeogenesis, energy metabolism and adiposity. Especially Sirtuin 1 has been shown to have a number of roles in white adipose tissue. (Beaudeux et al., 2010) So the increase in the adipsin production is well in line with that observed with ibuprofen and dexamethasone, given that higher adipsin concentration actually reflects the enhanced adipogenic development. However, results are contradictory with MCP-1 which was significantly increased when cells were exposed to resveratrol. It seems that the inflammatory response is provoked with this compound rather than suppressed. Further studies are definitely needed to explain this phenomenon.

MCP-1 secretion into the growth medium was significantly decreased with dexamethasone, ciglitazone, quercetin and kaempferol and as mentioned, increased with resveratrol. It is not surprising that dexamethasone decreased MCP-1 levels as it is a potent anti-inflammatory drug as mentioned earlier. Ciglitazone belongs to thiazolidinediones and is a ligand for PPARγ. Some cancers have shown to be responsive for TZD treatment, but this effect is unlikely to be mediated through PPARγ (Wei et al., 2009). Since SW872 is a liposarcoma cell line, it might be that this particular cancer is responsive to ciglitazone. It would again be interesting to test the effect of other TZDs (rosiglitazone, troglitazone and pioglitazone) on MCP-1 production. It has been shown in mouse 3T3-L1 adipocytes that resveratrol and quercetin decrease the viability of these cells and increase their apoptosis (Yang et al., 2008). It has been shown that quercetin and kaempferol effects are partly mediated through NF-κB (Gonzalés-Gallero et al., 2007) so it is probable that the decrease

in MCP-1 production with quercetin and kaempferol is mediated through NF-κB as MCP-1 has NF-κB binding sequence in its promoter.

It is interesting that the other compounds tested had only minimal effect on MCP-1 levels. One might have suspected that ibuprofen or rofecoxib would have an effect but no effect was observed.

6.4 SW872 and the differences between other adipocyte models

SW872 cell line was not an obvious choice to study adipocytes. However, despite of its liposarcoma background, this cell line is of human origin and that is an advantage. As expected, use of human cell line in research is more complex than the use of murine cell line. This is also reflected in the number of publications. Search in Pubmed gives 4464 articles for 3T3-L1, 458 articles for 3T3-F442A, but only 29 publications were found for SW872 (HTB-92), most of which are not concerned with differentiation or adipokine production at all. Therefore it might be that this type of study with this particular cell line is a rather unexplored territory or tested but left unpublished since this cell line was isolated at pretty much the same time as 3T3-L1 and 3T3-F442A cell lines in the mid-1970s.

Literature on these cells is scarce. In only 2 studies the SW872 cells have been grown over 10 days. There are also some major differences between this cell line and the most common ones. 3T3-L1 and 3T3-F442A cells were isolated from 17-19 day old mouse embryos. As a comparison, SW872 cell line was isolated from a 36 year old Caucasian male. Differences can also be found on transcriptional level. In 3T3-L1 cells, the expression of PPARγ was seen 24 hours after the induction to differentiate and it rose steadily for the following week (Salma et al., 2006). In SW872 the expression was high after 4 days but dropped during the next six days after which it rose again for the next seven days. The expression of PPARβ/δ rose steadily during the 2 week period. (Wassef et al., 2004)

6.5 Prospects

Most of the information concerning about the sequence of events in adipocyte differentiation and the key transcription factors has came from the studies with murine preadipocyte cell lines, namely 3T3-L1 and 3T3-F442A. Although very useful, these systems are limited in terms of intercellular and paracrine signaling between different cell types. After all, only 50% of the adipose tissue consists of adipocytes. Many cell lines fail to express certain adipocyte genes at *in vivo* levels and this might be a result from the lack of cellular environment. (Hwang et al., 1997) This might also be the case with SW872 cells. However, without the expression studies we cannot be sure of this. What we are sure is that with this differentiation regime the secretion of adipokines leptin and adiponectin is below the sensitivity limit of the methods used here.

There is evidence that differentiation and the recruitment of preadipocytes is integrated to adipose status through these paracrine and cell-cell interactions. It has been shown that the medium conditioned by preadipocytes from obese individuals increases the rat preadipocyte proliferation fourfold when compared to the medium from lean individuals. It has also been very nicely shown that the implantation of 3T3-F442A adipocytes into athymic mice develops into adipose tissue which is indistinguishable from endogenous white adipose tissue. These newly developed fat pads also expressed adipose-specific transcripts at levels comparable to normal tissue. (Hwang et al., 1997)

Because similar studies with this cell line are not available, this study hopefully serves as a stepping stone towards more detailed studies. Given the limited time, and the fact that cells had to be grown for 2 weeks, great deal of work is needed before the important aspects on differentiation, growth medium and culture conditions are clear and the process is well controlled.

7. Conclusions

Prevalence of obesity has grown into epidemic proportions in the western world and it has become a major health problem with linked comorbidities such as type 2 diabetes, atherosclerosis and non-alcoholic fatty liver disease. The current view is that the obesity can be considered as low-grade inflammation, without the classical characteristics of inflammation. Adipose tissue has been shown to secrete myriad of proteins, termed collectively as adipokines or adipocytokines. With obesity the expression profile of adipo(cyto)kines is altered towards more inflammatory state and with weight reduction the healthy expression profile returns. Number of preadipocyte/adipocyte cell lines have been developed to study the events in fat cells and these models have provided most of the current knowledge. Studies with transgenic mice have also provided valuable insights into the development of this tissue. In mammals the adipose tissue can be found around the body and these different compartments have their own distinctive behavior which is dependent on surrounding tissue. These behavioral patterns are very difficult to approach with cell line studies.

Current pharmacological treatments for obesity and related metabolic disorders are limited and ineffective (Cao, 2010). It has become clear that angiogenesis is crucial to the development of fat. Therefore limiting angiogenesis might be one way of treating obesity. However, limiting angiogenesis might be detrimental in some patient populations, such as diabetics. One treatment option might be to direct the excess energy to brown adipocytes. This cell type is dedicated to heat production from fat. Unfortunately, there are only few models available for this study and the hormonal regimen for differentiation of brown adipocytes is not known. Another approach is to use pharmacological interventions to balance adipocytokine production in adipocytes towards the healthy condition to treat obesity and prevent comorbidities.

In this study, we used human liposarcoma cell line SW872 as an adipocyte model. Differentiation of the cell line was studied in various culture conditions in order to achieve best combination for the cell line to produce adipocytokines. Culturing time varied between

few days to 3 weeks. Exposure to different compositions of differentiation medium was studied with two differentiation times. These procedures were successful as the cells showed adipocyte phenotype and produced increased levels of adipokine adipsin and reduced amounts of chemokine MCP-1. Adipocytokine secretion was also modulated with pharmacological agents and especially ciglitazone, resveratrol, quercetin and kaempferol showed interesting results.

The cell line and differentiation protocol developed in the present study can be used in further studies to investigate more detail the differentiation process and pathophysiological and pharmacological regulation of adipocytokine production in human adipocytes.

8. References

Ahima RS, Qi Y, Singhal NS, Jackson MB, Scherer PE. Brain adipocytokine action and metabolic regulation. Diabetes 2006;55:S145-54.

Armani A, Mammi C, Marzolla V, Calanchini M, Antelmi A, Rosano GM, Fabbri A, Caprio M. Cellular models for understanding adipogenesis, adipose dysfunction, and obesity. Journal of Cellular Biochemistry 2010;110:564-572.

Beaudeux JL, Nivet-Antoine V, Giral P. Resveratrol: A relevant pharmacological approach for the treatment of metabolic syndrome? Current Opinion in Clinical Nutrition and Metabolic Care 2010;13:729-736.

Cao Y. Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. Nature Reviews.Drug Discovery 2010;9:107-115.

Carmel JF, Tarnus E, Cohn JS, Bourdon E, Davignon J, Bernier L. High expression of apolipoprotein E impairs lipid storage and promotes cell proliferation in human adipocytes. Journal of Cellular Biochemistry 2009;106:608-617.

Duffaut C, Zakaroff-Girard A, Bourlier V, Decaunes P, Maumus M, Chiotasso P, Sengenes C, Lafontan M, Galitzky J, Bouloumie A. Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. Arteriosclerosis, Thrombosis, and Vascular Biology 2009;29:1608-1614.

Esterbauer H, Krempler F, Oberkofler H, Patsch W. The complement system: A pathway linking host defence and adipocyte biology. European Journal of Clinical Investigation 1999;29:653-656.

Fantuzzi G. Adipose tissue, adipokines, and inflammation. The Journal of Allergy and Clinical Immunology 2005;115:911-9; quiz 920.

Galic S, Oakhill JS, Steinberg GR. Adipose tissue as an endocrine organ. Molecular and Cellular Endocrinology 2010;316:129-139.

Gnacinska M, Malgorzewicz S, Stojek M, Lysiak-Szydlowska W, Sworczak K. Role of adipokines in complications related to obesity: A review. Advances in Medical Sciences 2009;54:150-157.

Gonzalés-Gallero J, Sánchez-Campos S, Tuñón MJ. Anti-inflammatory properties of dietary flavonoids. Nutrición Hospitalaria 2007;22:S287-93.

Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiological Reviews 1998;78:783-809.

Gualillo O, Gonzalez-Juanatey JR, Lago F. The emerging role of adipokines as mediators of cardiovascular function: Physiologic and clinical perspectives. Trends in Cardiovascular Medicine 2007;17:275-283.

Gullett NP, Ruhul Amin AR, Bayraktar S, Pezzuto JM, Shin DM, Khuri FR, Aggarwal BB, Surh YJ, Kucuk O. Cancer prevention with natural compounds. Seminars in Oncology 2010;37:258-281.

Guo Y, Xie J, Rubin E, Tang YX, Lin F, Zi X, Hoang BH. Frzb, a secreted wnt antagonist, decreases growth and invasiveness of fibrosarcoma cells associated with inhibition of met signaling. Cancer Research 2008;68:3350-3360.

Gustafson B. Adipose tissue, inflammation and atherosclerosis. Journal of Atherosclerosis and Thrombosis 2010;17:332-341.

Guzik TJ, Mangalat D, Korbut R. Adipocytokines - novel link between inflammation and vascular function? Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society 2006;57:505-528.

Hacke K, Rincon-Orozco B, Buchwalter G, Siehler SY, Wasylyk B, Wiesmuller L, Rosl F. Regulation of MCP-1 chemokine transcription by p53. Molecular Cancer 2010;9:82.

Hamza MS, Pott S, Vega VB, Thomsen JS, Kandhadayar GS, Ng PW, Chiu KP, Pettersson S, Wei CL, Ruan Y, Liu ET. De-novo identification of PPARgamma/RXR binding sites and direct targets during adipogenesis. PloS One 2009;4:e4907.

Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444:860-867.

Hwang CS, Loftus TM, Mandrup S, Lane MD. Adipocyte differentiation and leptin expression. Annual Review of Cell and Developmental Biology 1997;13:231-259.

Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M, Shimomura I. Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 2003;52:1655-1663.

Izem L, & Morton RE. Cholesteryl ester transfer protein biosynthesis and cellular cholesterol homeostasis are tightly interconnected. The Journal of Biological Chemistry 2001;276:26534-26541.

Izem L, & Morton RE. Possible role for intracellular cholesteryl ester transfer protein in adipocyte lipid metabolism and storage. The Journal of Biological Chemistry 2007;282:21856-21865.

Karastergiou K, & Mohamed-Ali V. The autocrine and paracrine roles of adipokines. Molecular and Cellular Endocrinology 2010;318:69-78.

Kita A, Yamasaki H, Kuwahara H, Moriuchi A, Fukushima K, Kobayashi M, Fukushima T, Takahashi R, Abiru N, Uotani S, Kawasaki E, Eguchi K. Identification of the promoter region required for human adiponectin gene transcription: Association with CCAAT/enhancer binding protein-beta and tumor necrosis factor-alpha. Biochemical and Biophysical Research Communications 2005;331:484-490.

Lago F, Dieguez C, Gomez-Reino J, Gualillo O. Adipokines as emerging mediators of immune response and inflammation. Nature Clinical Practice.Rheumatology 2007;3:716-724.

Lee MJ, Wu Y, Fried SK. Adipose tissue remodeling in pathophysiology of obesity. Current Opinion in Clinical Nutrition and Metabolic Care 2010;13:371-376.

Ma W, Gee K, Lim W, Chambers K, Angel JB, Kozlowski M, Kumar A. Dexamethasone inhibits IL-12p40 production in lipopolysaccharide-stimulated human monocytic cells by down-regulating the activity of c-jun N-terminal kinase, the activation protein-1, and NF-kappa B transcription factors. Journal of Immunology (Baltimore, Md.: 1950) 2004;172:318-330.

Matarese G, Procaccini C, De Rosa V, Horvath TL, La Cava A. Regulatory T cells in obesity: The leptin connection. Trends in Molecular Medicine 2010;16:247-256.

Montague CT, Prins JB, Sanders L, Zhang J, Sewter CP, Digby J, Byrne CD, O'Rahilly S. Depot-related gene expression in human subcutaneous and omental adipocytes. Diabetes 1998;47:1384-1391.

Moriuchi A, Yamasaki H, Shimamura M, Kita A, Kuwahara H, Fujishima K, Satoh T, Fukushima K, Fukushima T, Hayakawa T, Mizuguchi H, Nagayama Y, Abiru N, Kawasaki E, Eguchi K. Induction of human adiponectin gene transcription by telmisartan, angiotensin receptor blocker, independently on PPAR-gamma activation. Biochemical and Biophysical Research Communications 2007;356:1024-1030.

Ntambi JM, & Young-Cheul K. Adipocyte differentiation and gene expression. The Journal of Nutrition 2000;130:3122S-3126S.

Okada T, Nakai M, Maeda H, Hosokawa M, Sashima T, Miyashita K. Suppressive effect of neoxanthin on the differentiation of 3T3-L1 adipose cells. Journal of Oleo Science 2008;57:345-351.

Rabe K, Lehrke M, Parhofer KG, Broedl UC. Adipokines and insulin resistance. Molecular Medicine (Cambridge, Mass.) 2008;14:741-751.

Rangwala SM, & Lazar MA. Transcriptional control of adipogenesis. Annual Review of Nutrition 2000;20:535-559.

Richardson MA, Berg DT, Johnston PA, McClure D, Grinnell BW. Human liposarcoma cell line, SW872, secretes cholesteryl ester transfer protein in response to cholesterol. Journal of Lipid Research 1996;37:1162-1166.

Rosen ED, & Spiegelman BM. Molecular regulation of adipogenesis. Annual Review of Cell and Developmental Biology 2000;16:145-171.

Rosen ED, & Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature 2006;444:847-853.

Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. Genes & Development 2000;14:1293-1307.

Rutkowski JM, Davis KE, Scherer PE. Mechanisms of obesity and related pathologies: The macro- and microcirculation of adipose tissue. The FEBS Journal 2009;276:5738-5746.

Salma N, Xiao H, Imbalzano AN. Temporal recruitment of CCAAT/enhancer-binding proteins to early and late adipogenic promoters in vivo. Journal of Molecular Endocrinology 2006;36:139-151.

Salma N, Xiao H, Mueller E, Imbalzano AN. Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor gamma nuclear hormone receptor. Molecular and Cellular Biology 2004;24:4651-4663.

Sell H, Dietze-Schroeder D, Kaiser U, Eckel J. Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. Endocrinology 2006;147:2458-2467.

Tarnus E, Wassef H, Carmel JF, Rondeau P, Roche M, Davignon J, Bernier L, Bourdon E. Apolipoprotein E limits oxidative stress-induced cell dysfunctions in human adipocytes. FEBS Letters 2009;583:2042-2048.

Tilg H, & Moschen AR. Adipocytokines: Mediators linking adipose tissue, inflammation and immunity. Nature Reviews.Immunology 2006;6:772-783.

Trayhurn P. Endocrine and signalling role of adipose tissue: New perspectives on fat. Acta Physiologica Scandinavica 2005;184:285-293.

Trayhurn P, & Beattie JH. Physiological role of adipose tissue: White adipose tissue as an endocrine and secretory organ. The Proceedings of the Nutrition Society 2001;60:329-339.

Trayhurn P, & Wood IS. Adipokines: Inflammation and the pleiotropic role of white adipose tissue. The British Journal of Nutrition 2004;92:347-355.

Vassiliou G, Benoist F, Lau P, Kavaslar GN, McPherson R. The low density lipoprotein receptor-related protein contributes to selective uptake of high density lipoprotein cholesteryl esters by SW872 liposarcoma cells and primary human adipocytes. The Journal of Biological Chemistry 2001;276:48823-48830.

Wassef H, Bernier L, Davignon J, Cohn JS. Synthesis and secretion of apoC-I and apoE during maturation of human SW872 liposarcoma cells. The Journal of Nutrition 2004;134:2935-2941.

Wei S, Yang J, Lee SL, Kulp SK, Chen CS. PPARgamma-independent antitumor effects of thiazolidinediones. Cancer Letters 2009;276:119-124.

Westerink J, & Visseren FL. Pharmacological and non-pharmacological interventions to influence adipose tissue function. Cardiovascular Diabetology 2011;10:13.

White RT, Damm D, Hancock N, Rosen BS, Lowell BB, Usher P, Flier JS, Spiegelman BM. Human adipsin is identical to complement factor D and is expressed at high levels in adipose tissue. The Journal of Biological Chemistry 1992;267:9210-9213.

White UA, & Stephens JM. Transcriptional factors that promote formation of white adipose tissue. Molecular and Cellular Endocrinology 2010;318:10-14.

Wozniak SE, Gee LL, Wachtel MS, Frezza EE. Adipose tissue: The new endocrine organ? A review article. Digestive Diseases & Sciences 2009;54:1847-1856.

Yang JY, Della-Fera MA, Rayalam S, Ambati S, Hartzell DL, Park HJ, Baile CA. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. Life Sciences 2008;82:1032-1039.

Zou C, & Shao J. Role of adipocytokines in obesity-associated insulin resistance. Journal of Nutritional Biochemistry 2008;19:277-286.